A Light Microscopic Study of Chemically Induced Renal Papillary Necrosis and Upper Urothelial Carcinoma in Animal Models

by

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Submitted in partial fulfilment for the degree of Doctor of Philosophy in the Robens Institute of Health and Safety, in the Faculty of Science, University of Surrey, Guildford, Surrey.

December 1989
Renal papillary necrosis (RPN) and upper urothelial carcinoma (UUC) can both be caused by the long-term abusive consumption of analgesics and non-steroidal anti-inflammatory drugs (NSAID). A "cause-and-effect" relationship between RPN and UUC has been proposed. However, despite a considerable amount of clinical data and experimental evidence this relationship has not been proven nor is it widely accepted.

Experimental models of RPN and UUC have been difficult to establish using analgesics and NSAID because of the chronic periods of dosing required with these compounds which frequently have extra-renal toxicities and there is a large biological variation in response. These problems have lead to the use of alternative models using chemicals with known papillotoxic potential such as 2-bromoethanamine (BEA) hydrobromide. This compound will induce a dose related RPN in the rat in 24-48 hr.

Studies to determine which was the earliest cellular change occurring as a consequence of BEA-induced RPN were performed in a number of different species (rat, mouse, marmoset and pig), and to determine whether this acute RPN lesion was relevant to the chronic clinical situation in man. High resolution light microscopy using semithin glycolmethacrylate sections in conjunction with routine and enzyme histochemical staining was used throughout these studies to assess the histopathological changes. Comparison with renal tissue obtained from human analgesic abusers was also performed.

The results from these investigations show for the first time that the earliest cell type affected, in BEA-induced RPN in the rat and mouse is the medullary interstitial cell in the renal papilla. These cells
were also affected in the marmoset and pig where only terminal studies were undertaken. A degenerative cascade results affecting the medulla, cortex (in rats and nude mice) and a pronounced hyperplasia of the urothelium of the pelvis and ureter occurs in the rat and pig. Long-term changes in the rat closely resembled the changes observed in tissues from human analgesic abusers.

A series of investigations was performed to try and establish the link between RPN and UUC using the classical 2-stage "initiation-promotion" model for experimental carcinogenesis.

A specific urothelial carcinogen N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN) was used to initiate the urothelium of Wistar rats, followed by an acute BEA-induced RPN. The development of pre-neoplastic papillary and nodular hyperplasias (within 13 weeks of this regimen) and macroscopic transitional cell tumours in the renal pelvis and upper ureter (at 30-40 weeks) suggest that the localised injury associated with an acute RPN can "promote" an already initiated urothelium to induce pre-neoplastic and neoplastic changes. Studies where single analgesics were superimposed on to this BBN/BEA regimen produced results that suggest that analgesics may exacerbate these RPN-related changes to induce more severe pre-neoplastic and neoplastic changes earlier than with BBN/BEA alone.

A number of possible mechanisms are discussed in the light of the data from these investigations to link RPN and UUC. Peroxidative metabolism of potential carcinogenic compounds seems to be the most likely mechanism although the actual mechanistic process is probably a complex one with a multifactorial basis.
I dedicate this thesis to my wife
and to my parents.
ABBREVIATIONS

alk.phos. - alkaline phosphatase
ASP - aspirin
ATPase - adenosine triphosphatase
BBN - N-butyl-N-((4-hydroxybutyl)-nitrosamine
BBN-Gluc - beta-glucuronide of BBN
BCPN - N-nitroso-butyl-(3-carboxypropyl)-amine
BEA - 2-bromoethanamine hydrobromide
BEN - Balkan endemic nephropathy
BW - body weight
cAMP - cyclic adenosine monophosphate
Cas - chemical abstracts identification number
Ca^{2+} - calcium ion
cGMP - cyclic guanosine monophosphate
Cl^- - chloride ion
cm - centimetre (10^-2 metre)
DNA - deoxyribose nucleic acid
ECU - european currency unit
EEC - european economic community
El - ethylenimine
ERPF - effective renal plasma flow
ESRD - end-stage-renal disease
FANFT - N[4- (5-nitro-2-furyl-thiazoyl]-formamide
G - gram
GFR - glomerular filtration rate
GTT - gamma-glutamyl-transpeptidase
GLP - good laboratory practice
Gy - Gray (unit of dose of radiation)
Ha-ras - Harvey sarcoma virus oncogene
hr - hour(s)
H&E - Haemotoxylin and Eosin
IARC - International agency for research on cancer
ip - intraperitoneal
iv - intravenous
kg - kilogram
KW - kidney weight
K^+ - potassium ion
L - litre
M - molar
mg - milligram
MGT - mouse grown tumour
mg/kg - milligrams per kilogram
mg/ml - milligrams per millilitre
min - minute(s)
ml - millilitres
mmol - millimolar solution (Molar x 10^-3)
mm^3 - cubic millimetre
MW - molecular weight
NaNPAA - sodium salt of N-phenylanthranilic acid
Na^+ - sodium ion
NMR - nuclear magnetic resonance
NPAA - N-phenylanthranilic acid
NSAID - non-steroidal anti-inflammatory drug(s)
Abbreviations

OHT - original host tumour
PARA - paracetamol
PAS - periodic-acid-Schiff
PGH - prostaglandin hydperoxidase
PO_{4}^{2+} - phosphate ion
rpm - revolutions per minute
RPN - renal papillary necrosis
SD - standard deviation
SE - standard error
TCC - transitional cell carcinoma
TEM - transmission electron microscopy
um - micrometre (10^{-6} metre)
UTI - urinary tract infection
UUC - upper urothelial carcinoma
v/v - volume to volume
w/v - weight to volume
°C - degrees Celsius
yrs - year(s)

Abbreviations in Figure legends

CD - collecting duct
CE - covering epithelium of papilla
Cx - cortex
G - glomerulus
Gh - "ghost" papilla
LP - lamina propria
M - medulla
Mu - lamina muscularis
Pap - papilla
PE - pelvic epithelium
Acknowledgements

I am greatly indebted to Dr Peter Bach, who provided guidance, motivation, much valued critical comments on this thesis and "good news, bad news" throughout this study. I particularly appreciated the freedom he gave me to explore new avenues.

I am indebted to Dr Mike Robbins for his enthusiasm and commitment throughout the pig studies, and grateful to both him and his colleagues at the CRC Research Institute, Churchill Hospital, Oxford for making available the facilities and their time.

I am extremely grateful to Dr Marie Coates and Dr Frank Ward and their colleagues who provided the facilities and excellent tuition in the skills of gnotobiotics and isolator work.

I am grateful to Mrs Pauline Fullbrook, Mr Peter Scobie-Trumper and staff of the Animal Unit for ensuring the animals were of excellent quality throughout the studies, their dedication in maintaining the animals, and for imparting their skills in animal handling techniques.

I am grateful to Dr R.S. Tudor and his colleagues, of the Animal Unit, D.H.S.S Department of Toxicology, St Bartholomew's Hospital, London for performing the marmoset studies.

I am grateful to Dr Enoch Kwizera and Dr Martin Wilks for their advice and assistance during the in vitro studies, and to Dr Wilks for his invaluable translations of German clinical histories and for critical comments during the proof reading of this thesis.

I would like thank Dr Anke Schwarz, of the Freie Universitat, Berlin for her efforts in obtaining and providing specimens of renal tissue from analgesic abusers and her valuable comments.

I appreciate the help and tuition in the skills of word processing provided by Ms. Lisa Breitner and for her help, together with that of Gail Sutherland, Monique Rivet and Penny, in typing much of this thesis.

I am grateful to Dr Terry Hardy, of Beecham Pharmaceuticals, Research Division, for his much valued advice given throughout these studies and for the loan of his personal microscope for my personal use at home.

I am grateful to Ms. Patricia Ijomah for her assistance in the initial carcinogenesis studies.

I would like to thank the University of Surrey AVA unit, for their excellent service which ensured that the photographs included in this thesis were of the highest quality.

I would like to express my thanks to Ms. Julie Scarlett, a former M.Sc Toxicology student who permitted me to include some of her data in this thesis.
I would like to thank Mrs Sally Nichol for providing much appreciated technical assistance in the latter stages of this study.

I would like to express my thanks to colleagues, past and present, of The Robens Institute, for advice and assistance given freely throughout these studies.

I am grateful for the support of my family and friends, in particular the support of my wife Karen, without whose love and tolerance of a frequently absent husband, none of this would have been possible.

Finally, I am indebted to Cancer Research Campaign for providing the majority of the financial support throughout these investigations, and in part to the International Agency for Research on Cancer, the Commission of the European Communities and The Wellcome Trust for providing additional financial support.
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CHAPTER 1
INTRODUCTION TO NEPHROTOXICITY AND SUMMARY OF INVESTIGATIONS UNDERTAKEN

1.1 NEPHROTOXICITY.

Many chemicals target for specific organs, e.g. kidney, and cause a lesion. Organs such as the liver, kidney and lung (which are important sites for xenobiotic metabolism and elimination) are, frequent targets for chemical toxicity. Targeting may be specifically located to a single or discrete cell types within that organ, and could possibly be related to the specific functional or metabolic processes within these organs or cells.

Nephrotoxicity has been defined as adverse functional and structural changes induced directly or indirectly in the kidney by chemical and biological agents (or their metabolites) that are inhaled, ingested or otherwise absorbed (CEC-IPCS, 1989).

1.2 THE KIDNEY AND NEPHROTOXICITY.

Anatomically and functionally the kidney is an extremely complicated organ and these aspects, and many others, have formed the basis for a vast amount of literature. For a basic introduction and comprehensive reviews on the kidney and nephrotoxicity see Brenner & Rector, (1981), Bach et al, (1982), Bach & Lock, (1985, 1987, 1989), Hook & Hewitt, (1986) and CEC-IPCS (1989). The primary role of the kidney is the regulation of total body homeostasis, particularly the water-solute balance, but it also plays a significant role in the excretion of metabolic wastes and xenobiotics. The basic functional unit of the kidney is the nephron (see Figure 1.1). Any chemical toxicity which affects the kidney as a whole will often be initiated at a specific
FIG. 1. This scheme depicts a short-looped and a long-looped nephron together with the collecting system. Not drawn to scale. Within the cortex a medullary ray is delineated by a dashed line. 1, Renal corpuscle including Bowman's capsule and the glomerulus (glomerular tuft); 2, proximal convoluted tubule; 3, proximal straight tubule; 4, descending thin limb; 5, ascending thin limb; 6, distal straight tubule (thick ascending limb); 7, macula densa located within the final portion of the thick ascending limb; 8, distal convoluted tubule; 9, connecting tubule; 9*, connecting tubule of the juxtamedullary nephron that forms an arcade; 10, cortical collecting duct; 11, outer medullary collecting duct; 12, inner medullary collecting duct.

Figure 1.1 Diagram illustrating the anatomical subdivisions and arrangement of different nephrons within the kidney. From Kriz & Bankir (IUPS), (1988).
region of the nephron. The nephron is a heterogenous structure and is divided into several regions: glomeruli, proximal tubule, loops of Henle, distal tubule, collecting duct. Some of these i.e. $P_1$, $P_2$ and $P_3$ regions of the proximal tubule may be further subdivided. Some 20 morphologically distinct cell types are found along the length of the nephron, there is also heterogeneity between nephrons (Figure 1.1). This cellular heterogeneity exists at the biochemical and functional levels, hence it is not surprising to find chemical toxins often target for particular groups of cells or specific cells and do not affect the adjacent cells. This target cell specificity of different chemical nephrotoxins can be seen schematically in Figure 1.2.

There are several reasons for renal sensitivity to chemicals. The kidneys comprise only 0.4% of the total body weight in most mammals (Rush et al, 1984), but receive 20% of the resting cardiac output. This high blood flow dictates that large quantities of systemically circulating xenobiotics will be delivered to the kidneys, especially in the cortex which receives over 90% of the renal blood flow. The kidneys are metabolically very active, performing many important biochemical and functional processes and they have the second highest oxygen consumption rate per gram/tissue of the large parenchymal organs (Cohen & Kamm, 1981). These are some of the factors that predispose the kidney to damage by potential nephrotoxic substances.

The renal medulla receives approximately 10% of the total renal blood flow, thus the total amount of chemicals reaching the region may be significantly less than the cortex. However this region of the kidney is concerned with water and salt reabsorption via the counter current mechanism. This may lead to "trapping" of chemicals in the medulla,
Figure 1.2 From Darmady & McIver, (1980).
which results in a high and potentially toxic concentration of certain chemicals.

A chemical may act directly or indirectly on the kidney cells to produce a toxic response. A schematic representation of three possible mechanisms for renal damage from chemical toxicity (Figure 1.3) has been suggested (Rush et al, 1984). First, a chemical may enter renal cells and interfere directly with an essential metabolic or functional process, resulting in cellular damage (Scheme I). Second, a chemical may be metabolised to a highly reactive intermediate that may bind covalently to protein or initiate active oxygen species or lipid peroxidation and thus cause cellular damage (Scheme II). Finally, a chemical may be metabolised extra-renally to a stable metabolite that may enter the systemic circulation (Scheme III) and be transported to the kidney to cause toxicity via either scheme I or II (Rush et al, 1984).

1.2.1 Consequences of Nephrotoxicity.

Over the last decade, the number of toxic nephropathies resulting as a direct consequence of exposure to therapeutic agents or environmental chemicals has become increasingly discernible. The cellular response to a toxic insult may vary from a subtle biochemical aberration to cell death, resulting in necrosis, fibrosis and end stage renal disease. Functionally, toxicity may be reflected as a minor alteration in transport capability (eg. transient glucosuria, aminoaciduria), as polyuria with decreased concentrating capacity, or as frank renal failure with anuria and elevated blood urea nitrogen. Depending on the magnitude of the insult, these changes may be reversible, permanent or lethal. One renal cell type
FIGURE 1-3 Schematic representation of mechanisms of toxic injury induced by xenobiotics.

Figure 1.3 From Rush et al, (1984).
may be the primary site of nephrotoxic damage, but the consequence of
the loss of this cell may lead to a change in the maintenance of
normal renal function resulting in a cascade of degenerative changes.
These eventually result in secondary effects, many of which may affect
other distant areas of the kidney and/or cause extra-renal
abnormalities (Gregg et al., 1990a). Chemically induced acute renal
papillary necrosis (RPN) is a good example of this nephrotoxic
sequence of events. The medullary interstitial cell has been
suggested to be the primary site of damage (Bach & Bridges, 1985), the
structural and functional loss of which is thought to lead to a total
medullary necrosis with cortical changes only developing later (Gregg
et al., 1990a). In patients with analgesic associated RPN there is a
high incidence of upper urothelial carcinoma (UUC) which is thought to
be the secondary consequence of RPN and analgesic abuse. This high
association between analgesic induced RPN and UUC suggests a "cause-
and-effect" relationship (Bengtsson et al., 1978; McCredie et al.,
1982a,b; Prescott, 1982), but this has not been proven (Bach &
Bridges, 1985).

1.2.2 Socioeconomic consequences of Nephrotoxicity.
The gravity of the socioeconomic impact of end-stage renal disease
(ESRD) has just begun to be documented in terms of the annual addition
of more than 500,000 patients worldwide (CEC-IPCS, 1989). Thus, an
average of 50-100 new patients are added per million population per
year in the developed world. There is a scarcity of information from
developing countries, however it is known that they have a lack of
treatment facilities. Individual haemodialysis patients presently
cost the EEC 50,000 European Currency Units (ECU) (£33,000) annually.
By the end of 1986 there were 65,000 haemodialysis patients in the EEC, giving an annual bill of 3,250,000,000 ECU (£2200 million). Peritoneal dialysis for 7500 patients costs amounted to 165,000,000 ECU (£99 million) and the costs of transplantation has been calculated at 127,300,000 ECU for the first year of treatment. These sums include all hospitalisation periods, medication, but does not account for the loss of economic contribution of each patient. It is envisaged that the numbers of patients will continue to increase, thus increasing the already large burden on the medical and health services. Up to 20% of ESRD has been estimated to have a drug or chemical aetiology with 50% of all ESRD having no known aetiology it has been suggested that some of this unknown aetiology is probably due to chemical associated nephropathies (Dieperink, 1989). Analgesic associated RPN is a serious nephrotoxic condition, which may lead to end-stage renal disease requiring long-term dialysis treatment. It is, however, one of a few toxic related nephropathies which is preventable. An understanding into the mechanism of pathogenesis, identifying all possible causative agents, any risk factors that may predispose a patient to developing analgesic-associated RPN could help reduce the numbers of patients presenting with analgesic-associated RPN and the necessity to provide costly health care measures.

1.3 RELEVANCE OF ANIMAL MODELS TO MAN IN TOXICOLOGICAL STUDIES.

Animal models are utilised in toxicological studies in two ways:–

I. To evaluate the general overall effects of compounds on the animals, and within the category of acute, subchronic and chronic period of administration to determine the order of safety of a particular compound i.e. pharmaceutical drugs for therapeutic use.
II. To elucidate toxicity mechanism(s) at a molecular level in order to help provide a rational basis for risk assessment, methods to improve efficacy or safety and also to understand disease processes.

The following guidelines have been suggested by Weil, (1972) for the use of experimental animals with regard to toxicological evaluation with relevance to man.

a. The use of one or more species that biologically handle the material qualitatively as similarly as possible to man.

b. Use several dose levels on the principle that all types of toxicologic and pharmacologic actions in man and animals are dose related.

c. Choose administration routes to test animals which are the same as those to which man will be exposed if possible.

There are few recommendations regarding the selection of specific species of animals for toxicologic tests but much has been shaped by economics, practicality and tradition. In the absence of some specific reason for the use of exotic or expensive species such as non-human primates, and large animals such as pigs, most toxicity studies are performed on rats, mice, (and some on guinea pigs and rabbits and at least one non-rodent species such as the dog). These species are relatively inexpensive, easily obtainable, easily handled and there is a considerable amount of base-line biological and toxicological information available regarding their handling of many classes of chemical compounds. The use of genetically identical (inbred) animals in toxicology has been strongly advocated as a means of minimising the experimental variance attributed to the animals used (Festing, 1979). The use of strains which are sensitive or resistant to particular drugs or chemicals may offer valuable information about the mechanism
of toxicity which could be relevant to man (Festing, 1981). The region of the nephron affected by the nephrotoxin may have a bearing on the choice of species since the structural and functional characteristics of the nephron may prove to be a more suitable in a particular species to investigate the mechanism of nephrotoxicity (Schmidt-Nielsen, 1989).

The rat has several advantages which make it the primary choice of animal model for nephrotoxicity studies. It has a relatively simple renal morphology (unipapillate kidney compared to the multipapillate kidneys of man and pig) and several nephrotoxicity models are already well established and widely reported. The evaluation of the effects of a chemical on normal renal function can be determined using a broad spectrum of experimental investigative techniques. Abnormalities of total renal function can be determined using a battery of tests (all used clinically) on collected urine samples such as measurement of urine volume, urinary pH, urinary osmolality, electrolyte excretion, sugar excretion, protein excretion, and the appearance of enzymes in urine. Enzymuria can pinpoint a specific site of injury (eg trehalase, maltase identifying proximal tubular damages) but one must be aware of possible extra-renal sources of any enzymes. Specific effects on the kidney function can be determined on anaesthetised animals such as the assessment of glomerular filtration rate using inulin or $^{99m}\text{Tc}$-DTPA renography, and renal blood flow using para-aminohippurate clearance or $^{131}\text{I}$-hippuran renography. Histopathological examination of renal tissues after terminal sacrifice can provide vital information with regard to renal integrity and where the site of damage occurred. Histopathology may often
identify a lesion where no apparent functional changes were observed from functional tests. In renal papillary necrosis, histopathological examination currently offers the best means of identifying the early onset of this lesion, due to the focal nature of this lesion and the lack of any specific functional changes.

In a review concerned with the relevance of animal models to analgesic-associated renal papillary necrosis in humans, Bach & Hardy (1985), suggest "Animal models offer a potentially important means by which to understand the pathomechanism of RPN and provide a model system to improve the early diagnosis of the lesion to identify which analgesic(s) have the greatest papillotoxic potential and what factors exacerbate its development or contribute to the secondary consequences of papillary damage".

Analgesic abuse leads to the development of RPN (see Chapter 2, section 2.1) and may cause upper urothelial carcinoma (UUC) (see Chapter 2, section 2.2), the mechanisms of the pathogenic changes are not fully understood. Medical ethics dictate against the use of man. The answer is, therefore, to study reproducible animal models of RPN in the laboratory situation which can be utilised to determine the mechanism of the lesion. There are many questions to be evaluated such as:-

I. The pathogenic changes occurring during the onset of RPN.
   a) what are the earliest necrotic changes?
   b) is RPN initiated by similar mechanisms in different species?
   c) what other factors affect the progression of RPN?

II. The relationship between RPN and UUC.
   a) Does RPN progress to UUC?
   b) is it possible to produce a model of UUC from an RPN model?
III. Is it possible to evolve preventive and therapeutic procedures? These questions have formed the focus of investigations described below.

1.4 SUMMARY OF INVESTIGATIONS.

1.4.1 Chemically induced RPN in the rat

A time course study of a 2-bromoethanamine (BEA) hydrobromide induced RPN lesion in the Wistar rat (see Methods section 3.6.1), was conducted to answer some of these questions.

Previous studies on the development of BEA-induced RPN used relatively few time points and routine wax embedded tissue sections (> 5um) often precluded a detailed morphological evaluation. There is also a paucity of data on enzyme histochemical changes associated with the development of RPN.

This time course study has allowed the re-evaluation of an acute papillary necrosis using glycolmethacrylate embedded sections to provide unprecedented detail of the pathological changes. Furthermore, the ability to combine ‘routine’ staining with selective enzyme histochemistry on serial semi-thin (lum) sections enhances the ability to inter-relate changes in different cell types during the course of the development of degenerative processes that lead to RPN.

The earliest histopathological changes (see Results section 4.1.1) occurred in the medullary interstitial cells in the papilla tip, and in the interstitial matrix. A cascade of necrotic changes followed; producing frank RPN. Hyperplasia of the pelvic and ureteric urothelium occurred as a secondary consequence of this lesion.

1.4.2 Chemically induced RPN in the mouse.

For experimental details see Methods sections 3.6.2.1 and 3.6.2.3.
C57B1/6 mice have been reported to metabolise phenacetin via a similar pathway as in man, and are also prone to developing renal papillary necrosis (RPN) after dosing with phenacetin (Macklin & Szot, 1980). An investigation into whether this mouse strain was sensitive to the papillotoxin, 2-bromoethanamine (BEA), was undertaken to determine if it would be a suitable animal model for studies into the pathogenetic mechanism of analgesic nephropathy. Obese mice (ob/ob) are a random bred mouse strain derived from C57B1/6 mice which have the genetic potential to become obese, and they may also develop diabetes (Flatt & Bailey, 1981). Diabetics are prone to developing a number of nephropathies (including RPN). It was of interest to use the ob/ob strain to see whether they were more sensitive to BEA and whether there was any difference in sensitivity between the random outbred strain (ob/ob) and the inbred parent strain (C57B1/6).

In the mouse (see Results section 4.2.2) BEA caused necrosis of renal medullary interstitial cells associated with increased staining for interstitial cell matrix. Frank RPN was not observed at the time points studied. However, cortical changes not normally observed in the Wistar rat until after the onset of total RPN were evident in the mouse strains. A strain difference in susceptibility to BEA was seen: C57BL/6 > Obese > BALB/c. These strain differences in BEA toxicity are suggested to be due to metabolic differences between the strains. Genetic differences may also influence this sensitivity; obese mice are derived from the C57Bl/6 strain so out-breeding may confer some protection possibly due to hybrid vigour.

1.4.3 Chemically induced RPN in nude mice.

For experimental details see Methods section 3.6.2.2 and Results
By contrast the nude mouse showed the potential of the role of the immune system in renal papillary necrosis. Unexpectedly the Nude mouse develops both an RPN and a cortical necrosis of the $P_2$ and $P_3$ segments. This unique response could be exploited to better understand the role of biochemical characteristics along the nephron in the targeting of BEA, and the role of the immune system in nephrotoxicity.

1.4.4 Chemically induced RPN in the marmoset.

The marmoset is the smallest living primate and has a unipapillate kidney. It combines the attributes of having a simple kidney structure to examine pathologically and also being a primate therefore being distantly related to man. As such it offers the potential to be a novel animal model on which to investigate acutely induced RPN in an attempt to extrapolate the findings to the clinical condition analgesic nephropathy. The administration of BEA (see Methods section 3.6.3) at doses of 50 and 100 mg/kg did not induce frank RPN (see Results section 4.3), only minimal changes in the capillaries and matrix which would be concomittant with an early RPN lesion in the rat. The most prominent change was a hyperplasia of the covering epithelium of the papilla ridge. With only two animals in this study the results are interesting but without control data one cannot postulate too strongly on the significance of these few results.

1.4.5 Chemically induced RPN in the pig.

1.4.5.1 BEA induced RPN.

These studies (see Methods sections 3.6.4.2-3.6.4.5) showed that BEA caused a sub-acute RPN in the pig (see Results section 4.4.3) which took 7-14 days to develop compared to 24-48 hr in the rat. Moreover the lesion did not progress to frank necrosis as in the rat.
1.4.5.2 Effect of superimposing analgesic dosing on a BEA induced RPN lesion.

RPN was induced acutely in pigs using BEA, and these animals were dosed for 28 days with paracetamol (acetaminophen) to assess the effect of superimposing this analgesic drug on porcine renal function (see Methods section 3.6.4.6).

Necrosis of interstitial cell nuclei, interstitial matrix and thick ascending limb together with hyperplasia of the pelvic and ureteric urothelia with extensive vacuolation was observed (see Results section 4.4.3) after BEA and BEA/paracetamol treatment. These animals also exhibited a significant increase in effective renal plasma flow compared with that observed in the age-matched controls. There was no significant difference in the overall response in glomerular flow rate between the groups. These findings suggest that subsequent paracetamol dosing does not enhance BEA-induced RPN in the pig. BEA induces urothelial hyperplastic changes despite the lack of a gross RPN lesion. The pig is thus less sensitive to papillotoxins than the rat. The multipapillate pig kidney is more similar to that of man, implying that the use of acute RPN rodent "models" for analgesic nephropathy may not be justified.

1.5 EXPERIMENTAL UPPER UROTHELIAL CARCINOGENESIS STUDIES.

Studies (see Methods 3.6.5) into how RPN progresses to UUC, were based on the 'classical' 2-stage initiation-promotion protocol used in skin and bladder experimental carcinogenesis (see section 2.3.4). A marked hyperplasia of the pelvic and ureter urothelium occurs after BEA induced RPN, atypical urothelium may persist up to 40 weeks after BEA but UUC does not develop. Results (Chapter 5, section 5.1-5.5) showed
that 6 weeks after this protocol papillary/nodular hyperplasia occurred in the ureteric urothelium, this had progressed by 13 weeks to include ‘invasive’ foci which were lacking alkaline phosphatase (which is reported to be a preneoplastic ‘marker’ change in urothelium (Kunze, 1979). By 21 weeks bladder tumours were present, and pelvic tumours with dyplasia and upper urothelial tumours were observed at 40 weeks.

These results suggest that an acute RPN lesion can promote urothelial malignant changes including full blown tumours. This system has been used to evaluate the effects of chronic dosing of analgesics on the development of upper urothelial tumours (see Methods sections 3.6.5.3 and 3.6.5.5).

Early results (see Results section 5.3) with paracetamol and aspirin dosing suggest that subsequent analgesic dosing exacerbates the progression of UUC.

Attempts to increase the yield of tumour tissue by xenografting into nude mice (see Methods section 3.6.6) or growing tumour cells in culture from tumour explants were undertaken. Tumours were successfully transplanted (see Results section 5.6) and growth of the tumours occurred in 2/21 animals transplanted, but failed to progress beyond one passage step. Tumours were slow growing but identical to the donor tumours with respect to morphology (at the light and ultrastructural level) and histochemistry. Although tumour cells initially grew successfully in culture, bacterial contamination meant that no cells survived to establish a tumour cell line.

Chapter 2 reviews the literature on analgesic associated renal papillary necrosis and upper urothelial carcinoma, and chemical carcinogenesis.
CHAPTER 2

RENSAL PAPILLARY NECROSIS, UPPER UROTHELIAL CARCINOMA AND CHEMICAL CARCINOGENESIS

2.1 RENAL PAPILLARY NECROSIS (RPN).

2.1.1 Introduction.

Renal papillary necrosis (RPN) is a clinicopathological syndrome which was first described over one hundred years ago by von Friedreich (1877) and Turner (1888). Subsequently RPN was also frequently associated with diabetes mellitus and obstructive uropathy (Gunther, 1937).

Mandel (1952) reviewed 180 cases of RPN and agreed with the findings of Gunther and Froboese, but also suggested that bacterial toxins released into the urinary tract may have a significant role to play in cell death and necrosis. RPN may have a number of other possible different causes (Table 2.1).

However, since then it has become clear that the abusive intake of mixed analgesics is by far the most frequent cause of RPN. Spuhler & Zollinger (1953) first drew attention to this when they reported on the increased incidence of chronic interstitial nephritis associated with an abusive intake of mixed analgesics. Phenacetin was found to be common to all these mixed analgesics and similar findings by other researchers led the condition to be dubbed "phenacetin kidney". The assumption that phenacetin was the single common denominator that caused RPN was a concept that was to retard research into RPN for many years.

It soon became apparent that other analgesics had the potential to cause RPN (Gilman, 1964). Together with the suggestion that the
**Frequently reported**

Diabetes mellitus  
Analgesic abuse  
High dose nonsteroidal anti-inflammatory drug therapy  
Upper urinary tract obstructive uropathy (may be of consequence)  
Recurrent urinary tract infection (may be of consequence)  
Sickle cell hemoglobinopathy  
Acute pyelonephritis (may be superimposed)  
Dehydrated newborn infants (frequently jaundiced)

**Less frequently reported**

Renal vein thromboses  
Chronic alcoholism  
Dehydration in children (diarrhea loss of Cl\textsuperscript{-}, also K\textsuperscript{+})  
Severe jaundice  
Calyceal arthritis  
Glomerulonephritis  
Systemic candidosis  
Trauma  
Prolonged hypotension  
Dapsone

---

Table 2.1 Factors relating to the development of RPN. From Bach & Bridges, (1985).

The initial lesion was in the papilla and the so called "interstitial nephritis" was due to the atrophy of nephrons affected by RPN (Kincaid-Smith, 1967). The concept that the primary lesion associated with analgesic abuse is RPN, and interstitial nephritis is a secondary consequence has now been widely accepted (Burry et al, 1977; Burry, 1978; Gloor, 1978).

The identification of the causative agent for RPN has been complicated by the fact that patients abused and/or were prescribed mixed analgesics and a number of non steroidal anti-inflammatory drugs (NSAID) (Cove-Smith & Knapp, 1973; Nanra & Kincaid-Smith 1975; Nanra et al, 1980). A reduction in the availability of phenacetin in mixed analgesics occurred in Finland, Sweden and Denmark from 1961 and other countries followed suite over the next 25 years. In the Federal Republic of Germany, for example, phenacetin has only just been banned.
in 1986. However as Prescott (1982) points out the withdrawal of phenacetin did not significantly reduce the number of deaths from RPN either early or advanced, where analgesic abuse continued on a large scale, also withdrawing a drug would not change the course of a disease already well advanced towards chronic renal failure. Paracetamol (acetaminophen) has largely replaced phenacetin in analgesic mixtures and several studies have implicated this compound as the initiating agent in the pathogenesis of RPN (Krikler, 1967; Axelsen, 1975; Masters & Krikler, 1973; Rosner, 1976; Duggin, 1977; Molland, 1978; Nanra et al, 1978; Dubach et al, 1983). Rosner (1976) formed the opinion that paracetamol was the initial link, because it is a major metabolite of phenacetin and concentrated in the medulla (where the first pathological changes appear) whereas phenacetin is not concentrated in the medulla to the same extent (Gault, 1971).

To date at least thirty-six (36) therapeutically used agents (see Table 2.2) have been implicated in causing RPN in animals and man. This list is likely to be larger as each year a few new classes of papillotoxins are reported eg 1-Napthol (Poole & Buckley, 1989) and L-triiodothyronine (L-T3) (Kennedy & Jones, 1989) and there are examples of new therapeutic agents currently undergoing toxicity screening which must remain confidential. Evidence also points to other extremely common (and easily available) drugs such as aspirin, also having the potential to be papillotoxic. Rheumatic patients find this drug a good pain reliever which can be purchased over the counter and is therefore easily open to 'abuse'. Abuse in context with rheumatoid arthritic sufferers is a bit of a misnomer, as large quantities of analgesics are often prescribed and may have to be
consumed to combat the pain. Nanra & Kincaid-Smith, (1975), found that 60% of rheumatic patients had RPN in a retrospective study of autopsy tissue.

<table>
<thead>
<tr>
<th>Acetaminophen</th>
<th>4-Isopropylbiphenyl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aclofenac</td>
<td>Ketoprofenbutazone</td>
</tr>
<tr>
<td>Aminopyrine</td>
<td>Ketoprofen</td>
</tr>
<tr>
<td>5-Aminosalicylic acid</td>
<td>Meclofenamic acid</td>
</tr>
<tr>
<td>Antipyrine</td>
<td>Mefanamic acid</td>
</tr>
<tr>
<td>Aspirin</td>
<td>Mono-N-methylaniline</td>
</tr>
<tr>
<td>Aspirin, phenacetin and codeine</td>
<td>Naproxen</td>
</tr>
<tr>
<td>Aspirin, phenacetin and caffeine</td>
<td>Niflumic acid</td>
</tr>
<tr>
<td>Aspirin and pentazocine</td>
<td>Oxyphenbutazone</td>
</tr>
<tr>
<td>Bucloxic acid</td>
<td>Phenacetin</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>Phenothiazine</td>
</tr>
<tr>
<td>Dapsone</td>
<td>Phenylalkanoic acid</td>
</tr>
<tr>
<td>Dextropropoxyphene</td>
<td>Phenylbutazone</td>
</tr>
<tr>
<td>Fenoprofen</td>
<td>Sudoxicam</td>
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<tr>
<td>Flufenamic</td>
<td>Sulfinpyrazone</td>
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<tr>
<td>Glaphenine</td>
<td>Tetrahydroxyquinoline</td>
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<tr>
<td>Ibruprofen</td>
<td>Tolfenamic acid</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>Tolmetin</td>
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</tbody>
</table>

Table 2.2 Therapeutically administered analgesics, NSAID, and other drugs and chemicals implicated with a direct papillotoxic potential. From Bach & Bridges, (1985)

Aspirin has also been implicated in causing RPN in arthritic children together with administered paracetamol and NSAID (Wortman et al, 1980; Allen et al, 1986; Bailie, 1986). Long-term feeding studies with aspirin and aspirin-containing compounds by (Nanra & Kincaid-Smith, 1970; Molland, 1978) showed that aspirin did cause RPN in rats and suggested it may be an important aetiological factor in the clinical syndrome. Prescott (1982) also concluded that aspirin caused RPN in experimental animals and despite the considerable of evidence available for the role of aspirin as a causative agent of RPN in man "there is a curious reluctance to accept the nephrotoxicity of aspirin" especially based on a NIH consensus conference (NIH, 1984). The contribution of therapeutic doses of NSAID to the development of
RPN has been increasingly apparent over the years, with a considerable amount of evidence that now supports this hypothesis (Shelley, 1978; Prescott, 1979, 1982; Robertson et al, 1980; Erwin & Boulton-Jones, 1982; Mitchell et al, 1982; Bach & Bridges, 1985).

Attempts have been made to explain the reasons behind analgesic abuse (Prescott, 1982). It was originally linked with the misguided use of these drugs in an attempt to increase productivity and reduce strain in working environments such as watch factories (Gsell, 1974; Kielholz, 1957), heavy industry in Sweden (Grimlund, 1963) and the textile mills of Atlanta where analgesics were also distributed free to the workers (Fellner & Tuttle, 1969).

Only a minority of patients with analgesic nephropathy have taken the drugs for genuine indications such as arthritis, headaches or migraine in most other cases, however, analgesics have been taken because of their "pick me up" value and frequently used inappropriately. However, the stigma of being identified as an abuser have often resulted in the patients providing unreliable or misleading data (Murray, 1974, 1978; Schwarz et al, 1988). The success of several of the recent epidemiological investigations has, in fact, depended on the close association between a sympathetic history taker and patients who did not feel threatened by being identified as an analgesic abuser (Sandler et al, 1989; Schwarz et al, 1984, 1988; Elseviers & De Broe, 1987, 1988). The origins of analgesic abuse are usually psychosocial and patients have been variously described as neurotic, dependant, immature, introverted, anxious or depressed. Many have been said to have personality disorders and up to 20% may have psychiatric illness. Other addictive habits such as smoking (Fellner & Tuttle, 1969; Clarkson & Lawrence, 1970; Murray, 1974),
alcoholism (Clarkson & Lawrence, 1970; Murray, 1974) and use of psychotrophic drugs and sleeping tablets have been associated with analgesic abuse. Women in their middle age years (40 - 50) are predominantly the largest group in populations who abuse analgesics and suffer RPN. Studies from DeBroe and coworkers in Belgium are unique in that they have a large group of co-operative analgesic abusers contributing to a prospective case control study. The patients are generally females of the lower socio-economic group, 60% of whom complained of headaches, although they admitted using these analgesic mixtures (especially powders) inappropriately, for 4-56 years. In 1983 the total sales volume of analgesic compounds in Belgium amounted to 26 million packages. On average this represents 2.6 packages or approximately 25 g per capita per year. While single analgesics were sold mostly in the south of the country, analgesic mixtures comprised 63% of the total sales and were largely formulated as tablets. These were sold almost equally throughout the country, whereas analgesic mixture powders were the most commonly abused product, especially in the north of the country (Elseviers & De Broe, 1987, 1988). Since 1972 the sales of analgesics containing phenacetin reduced progressively to 21% (in 1976), 9% (in 1983) and less than 3% in 1987. Nevertheless, the prevalence of analgesic nephropathy in dialysis patients in Belgium remained at the same level (18-20%) between 1979 and 1984. These data strongly support the concept that phenacetin was not the most important aetiological factor in the genesis of the lesion (Elseviers & De Broe 1987, 1988). Environmental factors have also been implicated in RPN such as
dehydration, high temperatures, bacterial infection and diet (Burry et al, 1977; Eknoyan et al, 1982; Molland, 1982; Thomas & Tange, 1985) it is clear that RPN is a complex disease state the pathogenic mechanism of which can only be postulated on and will be discussed later. Clinical evidence shows that the prognosis for patients who continue to abuse analgesics (after RPN has been diagnosed) is very poor. This rapidly leads to end-stage renal disease (Nanra & Kincaid-Smith, 1972; Murray & Goldberg, 1975; Burry et al, 1977; Bach & Bridges, 1985).

2.1.2 Analgesic Associated RPN in Man.

2.1.2.1 Epidemiological evidence on RPN.

The national prevalence of analgesic-associated end stage renal disease can be grouped into 3 classes: low < 5% (USA, Canada, France, Great Britain, Italy, Scandinavia, Spain); moderate 11-15% (Australia, Federal Republic of Germany) and high > 16% (Belgium, Switzerland). The occurrence of this condition may, however, be severely underestimated (Cove-Smith & Knapp, 1978, Bucchianti et al, 1987; Wing et al, 1989) in those countries where the prevalence is reported to be low. There is also a marked regional heterogeneity in the documented prevalence in some countries. For example, in the Federal Republic of Germany abuse is highest in Berlin and lowest in Bavaria (Pommer et al, 1986, 1989a,b; Schwarz, 1987) (Figure 2.1), whereas in Belgium dialysis units report the maximum frequency as 51% in the north of the country (Figure 2.2), but < 4% in some part of the south, giving a national average figure of about 20% (Elseviers & De Broe, 1987, 1988). Thus the national average does not reflect the pockets of high frequency.

Over the last decade there have been a number of carefully controlled
<table>
<thead>
<tr>
<th>Region</th>
<th>Prevalence (pmp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schleswig-Holstein</td>
<td>8.08</td>
</tr>
<tr>
<td>Hamburg</td>
<td>26.25</td>
</tr>
<tr>
<td>Bremen</td>
<td>20.0</td>
</tr>
<tr>
<td>Niedersachsen</td>
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</tr>
<tr>
<td>Berlin (W)</td>
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</tr>
<tr>
<td>Nordrhein-Westfalen</td>
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</tr>
<tr>
<td>Hessen</td>
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</tr>
<tr>
<td>Bayern</td>
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</tr>
<tr>
<td>Rheinland-Pfalz</td>
<td>7.22</td>
</tr>
<tr>
<td>Saarland</td>
<td>0.00</td>
</tr>
<tr>
<td>Baden-Württemberg</td>
<td>10.22</td>
</tr>
</tbody>
</table>

Fig. 2. Prevalence of terminal analgesic nephropathy in the Federal Republic of Germany (1986). Reproduced, with permission, from Ref. 12; pmp, per million population.

**Figure 2.1 From Pommer et al, (1986).**

Fig. 1. Analgesic nephropathy as a cause of end-stage renal failure in Belgium (1984). From Ref. 11. Population, 10 million; area, 30,515 km²; chronic dialysis units (●), 54; total numbers of patients under chronic dialysis, 2334; (○) and number, patients with end-stage renal failure caused by analgesic nephropathy expressed as % of patients treated in the dialysis unit(s).

**Figure 2.2 From Elseviers & De Broe, (1988).**
epidemiological investigations that have supported some of the "gut feelings" about the condition, but there does appear to be important national differences that may well occur in other parts of the world. For example, case-control studies from Germany (Pommer et al, 1989a,b) have confirmed an increased risk associated with the abusive consumption of either phenacetin or paracetamol in mixed analgesics or with phenacetin alone, but there was no data on NSAID. By contrast in Australia (McCredie & Stewart, 1988) found only phenacetin-containing mixed analgesics increased risk. Regular heavy use of analgesics is reported to be common in the Southeastern USA, especially North Carolina, where analgesic abuse has been implicated in at least 10% of end stage renal disease (Gonwa et al, 1981), which is much greater than the national prevalence for the United States. The reasons for such abuse included increased workplace and social environments which encourages heavy use of analgesics.

Recently published data from Sandler et al, (1989) reported on over 500 patients (30-79 years), 28% of whom had chronic renal disease, as assessed by a sustained elevation in serum creatinine (≥ 1.5 mg/dl or 130 umol). The diagnosed cause of the chronic renal disease was hypertensive nephrosclerosis - 19%, diabetic nephropathy - 20%, glomerulonephritis - 14%, and interstitial nephritis - 19%. These patients were compared to an age, race and sex matched control group (> 500) who were telephone screened to obtain information about specific analgesic and NSAID use. Daily users were defined as those who took analgesics for > 360 consecutive days and weekly users took pain reliever at least once a week for as long as a year. There was no reported consideration of either the quantity of analgesic consumed or the duration of abuse. Odds ratios were used to assess the
relative risk associated with daily or weekly use compared to infrequent use of a particular product. Daily use of any analgesic increased relative risk to 2.8. The relative risk was 3.2 with paracetamol, 5.1 with phenacetin, but only 1.3 for aspirin. Daily NSAID use in men showed a relative risk of 4.6, which increased to 16.6 if they were aged ≥ 65 years. The risk associated with NSAID use was higher for interstitial nephritis 3.9, renal insufficiency 2.5. If daily NSAID use was sub-stratified to males the risk of interstitial nephritis was 13.0. For the whole group the risk of chronic renal disease was 3.6 for daily users of ibuprofen, but only 1.6 for indomethacin despite the fact that it was more commonly used. Stratifying for the male sub-group, ibuprofen users had a relative risk of 9.0 compared to 3.7 for indomethacin. These data highlight older men as the population at greatest risk, but gives no indication of why this should be. The increased risk associated with NSAID consumption is consistent with a number of case-reports and with animal studies (Brandstetter & Mar, 1978; Kimberly et al, 1978; Wiseman & Reinert, 1975; Nanra et al, 1978; Wortman et al, 1980; Gunson, 1983; Adams et al, 1986; Allen et al, 1986; Bailie, 1986). The greater risk for interstitial nephritis is consistent with several proposed mechanisms for renal injury from NSAID (Clive & Stoff, 1984; Bender et al, 1984; Orme 1986), especially those that believe that the reduction of renal blood flow leads to ischaemic injury in the medulla. The increasing availability of over-the-counter NSAID will probably lead to markedly higher and inappropriate use of these drugs. At present there is no clear way of knowing if such excessive use of these compounds in the general population will cause renal papillary
necrosis, exacerbate existing renal disease or have no effect on it. Analgesic nephropathy accounts for 44% of patients in some German outpatient clinics where 21% of patients had paracetamol metabolites in their urine. Groups of patients who discontinued or continued analgesic abuse were followed for > 50 months (Pommer et al, 1986, 1989a,b; Schwarz, 1987). Renal function declined more rapidly in those who continued to abuse, they had recurrent urinary tract infection, severe hypertension was more common, and mortality was greater. Individuals who stopped abusing showed improved renal function (Cove-Smith & Knapp, 1978; Kimberly et al, 1978; Schwarz, 1987), but because of an increased survival these patients may be at greater risk of developing upper urothelial carcinoma.

2.1.2.2 Clinical symptoms and diagnosis of RPN.

The diagnosis of RPN is difficult because of the insidious nature in its development; it progresses silently, over prolonged long time periods (Gault et al, 1968; Duggin, 1977, 1980; Kincaid-Smith, 1979; Bach & Bridges, 1985) the clinical situation. One early clinical sign of analgesic nephropathy is the loss of urine concentrating capacity (Bengtsson, 1962; Dubach et al, 1975; Nanra et al, 1978; Nanra, 1980). Polyuria may, however, be a consequence of several nephropathies, and loss of the concentrating mechanism may have a number of renal and extra-renal causes. RPN is also associated with electrolyte disturbances. Cove-Smith & Knapp, (1973) reported a high incidence of sodium wastage and Jaeger et al, (1982) showed that patients were hypocalcaemic as a result of a urinary calcium ion (Ca$^{2+}$) loss. Patients with analgesic nephropathy have a pronounced defect in the urinary acidification mechanism following ammonium
chloride administration (Bengtsson, 1962; Steele et al, 1969; Krishnaswamy et al, 1976; Nanra et al, 1978; Nanra, 1980) suggesting that damage to the medulla might be synonymous with loss of effective urinary acidification and altered electrolyte balance. Other classical clinical biochemical parameters used to diagnose renal disease (such as creatinine, blood urea nitrogen) only identify incipient renal failure, by which time papillary necrosis has long since occurred and the secondary degenerative change that follow this lesion have progressed towards end-stage renal disease. There are few tell-tale clinical symptoms, (Table 2.3), none of which are pathognomonic of the condition.

Degenerative renal changes may be identified by radiology (Lindvall, 1978), but these are essentially indicative of an advanced lesion, and they may miss early, but frank RPN. The most dependable method of assessing analgesic related disease is by detailed patient histories, but the stigma of analgesic abuse normally leads to patients giving unreliable or misleading data on their drug usage (Murray, 1974, 1978).

New techniques such as ultrasound scanning and nuclear magnetic resonance (NMR) imaging and \(^1\)H-NMR spectroscopy of urine samples which are also non-invasive techniques may in the future broaden our perspective on RPN and lead to earlier diagnosis.

Pathology remains the most reliable method to diagnose RPN unfortunately it is generally performed at post mortem, and only serves to confirm that RPN-related end-stage-renal disease was the cause of death. However, the renal papillae are often inadequately sampled and examined since a coronal section of the kidney may only reveal a few papillae.
Clinical features associated with RPN

Early symptoms
Females predominate 3:1 to 8:1; most common 40 to 60 yr; psychiatric abnormalities: immaturity, dependence, emotional instability, anxiety, headaches, introversion and neurosis.
Upper gastrointestinal disease: peptic ulceration of stomach or duodenum and dyspepsia.
Anaemia: gastrointestinal bleeding, haemolysis, iron deficiency and cyanosis.

Intermediate symptoms
Urinary tract disease: bacteriuria, sterile pyuria, nocturia, dysuria, microscopic haematuria, ureteral colic, and lower back pains.
Urinalysis: defect in ability to concentrate and acidify urine, proteinuria, nocturia and uraemia.

Late symptoms
Hypertension
Cardiovascular manifestations, ischaemic heart disease and peripheral vascular disease
Renal calculi and bladder stones
Renal malfunction: decreased GFR, increased BUN, renal tubular manifestation and ESRD
Renal osteodystrophy and bone pain
Carcinoma of renal pelvis
Acute renal failure: especially after renal surgery

Incidence
Very high Up to 86%
Up to 60%
Up to 100%
Up to 100%
Up to 70%
High
High
High
High
High

a: possibly caffeine withdrawal related; b: direct secondary consequence of high dose analgesic intake; GFR: glomerular filtration rate; BUN: blood urea nitrogen; ESRD: end stage renal disease.

Table 2.3 Clinical features associated with RPN.

Lomax-Smith & Seymour, (1980a) recommend taking transverse sections also to expose the hidden papillae (see Figure 3.2, in Methods, section 3.6.4.2). To identify the focal lesions within the papillae it is necessary to take histological sections which include the papilla tip (with ducts of Bellini) in addition to the cortex and outer medulla, this makes the cutting of sections for the diagnosis of RPN very time consuming.

2.1.2.3 The Pathology of RPN in Man.

The pathological course of RPN has been well documented (Gloor, 1978;
Burry et al, 1977; Burry, 1978) and confirms that the lesion is primarily one which begins at the apex or tip of the papilla. Burry (1968) defined these pathological changes in terms of early, intermediate and total RPN.

a). **Early RPN**: is evident as necrotic/degenerative changes to interstitial cell nuclei, loops of Henle and capillaries in the papilla tip. Changes in staining intensity of matrix ground substance of the interstitium have been described; in terms of an increase in intensity (Burry, 1968; Burry et al, 1977) whereas Gloor (1978) reported a loss of this matrix ground substance. Accumulation of lipid material was also evident at this stage in collecting duct cells (see Figure 2.3a).

b). **Intermediate RPN**: occurs when more anatomical elements of the medulla become necrosed as the lesion progresses into the outer medulla, which may show signs of atrophy, sclerosis and inflammatory response. Calcium deposits become extensive in the necrotic tip areas which may lead to bone formation. The papilla is often termed a 'ghost papilla' because of the increasing loss of tissue integrity. The ghost papilla may slough, reside in the pelvic space and be subsequently calcified to become a pelvic calculi, or be excreted and found in the urine. Whether the papilla remains attached or sloughs, a re-epithelialisation at the junction of viable and necrosed tissue frequently occurs which is comprised of transitional epithelium (see Figure 2.3b)

c). **Total RPN**: these changes progress to affect the outer medulla often up to the cortico-medullary junction on a crescentric line. Cortical changes become apparent when the papilla is totally necrosed
Fig 2-3a Analgesic nephropathy: early renal lesions. Schematic representation of the early pathologic lesions of analgesic nephrotoxicity.

PATCHY NECROSIS OF
Interstitial Cells
loops of Henle
Capillaries
BASEMENT MEMBRANE
GROUND SUBSTANCE
COLLECTING DUCTS SPARED

Fig 2-3b Analgesic nephropathy: intermediate renal lesions. Schematic representation of the intermediate pathologic lesions of analgesic nephrotoxicity.

MONONUCLEAR CELLULAR INFILTRATE
OF CORTICO-MEDULLARY JUNCTION

PATCHY NECROSIS OF
Interstitial Cells
loops of Henle
Vasa Recta

PARTIAL NECROSIS OF PAPILLARY TIP
Obliteration of Capillaries, loops of Henle and Collecting Ducts
Atrophic Changes
Calcification

Fig 2-3c Analgesic nephropathy: advanced renal lesions. Schematic representation of the late pathologic lesions of analgesic nephrotoxicity.

HYPERTROPHY OF MEDULLARY RAYS

CORTICAL SCARRING
Interstitial Fibrosis
Tubular Atrophy
Periglomerular Fibrosis
Mononuclear Cell Infiltration

TOTAL NECROSIS AND ATROPHY
OF PAPILLA

Figures 2.3a-c From Eknoyan et al, (1982).
and depends on the degree of fibrosis and sclerosis at the line of sequestration (if the papilla is sloughed). A loss of proximal tubular enzymes are often detectable in the cortex, but these changes only occur late in the development of RPN. Histologically the cortical and medullary changes are characterized as "chronic interstitial nephritis" (see Figure 2.3c). Tubular dilatation, atrophy, basement membrane thickening, sclerosis, fibrosis, inflammatory cell infiltration and vascular degeneration are the typical histological changes associated with chronic interstitial nephritis in the cortex as a consequence of the underlying RPN.

In addition, histological changes in the pelvic, ureteric and bladder urothelium have been reported (Johansson et al, 1976; Gloor, 1978; Mihatsch et al, 1978, 1984; Fourie et al, 1982; Johansson et al, 1986) which supports the hypothesis that RPN and UUC are linked by a common initiating factor of analgesic abuse (see Section 3.3). These changes include thickening of capillary walls (Mithatsch et al, 1978, 1979), sclerosis of lamina propria together with an altered fat and collagen deposition. A range of disordered epithelial states from hyperplasia through to malignancy and tumour growth have been described (Erikson & Johansson, 1976; Johansson et al, 1976; Bengtsson et al, 1978; Lomax-Smith & Seymour, 1980a,b; Hicks, 1980, 1983a,b; Blohme & Johansson, 1981) – these are considered below (see section 2.2).

2.1.3 Animal models of RPN.

2.1.3.1 Spontaneous models of RPN.

RPN occurs spontaneously in animals as a result of a variety of conditions. These include age (Gorer, 1940) and amyloid (Dunn, 1944;
Cornelius, 1970) related changes in mice, and changes that are a consequence of medullary bilirubin deposition (and perhaps other biochemical effects) in the Gunn rat (Axelsen & Burry, 1972; Axelsen, 1973; Henry & Tange, 1982). In addition, systemic candidosis also causes necrosis of the medulla (Tomashefski & Abromowsky, 1981). It has previously been reported that vascular occlusion (Beswick & Schatzki, 1960), ureteral obstruction (Dziukas et al., 1982) and the injection of heterologous serum into rats (Ljungqvist et al., 1966, 1967) also cause RPN. A critical analysis of these data (Bach & Bridges, 1985), however, suggests that the medullary infarct associated with all of these models differs from the chemically induced RPN, and is more comparable to the 'warm ischaemic' renal lesion (Mason & Thiel, 1982). It would still be valuable for these lesions to be more fully studied by histochemical methods, at both the light and ultrastructural levels, to establish the nature of the changes, and where they may be similar to the chemically induced lesion. The long-term feeding of rats with a diet deficient in essential fatty acids (Molland, 1982) also causes RPN, but the widespread degenerative changes in most of the major organ systems makes this a most complex experimental model.

2.1.3.2 Experimental models of RPN.

2.1.3.2.A Analgesic and non-steroidal anti-inflammatory induced RPN.

When analgesics such as aspirin, phenacetin and paracetamol were administered to rats, RPN was often induced, but only after prolonged inappropriately high non-clinical doses (Prescott, 1982). Many months of dosing may be required to induce the lesion (Rosner, 1976) and even then biological variation within the groups resulted in animals being affected to different degrees (Table 2.4). High doses
of these drugs often resulted in fatalities in experimental animals due to extra-renal toxicity such as gastric ulceration and perforation (Kaump, 1966).

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Oral daily dose (mg/kg)</th>
<th>Duration (weeks)</th>
<th>RPN (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Compound analgesics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A + P + C/A + P</td>
<td>280-900</td>
<td>8-72</td>
<td>37.5-100^a</td>
</tr>
<tr>
<td>A + NAPA + C/A + NAPA</td>
<td>500-900</td>
<td>12-72</td>
<td>56.3-100^a</td>
</tr>
<tr>
<td>A + salicylamide + C</td>
<td>840</td>
<td>12-72</td>
<td>53.9</td>
</tr>
<tr>
<td>Antipyrine + P + C</td>
<td>840</td>
<td>12-72</td>
<td>36.4</td>
</tr>
<tr>
<td><strong>Single analgesics</strong></td>
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<td></td>
</tr>
<tr>
<td>Aspirin</td>
<td>200-700</td>
<td>SD</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>10-66</td>
<td>55.0^a</td>
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<tr>
<td></td>
<td>318-500</td>
<td>8-20</td>
<td>33.0-100</td>
</tr>
<tr>
<td>Phenacetin</td>
<td>137-3000</td>
<td>4-36</td>
<td>37.5-80</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>894-3000</td>
<td>8-36</td>
<td>42.9-60</td>
</tr>
<tr>
<td>Antipyrine</td>
<td>1000</td>
<td>12-30</td>
<td>12.5</td>
</tr>
<tr>
<td>Amidopyrine</td>
<td>500-1200</td>
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<tr>
<td>Phenylbutazone</td>
<td>50</td>
<td>SD</td>
<td>Majority</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>SD</td>
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</tr>
<tr>
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<td>10</td>
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<tr>
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<td>75</td>
<td>SD</td>
<td>60.0</td>
</tr>
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<td>12-30</td>
<td>28.6</td>
</tr>
<tr>
<td>Indeacetic acid</td>
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<td>**</td>
<td>+</td>
</tr>
<tr>
<td>Mefanamic acid</td>
<td>100</td>
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<tr>
<td>Flufenamic acid</td>
<td>50-100</td>
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<td>+</td>
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<tr>
<td>Meclofenamic acid</td>
<td>**</td>
<td>**</td>
<td>+</td>
</tr>
<tr>
<td>N-phenylanthranilic acid</td>
<td>**</td>
<td>**</td>
<td>+</td>
</tr>
<tr>
<td>Naproxen</td>
<td>**</td>
<td>**</td>
<td>+^a</td>
</tr>
<tr>
<td>Fenoprofen</td>
<td>40-305</td>
<td>72</td>
<td>+^a</td>
</tr>
<tr>
<td>Sudoxicam</td>
<td>2</td>
<td>**</td>
<td>+</td>
</tr>
</tbody>
</table>

A, aspirin; P, phenacetin; NAPA, paracetamol; C, caffeine; SD, single dose; ** no data; ++, RPN produced, no quantitative data; a, chronic interstitial nephritis.

Table 2.4 Variable incidence of experimental renal papillary necrosis induced by single analgesics, analgesic mixtures and NSAID. From Nanra & Kincaid-Smith, (1986).

For a concise review and discussion of experimental analgesic-associated RPN see Nanra & Kincaid-Smith, (1986). There are reports of RPN occurring in horses (Gunson, 1983; Faulkner et al, 1984), sheep
and cattle (Salisbury et al, 1969) after veterinary administration of
NSAID particularly phenylbutazone, and also after phenothiazine and
flunixin meglumine. In these veterinary reports RPN was exacerbated in
these animals in hot dry weather resulting in a dehydrated state.
The Gunn rat is a mutant Albino strain which lacks the hepatic enzyme
uridine diphosphate glucoronyl transferase resulting in a
hyperbilirubinemia. A spontaneous RPN develops progressively with age
in homozygous rats (Axelsen, 1973; Henry & Tange, 1982). The
incidence of RPN in this strain of rats has been found to increase
with the administration of analgesic (APC) mixtures; 90% in treated
rats compared to 12% in control rats (Axelsen & Burry, 1972, Axelsen,
1975,) see Table 2.5.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Oral daily dose (mg/kg)</th>
<th>Duration (weeks)</th>
<th>RPN (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin</td>
<td>175-1150 SD 250 4</td>
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<td>46.7</td>
</tr>
<tr>
<td>Phenacetin</td>
<td>175-1150 SD 320- 500</td>
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<td>6.7</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>268- 420 4</td>
<td></td>
<td>37.0</td>
</tr>
<tr>
<td>A+P+C</td>
<td>38- 500 1-5</td>
<td></td>
<td>70-100</td>
</tr>
</tbody>
</table>

A, aspirin; P, phenacetin; C, caffeine; SD, single dose

Table 2.5 Experimental renal papillary necrosis in homozygous Gunn

Axelsen (1975) reported that paracetamol produced RPN at a higher
frequency than phenacetin alone. Aspirin alone (Henry & Tange, 1983a)
and NSAID have also been reported to induce a RPN in this strain of

This strain of rat has been proposed as a new reproducible model of
analgesic nephropathy (Henry & Tange, 1983b). However, a number of extra-renal lesions also occur due to the inability of these rats to conjugate bilirubin which makes it inappropriate in the long-term study of NSAID and analgesic associated RPN. Despite this the Gunn rat probably has an important role to play in the study of RPN and factors which affect the initiation of the lesion. Recent studies by Thomas & Tange (1985) have shown the relationship between experimentally induced urinary tract infection (with *Proteus mirabilis*), pyelonephritis and RPN.

### 2.1.3.2.B Non-analgesic chemical induced RPN.

The difficulties with the use of spontaneous animal models of RPN and analgesic/NSAID induced models of the lesion led to the use of alternative approaches using chemicals which had a marked papillotoxic potential although they may not be therapeutically used analgesics/NSAID. The most commonly used chemical agents fall into two categories – i) NSAID analogues and/or metabolites, ii) non-analgesic compounds.

In the first group N-phenylanthranilic acid (NPAA) an analogue of the fenamic acid NSAID has been most extensively studied. (Hardy, 1970a,b, 1974; Hardy & Bach, 1984). A dose related apex limited lesion can be produced by oral gavage administration with 1-2 mmol/kg for 14 consecutive days, diphenylamine gives similar results (Powell et al, 1983). The morphological changes due to NPAA are similar to those reported for analgesic associated RPN with the interstitial cells, microvasculature and loops of Henle affected first (at doses of 0.5-1.0 mmol/kg), with a progressive destruction of the collecting ducts and covering epithelium of the papilla (at doses
of 1.0-2.0 mmol/kg) and the outer medulla and cortex affected (at doses of 3.0-5.0 mmol/kg). There is a loss of the mucopolysaccharide interstitial matrix staining following NPAA (Powell et al, 1983). Functional changes include an increased proteinuria, decrease in urine osmolality, diuresis, decrease in chloride ion (Cl−) excretion (Hardy & Bach, 1984; Powell et al, 1985).

In the non-analgesic group there are two related compounds ethylenimine (EI) and 2-bromoethanamine (BEA) hydrobromide which induce RPN in 24-48 hours after a single dose (Bach & Bridges 1985).

2.1.3.2.C Ethylenimine-induced RPN.

Ethylenimine (EI) has been studied extensively (Mandel & Popper, 1951; Davies, 1968, 1970; Davies et al, 1968; Ham & Tange, 1969; Sherwood et al, 1971; Ellis et al, 1973; Ellis & Price, 1975; Axelsen, 1978a; Davies & Tange, 1982) since it was first used by Levaditi in 1901. It causes a dose related necrosis (Axelsen, 1978a; Davies & Tange, 1982), first affecting the interstitial cells of the papilla tip which then progressed to affect the other fine anatomical elements of papilla and medulla (Ham & Tange, 1969). A number of pertinent functional changes have also been described including marked polyuria, low specific gravity and enzymuria (Mandel & Popper, 1951; Ellis et al, 1973; Ellis & Price, 1975). Unfortunately a number of problems are associated with the use of EI; i) it is a powerful alkylating agent and proven mutagen (Ninan & Wilson, 1969), ii) chemically unstable and may be explosive (Dermer & Ham, 1969) iii) it is no longer commercially available.

2.1.3.2.D 2-Bromoethanamine hydrobromide-induced RPN.

Oka (1913) showed that 2-bromoethanamine (BEA) hydrobromide produced lesion that was histologically almost indistinguishable to that
induced by EI. BEA has largely replaced EI as the model optimal papillotoxin for the following reasons: i) it is commercially available, ii) is a stable, water soluble crystalline solid material (though unstable in solution - light sensitive). BEA-induced RPN is dose related (Bach et al, 1983) and the lesion has been characterised in a variety of species and strains of laboratory animal (see Table 2.6).

The route of administration of BEA alters the intensity of the lesion relative to the dose. Intravenous administration produces the most intense response with intraperitoneal and subcutaneous injection being approximately equal, but with oral dosing producing the weakest response (Burnett, 1982; Bach et al, 1983). In vitro BEA cyclizes to EI under strongly alkaline conditions (Dermer & Ham, 1969), but although this has been proposed as a mechanism of BEA-induced RPN (Murray et al, 1972) there is no evidence to support this by urinary excretion of EI (Bach & Gregg, 1988; Bach & Gregg, 1990).

The choice of an animal model system for the investigation of a human clinical disease condition has to fulfill certain criteria. The generally accepted axiom in experimental medicine is that "the greater the concurrence between the pathophysiology of a model lesion and that which occurs in humans the more likely will experimental observations be related to an interpretation of clinical significance" (Bach & Hardy, 1985).

Table 2.7 shows the comparison of pathological changes in experimentally induced RPN and the analgesic induced lesion in humans to be very strong and correlates well.

The greatest advantage attributed by the use of BEA is the rapid
<table>
<thead>
<tr>
<th>Species/strain</th>
<th>Reference.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donjru</td>
<td>Murray &amp; von Stowasser, (1976)</td>
</tr>
<tr>
<td>Munich-Wistar</td>
<td>Reineck et al, (1980)</td>
</tr>
<tr>
<td>MICE Tatonic Farm</td>
<td>Thiele, (1974)</td>
</tr>
<tr>
<td>Balb/c )</td>
<td>Scarlett et al, (1990)</td>
</tr>
<tr>
<td>C57Bl/6) - Obese )</td>
<td>Gregg &amp; Bach, (1989)</td>
</tr>
<tr>
<td>HAMSTER Syrian</td>
<td>Carlton &amp; Englehardt, (1989)</td>
</tr>
<tr>
<td>PIG Large White</td>
<td>Gregg et al, (1989)</td>
</tr>
</tbody>
</table>

**Table 2.6** List of published papers which used BEA to induce an acute RPN in different species and strains.
<table>
<thead>
<tr>
<th>Morphological and functional changes</th>
<th>Type of renal papillary necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acutely induced (BEA and EI)</td>
</tr>
<tr>
<td></td>
<td>Early*</td>
</tr>
<tr>
<td>Primary site of lesion</td>
<td>Yes</td>
</tr>
<tr>
<td>Loop of Henle and interstitial cells</td>
<td>Yes</td>
</tr>
<tr>
<td>Intervening cells</td>
<td>Yes</td>
</tr>
<tr>
<td>To include outer medulla but not beyond medullary junction</td>
<td>No</td>
</tr>
<tr>
<td>Occlusion</td>
<td>No</td>
</tr>
<tr>
<td>Concentrating mechanism</td>
<td>Yes</td>
</tr>
<tr>
<td>Collecting ducts and covering epithelia</td>
<td>Yes</td>
</tr>
<tr>
<td>Staining of extracellular matrix</td>
<td>Yes</td>
</tr>
<tr>
<td>Injury response</td>
<td>Yes</td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>Yes</td>
</tr>
<tr>
<td>Re-epithelialization</td>
<td>Yes</td>
</tr>
<tr>
<td>Bladder calculi</td>
<td>Yes</td>
</tr>
<tr>
<td>Ureter infection</td>
<td>Yes</td>
</tr>
<tr>
<td>Ureter of transitional urothelia of pelvis, ureter, and bladder</td>
<td>No data</td>
</tr>
<tr>
<td>Renal defect</td>
<td>Yes</td>
</tr>
<tr>
<td>Total or Late*</td>
<td>Yes</td>
</tr>
<tr>
<td>Increased cortical changes overlying necrosed papilla, for ex. scarring</td>
<td>Yes</td>
</tr>
<tr>
<td>Dilatation</td>
<td>Yes</td>
</tr>
<tr>
<td>Ureter of transitional urothelia of pelvis, ureter, and bladder</td>
<td>No</td>
</tr>
<tr>
<td>After salt loading or arterial clipping</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 2.7 Comparison of pathological changes in experimentally induced RPN and the lesions found in humans. From Bach & Hardy, (1985).

Induction of a dose related reproducible RPN within 48 hr. The tremendous reduction in time needed to produce this lesion, compared to those induced using analgesics, NSAID or their analogues, (which may take weeks or months), has several logistically important advantages. Also BEA affects the medulla only first, whereas analgesics, NSAID often affect the cortex, stomach and spleen.
Table 2.7 Comparison of pathological changes in experimentally induced RPN and the lesions found in humans. From Bach & Hardy, (1985).

induction of a dose related reproducible RPN within 48 hr. The tremendous reduction in time needed to produce this lesion, compared to those induced using analgesics, NSAID or their analogues, (which may take weeks or months), has several logistically important advantages. Also BEA affects the medulla only first, whereas analgesics, NSAID often affect the cortex, stomach and spleen.
2.1.3.2.D.i Functional changes associated with BEA.

There are marked similarities between the renal functional changes and the pathomorphological progression of the lesion following BEA administration and those reported for the analgesics associated lesion in both experimental animals and man (Bach & Bridges, 1982; Bach & Bridges, 1985; Bach & Hardy, 1985). Prominent among these is the loss of urinary concentrating ability (Fuwa & Waugh, 1968; Wyllie et al, 1972; Murray et al, 1972; Shimamura, 1976; Sabatini et al, 1981, 1983; Vanholder et al, 1981; Bach et al, 1983), loss of nephron function and severe cortical degeneration (Sabatini et al, 1981, 1983; Bach & Bridges, 1982).

An increased medullary plasma flow 6hr after BEA administration has been reported by Solez et al, (1974) and an increased filling of the medullary microvasculature using colloidal carbon (Bach et al, 1983). Sabatini et al, (1981) reported a decrease in the number of filtering and thus presumably functioning juxtamedullary nephrons assessed by the Hansen technique after BEA induced RPN.

Immunohistochemical demonstration of Tamm-Horsfall glycoprotein (THG) by Bach & Bridges, (1982) after BEA-induced RPN showed a total loss from distal tubules and accumulation in collecting ducts and ducts of Bellini. The loss of THG corresponded with onset of early polyuria. Loss of other urinary electrolytes (Na\(^+\), Cl\(^-\), PO\(_4\)\(^{2-}\) and Ca\(^{2+}\)) has also been reported following BEA-induced RPN (Arruda et al, 1979; Sabatini et al, 1981). The measurement of urinary acidification capacity and electrolyte handling could therefore offer a simple way of monitoring for RPN in toxicology screening programs. Arruda et al, (1979) however failed to find any differences between control and BEA treated...
rats studied 24 hr after dosing, and Sabatini et al., (1982) found neither an acidosis nor a defective urinary acidification in response to ammonium chloride loading one month after dosing with BEA. Wilks et al., (1986) studied the renal functional changes within 3 hr of BEA administration. Their data showed that the excretion of urea increased (from 30 min) osmolality decreased from 90 min and sodium excretion increased at 3 hr, but potassium excretion was unchanged. Glomerular filtration rate and the clearance of the organic ion p-aminohippurate decreased over the period of investigation. These data reflect early functional abnormalities, and suggest that BEA may be less target selective for the medullary interstitial cells than has previously been thought. Alternatively, these changes may represent aspects of the complexity in the renal response to injury. They may reflect a rapid homeostatic consequence caused by the perturbation of the medullary cells.

Recently the application of $^1$H-NMR spectroscopy to urine samples from animals treated with BEA has elucidated some metabolic disturbances which are unique to RPN (Gartland et al, 1989). There was an early elevation of trimethylamine N-oxide and dimethylamine, then a later increase in acetate and succinate occurs together with a minor increase in the excretion of lactate and alanine 24-48 hr after the administration of BEA which may herald secondary cortical changes.

2.1.3.2.D.ii Histopathological changes associated with BEA.

The histopathological changes induced by BEA during development of RPN have been described at the light microscopic level (Murray et al, 1972; Axelsen, 1978b; Bach et al, 1983; Mattingley et al, 1985) and the ultrastructural level (Shimamura, 1972; Hill et al, 1972, Sabatini et al, 1982). These changes follow the same pattern of early,
intermediate and total RPN described by Burry, (1968) and are dose and time dependant. With low doses <50 mg/kg in female and male Wistar rats, interstitial cells, loops of Henle and blood vessel walls were necrosed corresponding to 'intermediate' RPN. At higher doses of 100 mg/kg collecting duct epithelia and cuboidal covering epithelium undergo necrosis too, resulting in 'total' RPN. The lesion frequently extended into outer medulla with papilla tip becoming a sloughing 'ghost' with re-epithelialisation of exposed viable tissue after 5 days (Axelsen, 1978b). Histochemical staining changes in interstitial matrix of papilla occurs with BEA-induced RPN (Bach et al, 1983). At 2 and 4 hr an increased intensity with pronounced granular appearance of the matrix at the papilla tip. These changes were more marked at 8 hr and at 12-24 hr areas of intermediate necrosis coincided with a decrease in matrix staining in papilla tip. Casts within lumen of necrosed collecting ducts and loops of Henle showed extensive deposition of mucopolysaccharides (assessed by colloidal iron staining). The medullary interstitial cells have a very high lipogenic potential and contain numerous lipid droplets rich in long-chain polyunsaturated fatty acids (Bojesen, 1974). Oil red O stains the lipid droplets in these cells heavily, but not other parts of the kidney (Bach & Gregg, 1988) ORO positive lipid material accumulates in kidneys of analgesic abusers (Munck et al, 1970; Burry et al, 1977; Burry, 1978) and similar changes occur in aspirin-induced (Molland, 1976) and essential fatty acid-deficient diet-induced RPN (Molland, 1982). Recent studies have shown that in an acutely induced papillary necrosis, early lipid changes take place in the capillaries followed by a marked accumulation of lipid in the epithelial cells.
Normally there is no Oil red O positive lipid material in these cells. The epithelial accumulation of lipid material extends into those areas of the outer medulla which were not affected by the papillotoxin and appeared to be normal by routine H&E staining (Bach et al, 1987). Other chemically induced lesions, such as those caused by hexachlorobutadiene, aminoglycosides, cis-platin and polybrene, do not produce these Oil red O lipid changes (Bach et al, 1987), which suggests that the capillary and epithelial deposits of lipid material may be pathognomonic of RPN.

Biochemically the lipid changes in the BEA-induced RPN represent a phospholipidosis, in which phosphatidyl- and lysophosphatidylcholine, -inositol and -ethanolamine were increased (Duffy and Bach, unpublished data). The increase in urinary free polyunsaturated fatty acids with C18, C20 and C22 suggests a large precursor pool for the synthesis of eicosanoid related products (Scholey and Bach, unpublished data). These changes are histochemically the same as those reported by Burry, (1978) and Gloor, (1978) in man. Hyperplasia of the pelvic and ureteric urothelium after BEA administration has also been reported (Bach & Bridges, 1985; Mattingley et al, 1985).

2.2 UPPER UROTHELIAL CARCINOMA (UUC).

2.2.1 Introduction.

Upper urothelial carcinoma (UUC) is a pathological condition which has been associated with RPN and analgesic abuse. This relationship was first reported by Hultengren et al (1965), subsequently Johansson et al, (1974) reported a 92% association between RPN, UUC and mixed analgesic abuse involving phenacetin, phenazone and caffeine. Since...
then epidemiological evidence has shown a strong association between RPN and UUC particularly in those countries such as Switzerland, Scandinavia and Australia where analgesic abuse is more common. (Bengtsson et al, 1968, 1978; Dubach, 1971; Johansson et al, 1974, 1976; Mahony et al, 1977; Mihatsch et al, 1979; Mihatsch & Knusli, 1982; McCredie et al, 1982a,b, 1983a), often after a latent period of 10-20 years after initiating analgesic abuse (Bach & Bridges, 1985). As yet there is no proof for a "cause-and-effect" relationship between analgesic abuse associated RPN and UUC (Bach & Bridges, 1985). Patients who discontinue the abuse of analgesic generally allay renal failure and stabilise (Bell et al, 1969; Dubach, 1975). These patients are, however, the ones who may, due to their prolonged survival time be at a greater risk of developing UUC. Greatly improved dialysis techniques have meant the survival of analgesic abusing patients who would otherwise have developed end stage renal disease, and subsequently died (Mihatsch et al, 1980). It has therefore been suggested that the incidence of UUC will increase.

2.2.2 Incidence of UUC.

Urothelial carcinoma is one of the class of epithelial malignancies which accounts for 90% of deaths due to carcinoma. (Bach & Bridges, 1985). UUC in the general population accounts for a relatively limited proportion of the total number of human malignancies with a background incidence of between 1:156,000-180,000 in Sweden (Bengtsson et al, 1978). UUC has usually been described as a predominantly male disease with a ratio of 2:1. In contrast the incidence of UUC among analgesic abusers is as high as 92% (Johansson et al, 1974) and females predominate in analgesic-associated UUC with
a ratio of 2.5 to 1 (Bengtsson et al., 1978), in keeping with the predominantly female ratio of analgesic abusers. In addition, analgesic abusers develop UUC at a younger age than non-analgesic abusers (Mihatsch & Knusli, 1982). The distribution of urothelial carcinomas in analgesic abusers also has a distinct pattern compared to non-analgesic abusers. Tumours of renal pelvis, ureter and bladder are respectively induced 77 times, 89 times and 7 times more frequently, than non-analgesic abusers. Also the tumours are typically multiple, diffuse, poor differentiated and rapidly spreading (Lomax-Smith & Seymour, 1980a,b; Mihatsch & Knusli, 1982). Typically UUC occurs following a latency of 20 - 30 years from the time the individual started to abuse analgesics (Bengtsson et al., 1978). However as pointed out in section 2.2.1 patients who discontinue to abuse analgesics frequently develop UUC. This fact confuses the already difficult diagnosis of UUC with patients having a stable renal function and not being documented as previous analgesic abusers (Bach & Bridges, 1985).

There are a number of well documented factors in addition to analgesic consumption which affect the incidence of UUC including cigarette smoking (McCredie et al., 1983b). Recently Ross et al (1989) performed a population-based case-control study by telephone interview on how analgesic consumption, cigarette smoking and caffeine consumption posed risk factors for the induction of UUC. The major risk factor was found to be cigarette smoking with a relative risk factor of 4.5, compared to nonsmokers. Mixed analgesic compounds (aspirin, acetaminophen and caffeine) had a relative risk factor of 2.0 for subjects who used the compounds for periods of 30 consecutive days or more. Caffeine alone was found to have a relative risk factor of 1.8
which dropped to 1.3 if the contribution of smoking was removed. Other factors which had identifiable risks, but were not statistically significant were: 1.5 for alcohol consumption (2 drinks/day), 1.6 for urinary tract stones, 1.2 for urinary tract infection. This latter factor has also been reported by Johansson & Walhqvist, (1977) who found that 80% of patients presenting with UUC had an association with recurrent urinary tract infection. Ross et al, (1989) also looked at other commonly used drugs and found that thyroid hormone supplementation and Milk of Magnesia presented a minor risk. The finding that thyroid hormone supplementation presents a small but identifiable risk is interesting because these compounds have also been found to induce RPN experimentally in rats (Kennedy & Jones, 1989).

RPN is not the only disease condition associated with UUC; the highest incidence of pelvic tumours (1:200) is in those patients with Balkan nephropathy (BEN) in certain endemic areas of Yugoslavia, Bulgaria and Romania (Chemozemsky et al, 1977; Sattler et al, 1977). Clinically BEN represents a slow progressive renal degeneration including anaemia, concentrating defect, proteinuria and ureamia, leading to end stage renal disease, which occurs more commonly in females than males. Pathologically tubular and/or glomerular lesions are the earliest changes and cortical tissue loss is greatest opposite the hilum (Hall & Dammin, 1978). Thus clinically BEN is similar to RPN, but pathological early changes of BEN are only evident (usually) in late stages of RPN.

Various environmental factors such a fungal toxin, plant toxins have been implicated in BEN (Krogh & Elling, 1976; Krogh, 1978; Austwick,
1983), together with a high familial incidence which may suggest a genetic disposition or susceptibility to cancer. This "cancer family syndrome" with transitional cell carcinoma of the renal pelvis has been reported in USA (Frischer et al, 1985; Orphali et al, 1986). Although a genetic factor has not been implicated in analgesic-associated RPN, it cannot as yet be discounted as a possible influential factor. It may be that a pathological/metabolic change common to BEN and RPN could result in the alterations in the urothelia environment which initiates the onset of UUC.

2.2.3 Clinical Symptoms and Diagnosis of UUC.

The diagnosis of UUC is difficult in the clinical situation because UUC (as with RPN) progresses silently with few clinical symptoms to indicate the early development of the malignant changes in the ureter and pelvis (Bengtsson et al, 1968, 1978; Dubach et al, 1971; Johansson et al 1974, 1976; Mahony et al, 1977; Mihatsch et al, 1979, 1982; Bach & Bridges, 1985). The main symptom is usually a haematuria, occasionally accompanied by pain in the flank (Nielsen & Ostri, 1988).

Diagnosis is more likely where analgesic abuse is common and the patient is being monitored for the condition and receiving treatment. Retrograde ureteropyelography has been used to diagnose 69.5% of ureteral carcinoma and 82% of pelvic carcinoma in a retrospective study of 40 patients (Oschner et al, 1974). Urinary cytology can be a useful diagnostic technique; Eriksson & Johansson (1976) found a 83% predictive correlation between histological findings and cytology with 94% of most advanced tumour grades being identified as being advanced malignancies. Computerised tomography is another diagnostic
which has been shown to be advantageous over radiographic procedure (Gatewood et al, 1982; Nielsen & Ostri, 1988). New techniques such as nuclear magnetic resonance imaging and ultrasound scanning may, in the future, improve the early diagnosis of UUC. Recently, DNA ploidy by flow cytometry has been used retrospectively to identify abnormal DNA nuclear patterns in low grade tumours (which normally would be associated with good prognosis) which seem to indicate poor prognosis (Blute et al, 1988).

The urothelium of renal pelvis, ureter and bladder are all derived from the same embryological structure; the ureteric bud. They have a similar histological appearance although they do differ in thickness (Melicow, 1945) and also in cell surface characteristics (Newman and Hicks, 1981). It is therefore common to grade UUC tumours at the gross and histological levels on the same basis as is internationally used for bladder carcinoma (Berquikst et al, 1965; Mostofi et al, 1973; Helpap et al, 1985).

2.2.4 Treatment of UUC.

The prognosis for the patients with UUC is poor, and only have a mean survival time of 22 months (Mihatsch et al, 1980) due to difficulty in diagnosis, compromised renal function of patients with RPN (Clark, 1961) and multifocal sites of invasion and metastases (Johansson et al, 1974, 1976). The survival rate appears to be dependant on the grade of tumour and degree of invasion, with patients with low grade tumours generally having a better survival rate (Nielsen & Ostri, 1988). UUC does not respond readily to chemo- or radio-therapy (Johansson & Wahlquivst, 1977), and re-occurrence with invigorated metastases often occurs after surgery (Hultengren et al, 1965;
Johansson et al, 1976). Blohme & Johansson (1981) recently suggested the radical surgical procedure of prophylactic bilateral nephroureterectomy, but the survival rate was only 51% after 2 years in a series of surgically treated patients reported by Mahony et al (1977). Other studies recommend the use of conservative surgical measures, however, only patients who have a good prognosis (those with low grade tumours) should be considered for conservative parenchymal sparing surgery (Reitelman et al, 1987).

2.2.5. Experimentally induced UUC.

Various animal experiments have been performed to elucidate whether the relationship between analgesic consumption, RPN and UUC in particular does phenacetin induce tumours in the urinary tract. Urothelial hyperplasia and microvasculature changes were the most prominent morphological changes (87%) in female Sprague-Dawley rats fed on 0.535% phenacetin in their daily diet for 96-110 weeks (Johansson & Angervall, 1976a,b). These changes have been associated with RPN in human situation (Burry et al, 1978; Mihatsch et al, 1979; Mihatsch & Knusli, 1982) Animals fed 2.5% phenacetin in their daily diet for 18 months developed transitional cell hyperplasia and UUC with a ratio of 3:1 male to female (Isaka et al, 1979). Similar experiments in B6C3F1 mice failed to induce RPN or pelvic tumours, but did increase bladder hyperplasia (Nakanishi et al, 1982).

Mixed analgesics were implicated in development of RPN (Spuhler & Zollinger, 1953) thus Johansson (1981) studied the effects of co-formulated phenacetin, phenazone, caffeine and paracetamol. Phenacetin and phenazone either alone or in combination induced renal pelvic tumours and bladder tumours. Mixtures of phenazone or
phenacetin with caffeine induced comparable numbers of bladder tumours and a lower number of pelvic tumours. Paracetamol alone induced comparable numbers of bladder tumours and 3/13 rats with pelvic tumours were found to have RPN also. Many other tumour types were also induced of the phenacetin studies including ear, nasal, liver mammary bladder and renal parenchymal tumours (Isaka et al, 1979; Johansson, 1981). Thus phenacetin does not on its own or in combination appear to produce a target-specific carcinogenic metabolite.

Phenacetin is an aromatic amide with N-hydroxylated or N-nitrosated metabolites which are potent carcinogens (Nery, 1971; Lijinsky, 1984). The wide spectrum of possible N-nitroso compounds produced by phenacetin or phenacetin containing mixtures would explain this wide range of tumour target sites. Ham & Calder (1983) found that phenacetin was carcinogenic but paracetamol was not, they also suggested that the metabolism of phenacetin to N-hydroxyphenacetin was the reason for the carcinogenicity of phenacetin. A 2 year feeding study with paracetamol by Hiragi & Fuji, (1985) also found that paracetamol was not carcinogenic. Nakanashi et al, (1978) dosed rats with N-butyl-N-(4-hydroxybutyl)nitrosamine together with phenacetin and caffeine either alone or in combination. A significantly higher frequency of bladder tumours was induced in the phenacetin alone group and papillary hyperplasias were observed in the pelvis. Table 2.8 shows the incidence of experimentally induced urothelial hyperplasias and tumours using analgesics and NSAID. Patierno et al (1989) have investigated in vitro the cytotoxicity, mutagenicity potential of phenacetin, acetaminophen and aspirin in
### Table 2.8 Experimental uroepithelial hyperplasia and tumours induced by single analgesics and analgesic mixtures. From Nanra & Kincaid-Smith, (1986).

C3H/10T1/2 Clone 8 mouse embryo cells. Both acetaminophen and phenacetin induced a low but dose-dependant number of morphologically transformed foci. None of the compounds caused base substitutions to ouabain resistance at highly toxic concentrations. Patierno et al, (1989) suggest that if acetaminophen and phenacetin are carcinogenic in humans and animals then other cocarcinogenic factors are required and/or metabolism to carcinogenic metabolites.

#### 2.2.6 Relationship between RPN and UUC.

Although the relationship between RPN and UUC has not been proven there is substantial clinical and experimental evidence connecting the abuse of analgesics and the development of urothelial tumours (Johansson & Angervall, 1976; Bengtsson et al, 1978; Bach & Bridges, 1985). Phenacetin has been implicated in the induction of urothelial
malignancies from studies of Hultengren et al (1965) described renal pelvic tumours in six patients five of whom were known to abuse phenacetin containing drugs. In an autopsy study covering 25 years 8.6% of phenacetin abusers had urothelial tract tumours which was statistically significant compared to control groups (Mihatsch & Knusli, 1982). Compounds containing phenacetin, phenazone and caffeine were found to produce renal pelvis and bladder tumours (Bengtsson et al, 1968; Angervall et al, 1969).

The progression of hyperplasia, through dysplasia to malignancy is now widely accepted; a series of changes in the development of carcinoma in epithelial cells in a number of different organs (Farber, 1976; Sporn, 1976). Such events may occur in the genesis of analgesic-associated carcinoma. There are well documented foci of hyperplastic ureteric epithelia, in addition to malignancies in patients with UUC (Lomax-Smith & Seymour, 1980a,b) and in analgesic abusers with RPN, but no diagnosed malignancies (Blohme & Johansson, 1981).

Urothelial hyperplasia frequently occurs as a secondary associated morphological change as a consequence of RPN and has been observed in animals given N-phenylanthranilic acid (Hardy, & Bach, 1984), aspirin (Molland, 1976), BEA (Bach & Bridges, 1985; Bach & Gregg, 1988) and L-triiodothyronine (Kennedy & Jones, 1989) but there was little published on how this hyperplasia could progress to upper urothelial tumours in the long-term or with repeated dosing. Using these compounds in conjunction with chemical carcinogens it may be possible to develop "models" of UUC.

These data taken in the light of the already very established high incidence of UUC in analgesic abusers (Bengtsson et al, 1968, 1978).
and the strong association between RPN and UUC (McCredie et al., 1982a,b, 1983a) highlights the possibility of these proliferative changes in the pelvic and ureter epithelial cell are premalignant.

2.3 CHEMICALLY INDUCED CARCINOGENESIS.

2.3.1 Introduction to Carcinogens and Carcinogenesis.

The literal definition of carcinogenesis is the development of cancer or carcinoma (which is a specific type of cancer). A more complete definition is that of Friedberg (1985) who defines the carcinogenic process as: "Neoplasia is a process by which the normal controlling mechanisms that regulate cell growth and differentiation are impaired, resulting in progressive growth".

The majority of human cancers are suspected to be caused by "environmental factors" (Williams & Weisburger, 1986). This term is fairly loosely defined in this context, and may apply to a wide range of agents encompassing the food chain, geographical location, social habits, (e.g. smoking, drug abuse), therapeutic agents and occupational exposure to hazardous chemicals and/or products. Chemicals are the common denominator that underly the majority of these environmental factors, occurring naturally in the environment and diet, or synthetically as specific products (see Table 2.9).

For a basic comprehensive introduction to some of the pertinent areas in carcinogenesis see Becker, (1982); Friedberg, (1985); Williams & Weisburger, (1986) and Tannock & Hill, (1988).

2.3.2 Mode of action of carcinogens.

Chemical carcinogens are defined operationally by their ability to induce tumours. Evidence of tumourigenicity have been classified by four responses as follows:
Table 2.9

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Origin</th>
<th>Exposure</th>
<th>Type of Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>Synthetic</td>
<td>Occupational</td>
<td>Marrow (leukemia)</td>
</tr>
<tr>
<td>2-Naphthylamine</td>
<td>Synthetic</td>
<td>Occupational</td>
<td>Bladder</td>
</tr>
<tr>
<td>Oestrogens</td>
<td>Natural</td>
<td>Hormonal</td>
<td>Breast, uterus</td>
</tr>
<tr>
<td>Diethylstilbestrol</td>
<td>Synthetic</td>
<td>Drug consump.</td>
<td>Vagina, breast</td>
</tr>
<tr>
<td>Androgens</td>
<td>Natural/</td>
<td>Hormonal/drug</td>
<td>Liver</td>
</tr>
<tr>
<td>Anabolic steroids</td>
<td>Synthetic</td>
<td>consumption</td>
<td></td>
</tr>
<tr>
<td>Polycyclic hydro</td>
<td>Synthetic</td>
<td>Occupational</td>
<td>Skin, scrotum</td>
</tr>
<tr>
<td>carbon in tar, soot</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>and oil</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polycyclic hydro</td>
<td>Synthetic</td>
<td>Social -</td>
<td>Lung</td>
</tr>
<tr>
<td>carbon in</td>
<td></td>
<td>smoking</td>
<td></td>
</tr>
<tr>
<td>tobacco smoke</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aflatoxins</td>
<td>Natural</td>
<td>Food consump.</td>
<td>Liver</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>Natural</td>
<td>Food consump.</td>
<td>Colon, breast</td>
</tr>
</tbody>
</table>

Data from Doll, (1977).

1. An increased incidence of the tumour types compared to controls
2. The earlier occurrence of tumours than in controls
3. The development of types of tumours not seen in controls
4. An increased multiplicity of tumours in individual animals

Carcinogens interact with numerous tissue constituents and produce a number of effects. A number of considerations derived from a vast body of experimental evidence supports the view that DNA is a critical target of carcinogens (see Table 2.10), either by direct (genotoxic) interaction with molecules of DNA, or by indirect (nongenotoxic or
epigenetic) mechanism through cellular protein or RNA interaction
(Williams & Weisburger, 1986).

1. Many carcinogens are or can be metabolized to electrophiles that react covalently with DNA.
2. Many carcinogens are also mutagens
3. Defects in DNA repair such as in xeroderma pigmentosum predispose to cancer development
4. Several heritable or chromosomal abnormalities predispose to cancer development
5. Initiated dormant tumour cells are persistent, consistent with a change in DNA
6. Cancer is heritable at the cellular level and, therefore, may result from an alteration of DNA
7. Most if not all, concern display chromosomal abnormalities
8. Many concern display aberrant gene expression

Table 2.10: Considerations indicating that DNA is a critical target for carcinogens. From Weisburger & Williams, (1986).

Chemical carcinogens can be classified according to their proposed mode of action which result in eight classes (see Table 2.11), but they fall into 2 broad classes according to whether they are genotoxic or nongenotoxic.

Genotoxic carcinogens comprise those chemicals that function as electrophilic reagents and alkylate DNA and alter directly the process of DNA replication.

Nongenotoxic carcinogens comprise of those substances for which there is no evidence for a direct interaction with DNA. Their carcinogenic effects often occur only with high and sustained levels of exposure that lead to prolonged physiological abnormalities, tissue injury or hormonal imbalances, e.g. changes in cellular processes and
Structures may (through the action of nongenotoxic carcinogens) lead to the onset of carcinogenesis in the cell.

<table>
<thead>
<tr>
<th>Type</th>
<th>Mode of action</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotoxic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Direct-acting or primary</td>
<td>Electrophile, organic compound, genotoxic, interacts with DNA.</td>
<td>Ethyleneimine, bis(chloromethyl)ether</td>
</tr>
<tr>
<td>carcinogen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Procarcinogen or secondary</td>
<td>Requires conversion through metabolic activation by host or in vitro to type 1</td>
<td>Benzo(a)pyrene, 2-naphthylamine, dimethylnitrosamine</td>
</tr>
<tr>
<td>carcinogen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 Inorganic carcinogen</td>
<td>Not directly genotoxic, leads to changes in DNA by selective alteration in fidelity of DNA replication</td>
<td>Nickel, chromium</td>
</tr>
<tr>
<td>Epigenetic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 Solid-state carcinogen</td>
<td>Exact mechanism unknown; usually affects only mesenchymal cells and tissues; physical form vital</td>
<td>Polymer or metal foils asbestos</td>
</tr>
<tr>
<td>5 Hormone</td>
<td>Usually not genotoxic; alters endocrine system balance and differentiation; often acts as promoter</td>
<td>Oestradiol, diethylstilbestrol</td>
</tr>
<tr>
<td>6 Immunosuppressor</td>
<td>Not usually genotoxic; mainly stimulates &quot;virally induced&quot; transplanted or metastatic neoplasms</td>
<td>Azathioprine, antilymphocytic serum</td>
</tr>
<tr>
<td>7 Cocarcinogen</td>
<td>Not genotoxic or carcinogenic enhances effect of type 1 or type 2 agent when given at same time, may modify type 2 conversion to type 1</td>
<td>Phorbol esters, pyrene catechol, ethanol, SO₂ n-dodecane</td>
</tr>
<tr>
<td>8 Promoter</td>
<td>Not genotoxic or carcinogenic but enhances effect of type 1 or type 2 agent when given subsequently</td>
<td>Phorbol esters, phenol anthralin, bile acids tryptophan metabolites saccharin</td>
</tr>
</tbody>
</table>

Table 2.11 Classes of carcinogenic chemicals. From Weisburger & Williams, (1986).

Alterations in surface membrane recognition antigens that prevent autonomous growth due to contact inhibition may be changed so that the
cell continues to grow despite encroaching on its neighbours. (Lock-Caruso & Trosko, 1985). Switching on the cell cycle of growth mitosis by removing 'stop' chemicals (chalones) or allowing oncogenes to exert their effect are all potential modes of action by nongenotoxic carcinogens.

Pre-neoplastic changes (ie changes which occur in cell or tissue which are indicative of transformation to a neoplastic state later) have been recorded and include cellular hyperplasia (Farber & Sporn, 1976), functional changes indicative by loss of enzymes (Tsuda et al, 1985) and an increase in mitotic index (Sporn, 1976).

2.3.3 Experimental Carcinogenesis.

2.3.3.1 Justification for use of animals.

At present there seems to be no alternative to the use of animals as a means of screening for the long-term chronic exposure hazard of chemicals. The short-term in vitro assays using bacteria or mammalian cell lines may help initially to screen out some compounds for mutagenic potential. However, there are some limitations to these in vitro assays which make them inappropriate to long-term studies and extrapolation of the results to the in vivo and the clinical situation. Controversy exists as to whether there is any meaningful relationship between short-term tests as against long-term chronic studies. This is very relevant to the whole question of using animals to test for the carcinogenesis potential of chemicals. The fact that rats and mice have more similarities than differences to humans in terms of anatomy, physiology and biochemistry would seem to satisfy the assumption that one is justified in extrapolating from rodent species to man.
The purpose of carcinogenicity studies are to screen chemicals or other exposures (radiation) for carcinogenicity potential, elucidate the mechanism of known cancer-causing agents and are frequently performed as part of risk assessment to satisfy legislative requirements on new chemicals and drugs (IARC, 1986). Scientists are compelled to use experimental animals to screen for potential human carcinogens since the vast array of existing (and continual development of new) chemicals coupled with the longevity of man means a database derived from environmental and occupational exposure to these agents could never be completed (Moore, 1988).

Certain properties of tumours can only be studied in animals, for instance, metastasis is as common a cause of death in humans as primary cancers and at present the study of tumour metastasis is only possible in experimental animals (Lijinsky, 1988). Time course studies in animals allow the study of progression of the tumour, its biological and behavioural properties. Animal experiments also allow the study of multiple exposure to different compounds to examine additive, synergistic or antagonistic interactions. Such experiments are difficult to perform adequately but probably are very relevant to carcinogenesis processes occurring in humans exposed to multiple potential carcinogens in their occupation or environment (Lijinsky, 1988). Short term in vitro assays by their very nature preclude against chronic multiple chemical exposure. They are, however, useful in providing mechanistic information about the carcinogenic process at the cellular level (Moore, 1988). Laboratory animals can predict human carcinogenicity, this has been shown by the years of toxicological testing and health and safety screening. Of the 23
chemicals classified by IARC as definite human carcinogens (see Table 2.12), 21 of these chemicals have produced cancer in animals with 18 causing cancer in an identical site to that seen in humans (Moore, 1988).

1. 4-aminobiphenyl
2. analgesic mixtures containing phenacetin
3. arsenic and arsenic compounds
4. asbestos
5. azathioprine
6. benzene
7. benzidine
8. N,N-bis(2-chloroethyl)-2-napthylamine (Chlornaphazine)
9. bis(chloromethyl)ether and technical-grade chloromethyl-methyl-ether
10. 1,4-butanediol dimethanesulphonate (Myleran)
11. certain combined chemotherapy for lymphomas
12. chlorambucil
13. chromium and certain chromium compounds
14. conjugated oestrogens
15. cyclophosphamide
16. diethylstilboestrol
17. melphalan
18. methoxsalen with ultra-violet A therapy
19. mustard gas
20. 2-napthylamine
21. soots,tars and oils
22. treosulphan
23. vinyl chloride

Table 2.12 Twenty-three chemicals identified as definite human carcinogens. From IARC, (1982).

It is possible to compare the potency of carcinogens with similar target organs and to determine species differences in their response to certain carcinogens (Lijinsky, 1988). Species differences can be very useful in providing a means to identify mechanisms by comparing the differing metabolic pathways within cells/tissues/organs in susceptible and resistant species (Rice & Frith, 1981). There are many other aspects of carcinogenesis; role of genetics, age, sex, diet, hormones, homeostasis, and environment which can only be studied in experimental animals (Lijinsky, 1988).
2.3.3.2 Practical criteria for experimental carcinogenesis.

2.3.3.2.A Choice of Species.

The purpose of a long-term carcinogenicity experiment is to determine if administration of a test substance to animals alters the normal pattern of tumour development in that species (IARC, 1986). Accordingly the primary criteria in determining the choice of test animal is that there should be a low spontaneous rate of tumour induction. Since a low spontaneous incidence means a small increase in tumour development will be statistically significant (IARC, 1986). Economy and the short natural life span of the rat and mouse (2-3yrs) as against the dog (9-12yrs) and monkey (20-30 yrs) means these two species are chosen for all long-term studies, since the spontaneous natural occurring tumour incidence has to be determined. Obviously it is more economical and easier to determine spontaneous tumour incidence (type and numbers) in the rat or mouse than in the dog or monkey. Practically it is also easier to maintain the large numbers of rats and mice required in these long-term studies.

2.3.3.2.B Choice of carcinogen.

The criteria which have been recommended as applicable to all toxicological studies are namely the route, dose, duration of exposure should be as similar to that for human exposure. Hence, chronic studies of 2 years are typically 2/3 of the natural life span of rats and mice which represents the typical working life (and occupational exposure) of humans (Kluwe et al, 1984).

The chemical should obviously induce tumours in the desired organ in the species chosen so that the experiment provides a "model" of the clinical situation eg bladder tumours.

N-nitroso compounds (including nitrosamines) which are some of the
simplest in terms of structure of all groups of carcinogens have opened up the field of experimental carcinogenesis (Lijinsky, 1987). These compounds have been found to induce cancer in all laboratory species so far tested and as such are unique among chemicals carcinogens. The same compound will often induce tumours in different organs in different species (Lijinsky, 1981). Perhaps the most important factor influencing the use of N-nitroso compounds in experimental carcinogenesis is that simple changes in chemical structure of the compound can have a pronounced effect on the target site of action (Lijinsky, 1984; Preussman & Wiessler, 1987). This has allowed the mechanism of action of these potent carcinogens to be evaluated in different species using the structure-activity and metabolism relationship (Lijinsky, 1984, 1987; Okada, 1984).

2.3.3.2.C Experimental Design.

A comprehensive review of various aspects of long-term carcinogenicity experiments, with particular reference to statistical considerations is discussed in IARC publication No 79, (IARC, 1986). Many long-term carcinogenicity studies use the time for visible tumours to appear, time of animal death, cause of death, histopathological diagnoses of affected organs as end point determinations for the induction of carcinogenic potential for a chemical. There are many variables which can be limiting factors affecting the interpretation of experimental studies. These include variable tumour latency, induction of lethal tumours, dormant tumour cells, occult tumours (non-visible tumours) and experimental observations (Rice & Frith, 1981). McKnight (1988) suggests that the development of practical non-invasive methods to diagnose internal tumours in live animals or
protocols that can assure comparable tumour-free death rates in all
groups are possible methods when occult tumours are found/expected as
in experimental bladder carcinogenesis studies. Alternatively one can
use the incidence of preneoplastic lesions (identified by morphology
and histochemistry in time course studies) as end points in
carcinogenesis studies (Bannasch, 1986a,b). These methods have
limitations in that they may not be predictive and require a vigorous
experimental design and appropriate statistical tests for occult non-
lethal tumours (preneoplasia) such as the Cochran-Mantel-Haenzel test
should be performed (IARC, 1986; McKnight, 1988).

2.3.4 Two Stage (Initiation and Promotion) Carcinogenesis.

Carcinogenesis experiments using rodents as the model animal have
utilised particularly the "two-stage" initiation and promotion model
of cancer (Boutwell, 1964; Scribner & Suss, 1978) originally developed
from experimental skin cancer. This designation of a two step
mechanism is a convenient way of indicating the usual number of
experimental manipulations performed by the operator. It is an
'operational' definition and not related to the actual number of
biological and chemical interactions which occur in the cell during
transformation to a cancerous cell. Since the in vivo carcinogenic
process is a multistep pathway with many stages and end points
including cell death, regression and the induction of benign lesions
(Shubik, 1984).

The criteria for a two stage initiation-promotion experiment according
to Berenblum (1974) are:

1. The design should ensure that the initiation and promotion stages
do not overlap in time.

2. Neither the initiating or promoting agents should be carcinogenic
3. Tumours should be induced when the initiator is followed by the promotor but not vice versa.

4. Increasing the interval between the administration of the initiation and promotion stages should not decrease the tumour yield.

5. Ultimately the tumour yield should be quantitatively related to the dose of the initiating agent whereas the tumour latency time should be determined by the promotor.

2.3.4.1 Definitions of Initiation and Promotion.

Initiation: is a change in target tissue or organ induced by exposure to a carcinogen that can be promoted or selected to develop local proliferations, one or more of which can act as sites of origin for the ultimate development of malignant neoplasia (Farber, 1982). Initiation is associated with a change in DNA and activation of one or more oncogenes and requires one complete cell cycle to "lock in" these changes (Farber, 1984).

Promotion: is the process whereby an initiated tissue or organ develops focal proliferations, one or more of which may act as precursors for subsequent steps in the carcinogenic process, (see Figure 2.4), resulting in hyperplasia, increased or decreased enzyme induction and many other biochemical changes.

Cohen, (1985) has the following definition: an initiator is a "subcarcinogenic" dose of a chemical administered singly or for a short period of time. The promoting agent is administered multiple times after the initiating agent. These definitions are based on the experimental administration of chemical carcinogens and still require the basic criteria of Berenblum (1974) to be followed.
Figure 2.4 Diagrammatic representation of initiation and promotion, as viewed with the rare event-selection hypothesis. The different symbols represent different types of functionally altered cells. According to this hypothesis amplification (or expansion) of appropriately altered cells is the first pre-requisite of promotion of an initiated cell. From Farber, (1982).

2.3.5 Bladder carcinogenesis.

Occupational bladder cancer was first noticed in the Aniline dye industry in Germany in the early 1900’s (Rehn, 1895), but it was only some years later that 2-naphthylamine was found to be a potent bladder carcinogen (Hueper, 1934; Hueper et al, 1938). Later benzidine was also implicated (Case et al, 1954; Meigs et al, 1986).

Aetiologically a single compound such as 2-naphthylamine may be sufficient to induce cancer, but it is more probable that multiple exposure to a variety of agents and factors is responsible for most cases of human bladder cancer that are encountered today (Cohen,
Risk factors that have been identified to significantly increase the incidence of bladder carcinoma are: smoking (Mommsen & Aagaard, 1983; McCredie et al, 1983b; Koch et al, 1986), consumption of alcohol (Kunze et al, 1986), and genetic factors (Berger et al, 1986; Mommsen & Aagaard, 1986).

The majority of exogenous chemicals implicated in bladder cancer are related to aniline dyes and 2-naphthylamine, i.e aromatic amines. This gave a chemical structure base from which to produce carcinogens with which to develop experimental animal models in order to study the pathogenesis of bladder cancer.

2.3.5.1 Spontaneous animal models of bladder cancer.

Experimental research on urinary bladder cancer is performed mainly on chemically induced tumours due to the rare incidence of spontaneous tumours in laboratory animals. There are, however, certain strains of rat that do have a high incidence of urothelia carcinomas (see Table 2.13).

The Brown Norway (BN/BiRij) rat strain have a high incidence (28%-35% male 2% female rats) of urinary bladder tumours, (Boorman & Hollander, 1974; Bolhuis et al, 1978; Burek, 1978), and have been suggested as a suitable model for the human disease (Boorman et al, 1977). Deerburg et al, (1985) reported on another strain incidence of spontaneous urinary bladder tumours in DA/Han rats 53.9% male and 14.4% female of this inbred strain developed bladder tumours with the peak incidence occurring between 25 and 39 months of the animals life span. All neoplasms (90%) were of epitheloid origin being papillomas or papillary transitional cell carcinomas in the BN/BiRij strain with
<table>
<thead>
<tr>
<th>Strain</th>
<th>Sex</th>
<th>No. of rats</th>
<th>No. of rats with tumours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sprague-Dawley/Charles River (CD)</td>
<td>M/F</td>
<td>535</td>
<td>2 - papillomas</td>
</tr>
<tr>
<td>Fischer-344</td>
<td>M</td>
<td>144</td>
<td>1 - transitional cell papilloma</td>
</tr>
</tbody>
</table>
| Fischer-344                                 | M   | 1794        | 1 - transitional cell papilloma
|                                             |     |             | 1 - carcinoma, undifferentiated              |
| Fischer-344                                 | F   | 1754        | 2 - transitional cell papilloma
|                                             |     |             | 2 - transitional cell carcinoma              |
| Fischer-344                                 | M   | 994         | 1 - papilloma                               |
| Wistar                                      | F   | 457         | 2 - leiomyoma                                |
|                                             |     |             | 1 - squamous cell carcinoma                 |
| ACI/segHapBR                                 | M   | 216         | 1 - transitional cell carcinoma             |
| ACI/N                                       | M   | 55          | 2 - papilloma                               |
| ACI/N                                       | F   | 209         | 8 - papilloma
|                                             |     |             | 2 - transitional cell carcinoma             |
| BN/BiRj                                     | M   | 104         | 29 - transitional cell carcinoma            |
| BN/BiRj                                     | F   | 265         | 6 - transitional cell carcinoma             |

M, male; F, female.

Table 2.13 Incidence of naturally occurring urinary bladder tumours in adult rats. From Stula, (1986).

Squamous differentiation and keratinization occurring in presence of bladder stones. In the DA/Han strain transitional cell carcinomas were the predominant tumours induced, incidence in male rats were: papillary (16.8%), solid (56.7%) and mixed types of transitional cell.
carcinoma (15.1%). In the female rats tumour incidences were: papilloma (38.4%), papillary transitional cell carcinomas (30.1%), solid (9.6%) and squamous cell carcinoma (9.6%) with 60% associated with bladder stones (Deerberg et al, 1985). Some tumour types such as inverted papillomas have only been previously reported in humans. It is suggested that these rat strains could be suitable models for human urinary bladder cancer. Deerberg et al, (1985) suggests that the DA/Han strain possesses a genetic predisposition for these neoplasms, and as such would be highly appropriate for the study of genetic, hormonal and occupational influences on urinary bladder carcinogenesis.

There are, however, problems with spontaneous models:

i) less than 50% incidence and long latent period of development is a waste of resources.

ii) variable development of tumours produces difficulty in staging tumours.

2.3.5.2 Experimentally induced urothelial cancer.

Experimental chemical carcinogenesis in the urinary bladder has helped our understanding of the development of such lesions in animals. These models have provided the basis for extrapolating the molecular concepts involved in the mechanism of pathogenesis to the development of the human cancer (Ito & Fukushima, 1986). There are several animal models of bladder and renal parenchymal carcinoma (Hicks & Chowaniec, 1978; Cohen & Friedell, 1979; Cohen 1983, 1985; Hard, 1986; Ito & Fukushima, 1986), but to date there has been relatively little development of an experimentally induced model of upper urothelial carcinoma. A selection of the chemicals which have been used successfully to induce urinary bladder tumours is shown in Table 2.14.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Sex</th>
<th>Lesions</th>
<th>Chemical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wistar</td>
<td>F</td>
<td>HY, TC, SC, AC, UC</td>
<td>N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN)</td>
</tr>
<tr>
<td>Wistar</td>
<td>M</td>
<td>TC, SC, UC</td>
<td>BBN</td>
</tr>
<tr>
<td>Wistar</td>
<td>M</td>
<td>HY, TP, TC</td>
<td>BBN</td>
</tr>
<tr>
<td>Fischer-344</td>
<td>M/F</td>
<td>HY, TP, TC, SC, AC</td>
<td>Methylalkylnitrosamines</td>
</tr>
<tr>
<td>Norwegian albino</td>
<td>M/F</td>
<td>TP, TC</td>
<td>Bracken fern</td>
</tr>
<tr>
<td>Sprague-Dawley/</td>
<td>M/F</td>
<td>TP, TC</td>
<td>Bracken fern</td>
</tr>
<tr>
<td>Charles River CD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Norwegian albino</td>
<td>M/F</td>
<td>TP, TC</td>
<td>Quercetin</td>
</tr>
<tr>
<td>Sprague-Dawley</td>
<td>F</td>
<td>HY, TC, SC</td>
<td>N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT)</td>
</tr>
<tr>
<td>Wistar</td>
<td>F</td>
<td>HY, TC</td>
<td>2-Napthyamine</td>
</tr>
<tr>
<td>Fischer-344</td>
<td>F</td>
<td>TP, TC, SC</td>
<td>p-Quinone dioxime</td>
</tr>
<tr>
<td>Fischer-344</td>
<td>M/F</td>
<td>TP, TC</td>
<td>o-Anisidine</td>
</tr>
<tr>
<td>Fischer-344</td>
<td>M/F</td>
<td>HY, TC</td>
<td>Nitrilotriacetic acid</td>
</tr>
<tr>
<td>Fischer-344</td>
<td>F</td>
<td>TC</td>
<td>o-Toluidine hydrochloride</td>
</tr>
<tr>
<td>Fischer-344</td>
<td>M/F</td>
<td>HY, TP, TC</td>
<td>4-Amino-2-nitrophenol</td>
</tr>
<tr>
<td>Fischer-344</td>
<td>M/F</td>
<td>HY, TC</td>
<td>N-Nitrosodiphenylamine</td>
</tr>
<tr>
<td>Fischer-344</td>
<td>M</td>
<td>TP</td>
<td>Allyl isothiocyanate</td>
</tr>
<tr>
<td>Fischer-344</td>
<td>M</td>
<td>HY, TC</td>
<td>Melamine</td>
</tr>
</tbody>
</table>

AC, adenocarcinoma; HY, hyperplasia; SC, squamous cell carcinoma; TC, transitional cell carcinoma; TP, transitional cell papilloma; UC, undifferentiated carcinoma; M, male; F, female.

Table 2.14 Various chemicals reported to induce bladder cancer in rats. From Stula, (1986).

Two of these chemicals; N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT) and N-Butyl-N-(4-hydroxybutyl)-nitrosamine (BBN) are probably the most widely used carcinogens that induce experimental models of
urinary bladder tumours in animals.

2.3.2.2.A N\[4-(5-nitro-2-furyl)-2-thiazolyl\]formamide (FANFT).

Formic acid 2-[4-(5-nitro-2-furyl)-2-thiazolyl] hydrazide, a nitro-
furan derivative was reported to induce carcinomas of kidney, breast, intestine and ear duct, but not the urinary bladder (Price et al, 1966). Nitrofurans can be reduced to aminofurans and possibly hydro-
xylamine derivatives in vivo (Taylor et al, 1951) which, being aromatic amines, would be expected to induce bladder neoplasms.

Erturk et al, (1967) used FANFT, (Figure 2.5a) dosed as a 0.188% mix (by weight) in the diet for 4  6  weeks in 30 female Sprague Dawley rats to specifically induce urothelial carcinomas.

Of the animals that lived 34 weeks or more, 29 developed gross bladder tumours and 3 animals developed carcinoma of the renal pelvis which invaded the kidney. Benign mammary tumours also developed in 15/30 animals. Further studies feeding 35 male Sprague Dawley rats on a 0.188% FANFT diet for 26 or 46 weeks by Erturk et al, (1969) resulted in a 100% incidence of gross bladder carcinomas.

Over half the 35 rats developed severe pelvic hyperplasia and 4 cases of renal pelvic carcinomas were reported which invaded the kidney. Seventy female Sprague Dawley rats were also fed for 26 or 46 weeks on a 0.188% FANFT diet and groups were autopsied at periodic intervals. The following histological changes were noted by Erturk et al (1969), during the carcinogenesis of bladder. Hyperplasia at 3 weeks was followed by mitotic activity and squamous metaplasia at 8 weeks. Microscopic papillae lesions throughout the bladder occurred at 9 weeks and progressed to small tumours at 12 weeks. By 25 weeks gross tumours were 2 cm in diameter and had enlarged to 3-7 cm by 45 weeks.
Figure 2.5a  N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT).

Figure 2.5b  N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN).
Metastases occurred late in the process and were very rare. Cohen & Friedell (1979) produced a succinct and comprehensive review of the FANFT induced animal model of bladder carcinoma. FANFT reproducibly induces 100% tumour incidence in the urinary bladder of rats, mice, hamsters and dogs without sex predominance. The pathogenesis develops through mild hyperplasia to invasive cancer. Hyperplasia is present in all rat strains at 2/3 weeks Sprague-Dawley (Erturk et al, 1969), Fischer (Tiltman & Friedell, 1971) and F344 (Jacobs et al, 1976), which increases in severity and by 8 weeks nodular and papillary hyperplastic lesions have developed. These lesions gradually enlarge, protrude into the lumen, and downward into stroma. By 25-30 weeks cellular and nuclear atypia are present together with invasion of the basement membrane which by 45-50 weeks is through to the muscle layer. Late development of ureter and renal pelvis tumours with obstruction of ureter and subsequent hydronephrosis occurs in Sprague Dawley but not Fischer rats. Since these early reports there has been little attempt to increase either the incidence or severity of upper urothelial tumours as a model of UUC.

FANFT-induced bladder carcinomas resemble the human disease in several ways; most of the lesions are papillary transitional cell carcinomas. As with human disease the FANFT-induced lesion progresses from epithelial hyperplasia to non-invasive carcinoma, invasive carcinoma and eventually distant metastases. The progress of the lesion can be followed by urinary cytology and haematuria is a frequent early sign of disease as with humans.

FANFT is often used as the initiator in two stage carcinogenesis experiments in order to evaluate potential promoter agents in bladder carcinogenesis. Table 2.15 shows a selection of agents which have
been "screened" using FANFT initiated bladder urothelium for promoter activity by determining an increase in tumour incidence, or decrease in tumour latency, or alteration in target specificity.

There are several disadvantages of using FANFT in a diet mix:

i) increased exposure risk to personnel

ii) level of dosing to animals is unknown

iii) it is an expensive chemical to use in the quantities required over the effective period

iv) it is very difficult to get supplies in the United Kingdom. It is only synthesised in the USA, but due to problems with synthesis from time to time, there has been a failure of the companies who produce it to keep up to the demand for supplies used by researchers.

v) finally big spenders seem to get first choice! as we have had an order for 200 g outstanding for 5 years.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Promoter</th>
<th>Action</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-Tryptophan</td>
<td>Y</td>
<td>(1)</td>
<td>Cohen et al, 1978</td>
</tr>
<tr>
<td>Saccharin</td>
<td>Y</td>
<td>(1)</td>
<td>Cohen et al, 1978</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hicks, 1978</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cohen, 1982</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Schoenig et al, 1985</td>
</tr>
<tr>
<td>Phenacetin</td>
<td>Y</td>
<td>(2)</td>
<td>Anderstrom et al, 1983</td>
</tr>
<tr>
<td>Mechanical perforation</td>
<td>Y</td>
<td>(2)</td>
<td>Anderstrom et al, 1983</td>
</tr>
<tr>
<td>E.Coli (06K131)</td>
<td>Y</td>
<td>(2)</td>
<td>Johansson et al, 1987</td>
</tr>
</tbody>
</table>

(1)-increased incidence of bladder tumours
(2)-increased incidence of pelvic tumours

Table 2.15: some agents screened for promoting potential in urothelium initiated with FANFT.
2.3.5.2.B N-Butyl-N-(4-hydroxybutyl)-nitrosamine (BBN).

N-Butyl-N-(4-hydroxybutyl)-nitrosamine (BBN) (Figure 2.5b) was first reported to be a specific urinary bladder carcinogen by Druckey et al, (1964). It is a metabolite of N-nitrosodi-n-butylamine (IARC, 1978) which itself is a potent carcinogen inducing tumours in liver, forestomach, lung, bladder and oesophagus. BBN has become widely used as a chemical carcinogen to induce urinary bladder tumours in experimental animal models (Becci et al, 1981; Cohen, 1985). Oral doses of 40 and 20 mg/kg b.w. of BBN given in the drinking water selectively induced multiple carcinomas of the urinary bladder in all animals in three different rat strains; Wistar (Ito et al, 1969; Kunze & Schauer, 1977), ACI/N (Herman et al, 1985) and Fischer 344 (King et al, 1984). Wistar rats were found to be more susceptible to BBN when compared with mice, hamsters and guinea-pigs (Hirose et al, 1976, Ito, 1976). However, mice developed a higher incidence of invasive carcinomas than rats (Ohtani et al, 1986).

A 100% incidence of urinary bladder carcinomas is induced in rats by continuous, prolonged administration of BBN in the drinking water (Ito & Fukushima 1986, see Table 2.16 and Figures 2.6a,b) which is dose related. The incidence of carcinomas is proportional to the total dose consumed (Ito & Fukushima, 1986), after a period of 40 weeks. Intravesicular instillation of a 2% BBN solution directly into the bladder of female ACI/N rats, three times weekly for a period of 20 weeks, resulted in 70% incidence of urinary bladder papilloma and 57% incidence in carcinomas (Hashimoto et al, 1974) thus showing the direct carcinogenic action of BBN on bladder urothelial cells.

The detailed morphology, classification and histogenesis of urinary
Table 2.16 Lesions of the urinary bladder in male rats treated with 0.05% BBN for different periods.

<table>
<thead>
<tr>
<th>Period (in weeks)</th>
<th>Effective No. of rats</th>
<th>PN hyperplasia</th>
<th>Papilloma</th>
<th>Carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>With BBN</td>
<td>Without BBN</td>
<td>No. of rats</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>38</td>
<td>18</td>
<td>12 (66.7)</td>
<td>6 (33.3)</td>
</tr>
<tr>
<td>4</td>
<td>36</td>
<td>11</td>
<td>11 (100)</td>
<td>8 (72.7)</td>
</tr>
<tr>
<td>6</td>
<td>34</td>
<td>17</td>
<td>16 (94.1)</td>
<td>15 (88.2)</td>
</tr>
<tr>
<td>8</td>
<td>32</td>
<td>10</td>
<td>10 (100)</td>
<td>9 (90)</td>
</tr>
<tr>
<td>12</td>
<td>28</td>
<td>9</td>
<td>9 (100)</td>
<td>9 (100)</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20 (100)</td>
<td>20 (100)</td>
</tr>
</tbody>
</table>

* Percentages are given in parentheses.

Table 2.16 Lesions of the urinary bladder in male rats treated with 0.05% BBN for different periods. From Ito & Fukushima, (1986).

Figure 2.6a Occurrence of lesions in the urinary bladder of rats given 0.05% BBN. PN, papillary or nodular. From Ito & Fukushima, (1986).

Figure 2.6b Occurrence of papillary or nodular hyperplasia in the urinary bladder of rats given BBN. From Ito & Fukushima, (1986).
bladder carcinomas induced by BBN in female Wistar rats (Kunze & Schauer, 1977; Kunze, 1979) showed 88% of the tumours to be papillary and non-papillary transitional cell carcinomas. Fukishima et al (1976) concluded that 95% of BBN-induced bladder tumours were transitional cell carcinomas in origin, the remainder being squamous cell carcinomas, undifferentiated carcinomas and carcino-sarcomas, and similar to characteristic tumours described for human bladders.

A series of studies by Becci et al (1979) established a quantitative dosing regime for the administration of BBN. Previous studies had almost exclusively used oral administration in the drinking water of varying concentrations of BBN, usually between 0.05% and 0.5% solution. This method of dosing has three main disadvantages: i) there is no means by which the quantity of BBN consumed by each animal can be controlled ii) there is a large exposure risk to technical staff in mixing and handling the large volumes of carcinogenic solutions iii) sawdust is contaminated with water spillage.

Oral administration of BBN (in a 20:80 ethanol:water solution so that each volume is contained in a 0.5 ml volume) using intubation directly into stomach has been successfully utilised to induce transitional cell carcinomas in female Fischer 344 rats (Becci et al, 1979) and male B6D2F1 mice (McCormick et al, 1981a). Oral gavage allowing quantification of administered BBN has allowed aspects of chemical carcinogenesis of urinary bladder tumours to be investigated such as dose schedule and total dose. McCormick et al, (1981a) determined that BBN induces bladder tumours in a dose related manner, with high organ specificity, short tumour latency. Frequent smaller doses induced a higher incidence of tumours than fewer large doses up to the same total dose (McCormick et al, 1981a).
The experimental procedures in administering BBN by oral gavage make it preferable to use than other carcinogens in diet and via drinking water.

Unilateral ureter ligation in male Wistar rats treated with 0.025% BBN in drinking water for 20 weeks induced a greater incidence of pelvic and ureteric papillomas and carcinomas together with enhanced hyperplasia of the pelvic and ureteric urothelium above the ligation (Ito et al, 1971).

BBN-induced bladder tumours in experimental animals have been frequently used to assess chemotherapeutic agents for possible clinical use. Retinoids, natural and synthetic, analogues of vitamin A have been used frequently to inhibit bladder cancer in experimental animals (Grubbs et al, 1977; Becci et al, 1981; Fukushima et al, 1981; McCormick et al, 1981b; Moon et al, 1982,1983). However the inhibition of carcinogenesis is not complete (McCormick et al, 1981b) and the use of BBN-induced tumours to investigate different chemotherapeutic protocols is continuing.

BBN is now used frequently as a bladder urothelium initiator to screen potential bladder urothelium promoters since bladder carcinogenesis is known to be a multifactorial process with many potential causative agents (Anderson et al, 1986; Fukushima et al, 1985; Miyata et al, 1985; Kitahori et al, 1985; La Voie et al, 1985). Table 2.17 shows a selection of chemicals which have been screened by this short term in vivo assay system. The marker change which is used to determine whether there is a promoting or carcinogenic potential is the development of pre-neoplastic (papillary/nodular) hyperplasia.

The pathogenetic pathway of experimental bladder carcinogenesis
Table 2.17: Some chemicals screened for promoting potential in the urinary bladder after BBN initiation.

<table>
<thead>
<tr>
<th>AGENT</th>
<th>PROMOTER (Y/N)</th>
<th>ACTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% sodium saccharin</td>
<td>Y</td>
<td>(1)</td>
</tr>
<tr>
<td>2% sodium-O-phenylphenate</td>
<td>Y</td>
<td>(1)+(2)</td>
</tr>
<tr>
<td>2% butylated hydroxyanisole</td>
<td>Y</td>
<td>(1)+(2)</td>
</tr>
<tr>
<td>5% sodium-L-ascorbate</td>
<td>Y</td>
<td>(1)+(2)</td>
</tr>
<tr>
<td>5% ascorbic acid</td>
<td>N</td>
<td>--</td>
</tr>
<tr>
<td>5% ascorbic stearate</td>
<td>N</td>
<td>--</td>
</tr>
<tr>
<td>5% sodium erythorbate</td>
<td>Y</td>
<td>(1)+(2)</td>
</tr>
<tr>
<td>0.8% ethoxyquin</td>
<td>Y</td>
<td>(1)+(2)</td>
</tr>
<tr>
<td>0.02% N-nitrospyrrolidine</td>
<td>Y</td>
<td>(2)</td>
</tr>
<tr>
<td>0.2% methylhydroquinone</td>
<td>N</td>
<td>--</td>
</tr>
<tr>
<td>0.2% hydroquinone</td>
<td>N</td>
<td>--</td>
</tr>
<tr>
<td>0.2% resorcinol</td>
<td>N</td>
<td>--</td>
</tr>
<tr>
<td>0.8% catechol</td>
<td>N</td>
<td>--</td>
</tr>
<tr>
<td>0.5% pyrogallol</td>
<td>N</td>
<td>--</td>
</tr>
<tr>
<td>0.6% carbozole</td>
<td>Y</td>
<td>(1)</td>
</tr>
<tr>
<td>0.1% quinoline</td>
<td>N</td>
<td>--</td>
</tr>
<tr>
<td>1% uric acid</td>
<td>N</td>
<td>--</td>
</tr>
<tr>
<td>2% trisodium nitritoltri-acetate monohydrate</td>
<td>Y</td>
<td>(1)+(2)</td>
</tr>
<tr>
<td>5% sodium citrate</td>
<td>Y</td>
<td>(1)+(2)</td>
</tr>
<tr>
<td>0.5% diphenyl</td>
<td>Y</td>
<td>(1)+(2)+(3)</td>
</tr>
<tr>
<td>0.2% allopurinol</td>
<td>Y</td>
<td>(1)</td>
</tr>
<tr>
<td>1.3% acetaminophen</td>
<td>N</td>
<td>--</td>
</tr>
</tbody>
</table>

(1) - Increased incidence papillary/nodular hyperplasia
(2) - Increased incidence papillomas in urinary bladder
(3) - Increased incidence carcinoma in urinary bladder
% - percentage of test chemical in diet


(induced by chemical carcinogens such as FANFT and BBN) is schematically represented in Figure 2.7 with simple hyperplasia being the initial response to variety of carcinogens.

Research into experimental bladder tumours using chemical carcinogens such as BBN and FANFT has elucidated pre-malignant changes in normal histological urothelial cells which subsequently become hyperplastic then malignant. Kunze (1979) showed the loss of alkaline phosphatase to be a marker change for pre-neoplastic cells after BBN treatment.
Normal urothelium. 

Simple hyperplasia. 


Papilla. 

(Dysplasia). 

Non invasive carcinoma. 

Invasive carcinoma. 

Metastatic carcinoma. (Modified from Cohen, 1983).

**Figure 2.7** Schematic representation of the mechanism of pathogenesis of urothelial carcinoma, derived from experimental urinary bladder carcinogenesis studies.

The increase in gamma-glutamyl transpeptidase (GGT) has been well established as a pre-malignant marker for hepatomas (Hanigan & Pitot, 1985) and Ozono et al (1985) has found similar changes in the urothelium of BBN treated animals.

The urothelia that lines the bladder, ureter, renal pelvis and that covers the renal papillae are all derived from the same embryological structure; (the ureteric bud) and have a similar histological appearance although they do differ in thickness (Melicow, 1945) and also in cell surface characteristics (Newman & Hicks, 1981).

This 'common embryological genesis' concept is the basis of the choice of BBN as the "initiating" carcinogen in developing an experimental model of UUC and its relationship to RPN (see Methods, section 3.6.5).
Several other factors biased the choice of BBN against FANFT: i) oral dosing regimes evolved allow "control" over frequency and total dose administered to animals ii) BBN has a greater specificity than FANFT with no benign "extra-urothelial" tumours induced e.g. mammary tumours iii) a supplier was found who could supply the necessary quantities required for the experimental procedures at a reasonable cost.

2.3.5.2.B.1 Metabolism, pharmokinetics and cellular changes induced by BBN.

BBN undergoes omega-oxidation by P-450 dependant monooxygenases (Gottfried-Anacker et al, 1985) to produce a number of metabolites the major one is N-nitroso-butyl-(3-carboxypropyl)amine (BCPN) which comprises 40% of the administered dose (Okada, 1984). The presence of the 4-hydroxybutyl group has been found to be necessary for selective bladder carcinogenesis (Okada, 1984). Suzuki & Okada, (1980) found that 80% of the N-nitroso moieties produced as a consequence of BBN metabolism were excreted within 48 hr. The hydroxylated metabolites are mainly excreted as beta-glucuronides (BBN-Gluc) according to Gottfried-Anacker et al, (1985), and the two metabolites BCPN and BBN-Gluc are detectable in the serum within 2 min after an intravenous injection of BBN (Bonfanti et al, 1988). BBN was found to disappear completely from the circulating blood 90 min, BBN-Gluc was detectable up to 40 min and BCPN was detectable up to 120 min after initial BBN injection. The body and renal clearance rates of BBN were 86.1 and 0.22 ml/min/kg respectively (Bonfanti et al, 1988). When either BCPN or BBN-Gluc were administered each on their own, only 41% and 50% respectively of these compounds were recovered in the urine suggesting
that they undergo further biotransformation to potential proximate carcinogens (Bonfanti et al., 1988). It has, however, been suggested that the species/strain differences reported by (Hirose et al., 1976; Ohtani et al., 1986; Okada, 1984) may be due to genetically determined differences in metabolic enzyme systems (Mori et al., 1987) which affects the metabolism of BBN and hence the potentially carcinogenic metabolites.

2.4 THE NUDE MOUSE AND EXPERIMENTAL CARCINOGENESIS.

2.4.1 History of Nude Mouse.

The nude mouse is a spontaneous hairless mutant which was discovered in a closed albino mouse colony in 1964 and first reported in the literature by Flanagan (1966). Flanagan showed that the mutation was due to a single autosomal recessive gene, to which he assigned the symbol nu. In studies where homozygous (nu/nu) male mice were mated with heterozygous (nu/+ ) female mice with resultant 1:1 ratio of nude to normal offspring (Flanagan, 1966).

In addition to hairlessness the nude mouse also has a short life span and lacks a complete thymus (Hansen, 1978). The "athymic" nature of the nude mouse meant that initially it became an important tool in immunological research, since the thymus plays a primary role in the immune response. However, the fact that homozygous (nu/nu) mice accepted transplants (xenografts) of tumour tissues (Rygaard & Polvsen, 1969) opened up a new era in cancer research. The lack of an immune 'host-graft' tissue rejection response allowed the use of tissue from human tumours to be used rather than spontaneous animal tumours or chemically induced "model" tumours. For reviews discussing the use of the nude mouse in cancer research see Sharkey & Fogh.
The factors which make the nude mouse an ideal host for tumour xenografts (namely athymic, lack of a host-graft rejection) also leaves the animal prone to bacterial and viral pathogens (Ediger & Giovanella, 1978). Although the nude mouse is not immunologically neutral, it can produce immunoglobulins and display Natural Killer (NK) cell reactivity (Sharkey & Fogh, 1984).

As a result of this susceptibility special precautions and maintenance facilities have to be provided in order to obtain and maintain healthy animals and consistent results. It is necessary to keep these animals in a gnotobiotic (germ-free) environment such as a bacteria-free isolators (see section 3.6.6) and ensure they remain isolated from other laboratory animal species (especially mice). Nude mice are especially susceptible to the murine hepatitis virus (which is lethal in nude mice, but not apparent in normal mice) and the Sendai virus; producing a fatal pneumonia rather than the mild "cold" symptoms induced in normal mice (Ediger & Giovanella, 1978). Personnel in contact with nude mice should not be working with other rodents (Sharkey & Fogh, 1984).

Nude mice are available from several commercial sources. The nude gene (nu) has been bred into a variety of background strains (Hansen, 1978; Sparrow, 1980). In order to assure a consistency of results, a minimum of 5 backcrosses should have been performed (Festing, 1979). A simple mean of assuring genetic consistency is to transfer skin grafts among heterozygous litter mates, which are often available as a by product of breeding heterozygous females to homozygous males (Ediger & Giovanella, 1978). If the skin grafts take then the colony is genetically homozygous, if not the animals may have to be subjected
2.4.2 Growth of Human Tumours in Nude Mice.

The athymic characteristics of the nude mouse has made it a frequently used vehicle for growing human and other tumour cells. The growth of human tumours was first reported by Rygaard & Polvsen (1969) after transplantation into nu/nu mice. Innoculation of cultured human cells was also successful (Giovanella et al, 1972). During the past 20 years many variables that affect the frequency and speed of growth of tumour xenografts have been identified including tumour origin, age, sex and genetic background of nude mouse host and site of tumour inoculation, see Table 2.18.

Table 2.18: Variables that affect transplantation.

<table>
<thead>
<tr>
<th>Host</th>
<th>Tumour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
<td>Tumour type</td>
</tr>
<tr>
<td>Age</td>
<td>Origin (primary, metastatic, recurrent)</td>
</tr>
<tr>
<td>Sex</td>
<td>Preparation of tumour tissue</td>
</tr>
<tr>
<td>Health of the animal</td>
<td>Histology</td>
</tr>
<tr>
<td>Transplant site</td>
<td>Differentiation</td>
</tr>
<tr>
<td>Life span of the animal</td>
<td>Proliferation</td>
</tr>
</tbody>
</table>


Approximately 50% of malignant transplantations are successful. The "take" rate is dependant on many variables including those listed above, and in addition, the degree of malignancy, and the degree of differentiation of the original tumour (Polvsen et al, 1982). Metastases grow considerably better than primary site tumours, and tumour-derived cell line inoculations do considerably better than surgically derived tissue specimens (Fogh et al, 1977). Reed & Manning (1978) report a metastatic bladder carcinoma xenograft which was extremely rapidly growing and required 6 passages in 29
weeks that then yielded 100g of tumour tissue from 18 mice where 1g of original tumour tissue had been implanted. This example illustrates how the nude mouse xenograft procedure can be utilised to increase the mass of malignant tissue from a rare or uncommon tumour (occurring in humans) and thus allow a variety of experimental procedures to be performed.

2.4.3 Nude Mouse in Urothelial and Renal Carcinoma Research.

Nude mice have been used to research into growth, histological, biochemical characteristics of urothelial and renal malignancies tumours to chemotherapeutic agents, cytotoxic drugs and novel cancer treatments using monoclonal antibodies as targeting molecules (Huland et al, 1985, 1986; Kyriazis et al 1985).

2.4.4 Stability of Xenograft Tumours.

To be of use the mouse grown tumour (MGT) must retain and display all biological characteristics found in the original human tumour (OHT). This question is an important argument for assessing the reliability of nude mouse tumour models particularly in drug sensitivity testing. Several structural, biochemical and functional criteria play an important part in the evaluation of any differences that occur between the OHT and MGT (Table 2.19). The choice of which criteria to use to evaluate the differences may explain the discrepancies among published observations (Sharkey & Fogh, 1984).

The inherent hazard in pathological/histological grading is that each pathologist has their own idea of what constitutes a particular pathological lesion; "one man's carcinoma is another man's hyperplasia", thus their observations are qualitative and subjective.
<table>
<thead>
<tr>
<th>Similarities</th>
<th>Differences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histology, ultrastructure</td>
<td>Cell cycle parameters</td>
</tr>
<tr>
<td>Hormone production</td>
<td>Growth rate</td>
</tr>
<tr>
<td>Production of tumour marker</td>
<td>Metastatic spread</td>
</tr>
<tr>
<td>Chromosomes</td>
<td>Invasive properties</td>
</tr>
<tr>
<td>DNA content</td>
<td>Stroma and vascularization of mouse origin</td>
</tr>
<tr>
<td>Chemotherapeutic sensitivity</td>
<td>Metabolism, pharmacokinetics</td>
</tr>
</tbody>
</table>

Table 2.19: Criteria which may be evaluated to establish similarities or differences between the donor tumour and the xenografted tumour. From Mattern et al, 1988.

Quantitative morphometric analyses has revealed that significant changes have occurred in histological differentiation when no changes were reported based on a purely subjective grading scheme (Sharky & Fogh, 1984).

Knofel et al (1987) studied 30 human renal cell carcinomas after several passages (10-50) in NMRI/nu/nu mice, and found that the majority remained stable with regard to histopathology, nuclear grade. Only one sixth (5 out of 30) altered their mitotic rate and DNA content (which was in fact increased). Fogh et al, (1978) compared a number of parameters for a melanoma tumour before (MeWo) and after growth (NuCuMeWo) in the nude mouse. They compared histological and ultrastructural morphology, in vitro growth rates, chromosomal analysis (human chromosome presence showed tumour cells derived from original tumour rather than mouse tissues), immune adherence tests and isoenzyme analysis: differing mobility patterns of human and mouse enzymes on starch gel electrophoresis of polymorphic human enzymes were used to show that NuCuMeWo cells were derived from original MeWo tumour and not mouse tissues.

A general summary is that tumours are stable after many passages at histological and biochemical levels, tumour cell kinetics tend to
increase and it is necessary to monitor phenotypic and genotypic
tumour features throughout the experimental period.

2.5 INVESTIGATIONS UNDERTAKEN.

A series of investigations were initiated based on these data reported
on RPN, UUC in the literature (described above). It was of interest
to determine: i) which was the earliest change occurring as a
consequence of BEA-induced acute RPN, ii) how relevant is the acute
RPN model in the unipapillate of the rat to the chronic clinical
lesion in the multipapillate human kidney; could it be reproduced in a
multipapillate animal species ie the pig, iii) using a classical
two-stage carcinogenesis model was it possible to induce UUC in an
initiated urothelium with an acute RPN injury as the "promoting" step,
iv) could this UUC lesion be affected by subsequently dosing with
analgesics and v) was it possible to develop an "in-vitro" cell line
of any chemically-induced UUC tumours or a passable tumour in Nude
mice. The methods, results and discussions of these investigations
undertaken form the remainder of this thesis: Methods (Chapter 3),
Results and Discussions of RPN studies (Chapters 4), Results and
Discussions of UUC studies (Chapter 5).
CHAPTER 3
MATERIALS AND METHODS

3.1 MATERIALS AND INSTRUMENTATION.

3.1.1 Chemicals.

All the chemicals used in these studies were of analytical grade and of the highest purity that was commercially available unless otherwise stated. Chemicals are listed according to supplier.

Aldrich, Gillingham, Dorset, UK.: 2-Bromoethanamine hydrobromide

British Drug Houses Ltd (BDH), Eastleigh, Hampshire, UK.: acetic acid, ammonium sulphide, calcium chloride, calcium nitrate, cobalt chloride, cobalt nitrate, disodium hydrogen orthophosphate, formaldehyde (40 v/v solution), Haemotoxylin (Harris'), n-hexane, hydrochloric acid, Giemsa, magnesium chloride, manganese chloride, methanol, N-phenylanthranilic acid, sodium acetate, sodium chloride, sodium hydrogen carbonate, sodium dihydrogen orthophosphate, sodium-beta-glycerophosphate, sodium hydroxide.

Intertox Chemicals, Cheshire, UK.: peracetic acid.

James Burroughs (FAD) Ltd., Witham, Essex, UK.: ethanol (100%)

Koch-Light Ltd., Haverhill, Suffolk, UK.: N-gamma-L-glutamyl-beta-napthylamide


Midwest Research Institute (MRI), Kansas City, Missouri, USA.: N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN), purity: 97% +, molecular weight: 174.3, CAS No.: 924-16-3.


Roche Products Ltd, Welwyn, Herts, UK.: Vitamin K supplement (Konatin 10)
Sigma Chemical Company Ltd, Poole, Dorset, UK: adenosine triphosphate, aspirin, carboxy-methylcellulose, Cyanocobalamin, Eosin, Fast garnet red, glycyglycine, naphthol-AS-Bl-phosphate, Paracetamol (4-acetomidophenol), periodic acid, Red violet LB, Schiff’s reagent, sodium barbitone, TRIS (Tris(hydromethyl)aminomethane), Triton X-100.

3.1.1.1 Synthesis of sodium salt of N-phenylantranilic acid

Procedure.

1. Add the minimum volume of 1.0 M NaOH (pH > 8.0) sufficient to dissolve 15 g of NPAA (Aldrich), typically 60-70 ml.

2. Evaporate solution using a rotary evaporator at 65°C and at 30 mm Hg vacuum pressure until grey-white sludge is obtained.

3. Product is then removed from the vessel and redissolved in minimum volume of 96% ethanol and crystallised at 60°C in an oven. Purification is performed by redissolving in minimum volume of 1:1 (v/v) ethanol:ether solution and recrystallize again.

4. Crystals can be ground to a fine powder using a mortar and pestle. [Caution: The product is a fine particulate compound that irritates the nasal and oral epithelia, necessitating the use of face mask and goggles.]

5. Compound can readily be dissolved in aqueous solutions which is the great advantage over the insoluble parent compound.

3.1.2 Materials.

H.A. Coombs Ltd, Wiltshire, UK.: 2.54 cm wide rubber band.


Flow Laboratories Ltd, Rickmansworth, Herts, UK.: Multiwell plates.

Gibco, Paisley, Scotland, UK.: RPMI-1640 media [10x], L-glutamine (200
mM) [100x], Foetal calf serum (heat inactivated), antibiotic-
antimycotic solution [10,000 units/ml], NUNC cryotubes.

Gillette Surgical, Isleworth, Middlesex, UK.: Needles (Luer fitting,
23G (0.6 x 30 mm), 25G (0.5 x 16 mm)).

Histolab & Cytolab, Hemel Hempstead, Hertfordshire, UK.: Histomount
mounting medium (D.P.X), tissue stubs.

AR Horwell Ltd, West Hampstead, London, UK.: Microscope slides (single
frosted end).

ICI, Macclesfield, Cheshire, UK.: Dry Ice (solid CO₂).

Ilford Ltd, Mabberley, Cheshire, UK.: Black and White photographic film
- Ilford FP4 [ISO 125].

Kodak Ltd, Liverpool, UK.: Colour photographic films : Kodak
EKTACHROME [Daylight, ISO 100].

Raymond A. Lamb Ltd, London, UK.: Coverslips (22 x 22 mm, 22 x 40 mm,
22 x 50 mm), coplin staining jars, staining racks, slide storage
trays, slide storage boxes, adhesive slide labels.

LRC Products Ltd, Malaysia.: Industrial Rubber gloves (Marigold).


3M UK Ltd, Bracknell, Berkshire, UK.: 3M dust masks, scotch tape,
white pressure sensitive tape, glass fibre tape.

Polysciences Ltd, Northampton, Northants, UK.: JB-4 Glycolmethacrylate
resin, embedding moulds (16 x 12 x 6mm, 19 x 13 x 5mm, 15 x 5 x 2mm,
12 x 6 x 5mm), tissue stubs.

Pressure Seal Plastics, Chesterfield, S. Yorkshire, UK.: plastic foam,
clear PVC plastic (0.2 mm thick), coloured PVC plastic (0.5 mm thick).

Dustmaster).
Sabre International Products Ltd, Reading, Berkshire, UK.: Gavage Needles: Luer fitting, 15-16G intra-venous needles modified by cutting to appropriate length and rounding off tip, Syringes (Luer fitting, 1 ml, 2 ml).

Sigma Chemical Company Ltd, Poole, Dorset, UK.: Gelatin mountant, Monastoral blue B.

Sterilin Ltd, Middlesex, UK.: Sterilin tubes.


Surgikos Ltd, Livingston, Scotland, UK.: Disposable surgical gloves (Microtouch):

TAAB Laboratory Equipment Ltd, Aldermaston, Berkshire, UK.: Dustoff fluorocarbon gas, TAAB Superglass (for Ralph knives), scalpel blades (N° 22), camel hair brush (N° 5), single edged razor blades, tissue stubs, embedding moulds for EM, Spurr EM resin embedding kit.

3.1.3 Instruments.

Galaxy G400D0 balance: (OHAUS Scale Corp., Flerham Park, NJ. USA)


Camera (s): Nikon UFX-II photographic systems and Nikon FE2 SLR, (Nikon UK Ltd)

Flow cabinet: Pathfinder, (Havant, Hampshire, UK) with activated charcoal filters suitable for formaldehyde fumes and autopsy odours.
Microtome: Bright 5030/WD/MR-CV, (Bright Instruments Co, Huntingdon, Cambridgeshire, UK).

Incubator: LEEC MKII Proportional Temperature Controller, (LEEC Ltd, Nottingham, Nottinghamshire, UK).

Knifemaker: LKB 2078 histoknifemaker, (LKB, Bromma, Sweden)

Sledge (fixed frozen sectioning) microtome: Reichert with freezing stage attached to Mectron thermocouple system (Frigistor Ltd, UK).

Hot plate, Slide drying cabinet, Water bath: (Raymond A. Lamb Ltd, Sunbeam Rd, London, UK).

Osmometer: Wescor Inc. 5100 Vapour Pressure Osmometer, (Chemlab Scientific Products Ltd, Hornchurch, Essex, UK).

Sterilising tower, (Sartorius, Gottingen, FDR).

Autoclave: Rodwell Series 32, (Rodwell Scientific Instruments, UK).

Thin plastic film Isolator: made "in-house" at the Robens Institute by Mr S. Tuttlebury (Trexler & Reynolds, 1957).


Halidehound (halide sniffer): (Ricca-Reddington Industries, Florida, USA).

Ear punch pliers: Junior Punch plier (3/64" hole), (Maun Industries, UK).

Surgical instruments: scalpel blades N° 22, single edged razor blades, scissors, forceps, fine point forceps (N° 5), (TAAB Laboratory Equipment Ltd, Aldermaston, Berkshire, UK).

Animal balance: Torbal Torsion balance (0-2 Kg reading to 1g), (K.T. Forrest & Co., Streatham, London, UK).

Radio frequency sealing machine: (STANELCO Ltd, Borehamwood, Herts, UK).
3.2 ANIMALS.

3.2.1 Licence Details.

All experimental procedures on rats and mice were performed under Licence (No. 61672, Certificate A1) issued by the Home Office under the Cruelty to Animals Act (1876) Licence to Experiment on Living Animals. This Act has now been replaced by the Animals (Scientific Procedures) Act (1986), however licences issued under previous Act remain valid. Since July 1988 all carcinogenesis studies commenced have been designed and performed according to United Kingdom Committee on Cancer Research Guidelines for the Welfare of Animals in Experimental Neoplasia (1988).

All experimental procedures involving pigs were performed by Dr M.E.C. Robbins at the CRC Normal Tissue Radiobiology Research Group, The Churchill Hospital, Headington, Oxford, according to the procedures stipulated on his licence issued under Acts mentioned above. Similarly all experimental procedures involving marmosets were performed according to guidelines in the Act for primate work under the supervision of Dr R.J. Tudor, D.H.S.S. Department of Toxicology, St. Bartholomew’s Hospital Medical College, London, UK.

3.2.2 Rats

Male Wistar rats (Porton strain, random bred closed colony maintained by Experimental Biology Unit, University of Surrey) were used. Rats were regularly handled prior to dosing, to minimise stress responses during experiments. The rats were given food (Spratts Laboratory Animal diet No. 1, Spratts Barking, UK) and tap water ad libitum.
They were housed in translucent "shoe-box" type cages; dimensions 60 x 35 x 18 cm (up to 9 rats per cage) on sterile soft wood shavings (Lee and Son, Chertsey, Surrey, UK) and acclimatized to a controlled environment (12 hours light : dark schedule with the light cycle beginning at 0730 hr GMT), temperature of room maintained at 23±1°C and humidity at 40±10% and 15 air changes per hour.

3.2.3 Mice.

Several strains [Schnider (Sch) (University of Surrey strain), Obese (Ob/Ob) (University of Surrey strain), C57Bl/6 and Balb/c (Bantam and Kingman Ltd, Hull, N. Yorkshire), Nude mice (MFl-nu/nu/Ola/Hsd) (Harlan Olac Ltd, Shaw's Farm, Blackthorn, Bicester, Oxfordshire, UK.)] were used in a number of studies, investigating differences in response to nephrotoxins (see section 3.6.2). All strains were housed in translucent mouse box cages, dimensions 32 x 25 x 16 cm (with no more than 12 mice per cage) on sterile soft wood shavings (Lee and Son, Chertsey, Surrey, UK) and acclimatized to a controlled environment as outlined in section 3.2.2 above. Germ free Nude mice were maintained in an isolator system (see section 3.6.6.4.D).

3.2.4 Pigs

The animals were maintained at the CRC Normal Tissue Radiobiology Research Group, (University of Oxford) Research Institute, Churchill Hospital, Oxford, under the supervision of Dr. Mike Robbins. Female Large White pigs (from the Pig Improvement Company, Oxford, UK.) were used. These were kept in pens (indoors) in a controlled environment of (12 hours dark/light schedule), temperature maintained at 18±2°C, humidity - not controlled: atmospheric. Animals were housed for a two week acclimatisation period prior to commencing any experimental
procedure to minimise any stress induced due to their change in environment. The animals were fed on a proprietary pig diet (Speedipork, BBO Ltd, UK) and allowed water from a demand/access system. In the initial experiments the animals were 6-7 months old with a body weight of 60-70 kg. The later experiments were performed on younger pigs aged 14 weeks and with body weights 20-30 kg. The reasons for using younger animals were: economical (pigs cost £40 each, and £1.50 a day to keep, therefore young pigs are more cost effective), and space (younger pigs are smaller and so it was possible to keep more in the limited amount of pen space that was available).

3.2.5 Marmosets.

Animals were housed in the Animal Unit of DHSS Department of Toxicology, St Bartholemew's Hospital, Medical College, London and maintained in a controlled environment with 12 hr light from 0700-1900 hr, temperature 23±3°C, relative humidity 50±10%. The animals were housed individually in metabolic cages, and fed SDS Marmoset diet and fruit, and allowed tap water ad libitum.

3.2.6 Animal Identification.

Animals (rats and mice) were identified using a numbering system. Each animal either had one or both ears punched using punch pliers according to the system illustrated in Figure 3.1. Four punch positions on the left ear represented single figures; 1, 2, 4 or 5, whereas equivalent positions on the right ear represented tens; 10, 20, 40 or 50, complete holes on the right ear represented hundreds; 100, 200. Thus it was possible using this system to give all animals individual identification numbers up to 299.
Figure 3.1 Schematic diagram illustrating the ear punch identification system for rats and mice. a) illustrates punch positions and their allocated numerical values; b)-e) illustrate examples of identification numbers constructed with different punch positions: b) 3, c) 6, d) 48 and e) 179.
In the carcinogenesis experiments each animal was given an individual number. In some of the short-term toxicity studies (especially with mice) all the animals in one experimental group were identified by the same number ie control = 1, low dose = 3, high dose = 4. The pigs were given individual identification numbers were on plastic "punch" tags which were attached to the animal’s ear.

3.3 DOSING PROTOCOLS.

3.3.1 Anaesthetics.

3.3.1.1 Rats and mice.

These were anaesthetised using sodium pentobarbitone (Sagatal) 60 mg/kg intraperitoneally (ip). To allow an accurate injection volume to be administered to mice, the Sagatal was freshly diluted 1:10 with physiological saline (0.9% NaCl w/v) immediately prior to injecting. Ether (diethyl ether) anaesthesia was used on occasions where only a postmortem examination was to be performed and no actual surgical procedures. This was the preferred method of anaesthesia (or pre-anaesthesia to reduce the stress for the large (600g plus, body weight) rats prior to administering the Sagatal), in the carcinogenesis experiments. A measured volume of ether (50 ml) was poured on to a cotton wool pad which was held in a wire holder within the bucket (with a secure lid) which was used as the anaesthetising chamber. Animals were then placed in this chamber for a suitable period until they were completely anaesthetised.

3.3.1.2 Pigs.

The pigs were anaesthetised with a gas mixture of 2-3% halothane, 30% nitrous oxide plus 70% oxygen as administered by means of a mask which covered the animals snout.
3.3.2 2-Bromoethanamine hydrobromide (BEA) MW 204.9 C₂ H₇ N Br₂
Animals were injected intraperitoneally (i.p.) with a single dose (50-100 mg/kg body weight) BEA freshly prepared (Bach et al, 1983) in physiological saline (100 mg BEA per ml of saline) at 0900 hours unless otherwise stated.

3.3.3 N-butyl-(4-hydroxybutyl)-nitrosamine (BBN).
BBN is a yellow-brown oil, chemical formula: C₈ H₁₈ N₂ O₂
MW 174.3, and is soluble in common organic solvents.
BBN is heat and light sensitive, therefore preweighed vials containing 2g BBN were wrapped in aluminium foil placed in outer "carcinogen" containers and stored at -20°C. Immediately prior to use 12.5 ml of 20:80 ethanol:water solution was added to 2g BBN to obtain a solution where 0.5 ml contained 80 mg BBN.
Animals were dosed twice weekly for 5 weeks to a total dose of 800 mg. Each dose of 80 mg was administered in a 0.5 ml volume of a 20:80 ethanol:water solution by oral intubation (Becci et al, 1979; McCormick et al, 1981a). Oral gavage dosing of this carcinogen was chosen because it has been shown by Becci et al (1979) and McCormick et al, (1981a) to be a more controlled method of quantitatively dosing animals, thus less animals are required overall. In addition there is a minimum risk of exposure to staff administering the BBN compared to dosing via dietary or drinking water methods and there is a very limited likelihood of control or other animals being affected.
Animals in experiment number 5 (section 3.6.5.5) received a total of 400 mg of BBN; they were dosed twice weekly for 2.5 weeks. This was an attempt to reduce the bladder tumour incidence in the positive control groups (i.e., BBN only treated).
3.3.3.1 **Safety Procedures for working with BBN**

BBN was weighed out according to GLP procedures for high risk chemicals instigated by Robens Institute (Protocol: GTX/METHODS/-CARCINOGENS).

1. Each worker involved in dosing BBN wore the following protective equipment to reduce the possible exposure risk:
   a) full length total cover elasticated cuff lab coat
   b) disposable face mask
   c) disposable paper coverall "J-suit" including slipover boots
   d) flow helmet
   e) 2 pairs gloves (1 disposable surgical, 1 industrial rubber)

2. The mixing of solutions and filling of syringes was performed in a plastic seed tray (40 x 25 x 3 cm) within a radioactive work tray (100 x 50 x 6 cm), both coated with benchkote (absorbent side up) and all equipment was labelled with carcinogen tape.

3. All dosing was carried out by oral gavage over an empty cage, syringes were disposed in a labelled Cinbin receptical which was "double bagged" when full and disposed of by Hazardous Waste procedures implemented by the University.

4. After dosing any BBN residues remaining were diluted in a 5% Decon 90 solution and the glass vial returned to carcinogen container. Both container and vial were be disposed of by approved Hazardous Waste procedures implemented by the University.

5. The cages were cleaned twice a week, sawdust etc placed directly into black bin bags. No more than three cage loads of waste per bag. This was placed inside a second bag and sealed with carcinogen tape for immediate incineration. (In BBN study number 5, the University
had changed its policy on disposing of carcinogenic bedding and it had to be disposed of through the Hazardous Waste procedure).

6. The empty cages were decontaminated using 5% (v/v) Decon 90 solution for at least 48 hr. These cages were then thoroughly rinsed and placed in the normal cage washing procedure.

7. The benches were covered with black plastic and benchkote, decontaminated after each dosing/cleaning session by a fine spray of 5% (v/v) Decon 90 and wiped with Kimwipes, all cleaning material was double bagged and incinerated.

8. The floor was cleaned after each session with 5% (v/v) Decon 90 solution.

3.3.4 Aspirin (Acetylsalicylic acid) MW 180.15 C₉ H₇ O₄

Animals were dosed twice weekly for 18 weeks by gavage with aspirin at 90 mg/kg. The aspirin was administered as a suspension (90 mg/ml concentration) in 1.25% (w/v) carboxy-methylcellulose. Each animal received 0.1 ml/100g body weight.

3.3.5 Paracetamol (Acetaminophen) MW 151.16 C₈ H₉ N O₂

3.3.5.1 Pilot study (see section 3.6.5.2)

Animals were dosed twice weekly for 18 weeks by gavage with paracetamol at 75 mg/kg. The paracetamol was administered as a suspension (75 mg/ml concentration) in 1.25% (w/v) carboxy-methylcellulose. Each animal received 0.1 ml/100g body weight.

3.3.5.2 Main study - BBN study 5. (see section 3.6.5.5)

Animals were dosed twice weekly for 18 weeks by gavage with paracetamol at 100 mg/kg. The paracetamol was administered as a suspension (100 mg/ml concentration) in 1.25% (w/v) carboxy-methylcellulose. Each animal received 0.1 ml/100g body weight.
3.3.6 N-Phenylantranilic acid (NPAA) MW 213.23 C_{13} H_{11} N O_2
Animals were dosed twice weekly for 18 weeks by gavage with NPAA at 107 mg/kg. The NPAA was administered as a suspension (107 mg/ml concentration) in 1.25% (w/v) carboxy-methylcellulose. Each animal received 0.1 ml/100g body weight.

3.3.7 Sodium N-phenylantranilate (NaNPAA) MW 235.23 C_{13} H_{10} N O_2 Na
Animals (pigs) were dosed with a single intravenous infusion (given over a 30 min period) of NaNPAA (117.5 or 235 mg/kg bodyweight) freshly prepared in distilled water (235 mg/ml concentration).

3.4 TISSUE PREPARATION.

3.4.1 Fixation.
The purpose of fixation is to preserve the morphology of tissue as near as possible to that in life minimising shrinkage and distortion artefacts. The choice of fixative is critical when the detection of enzymes and antigens is attempted. The process of morphological preservation and retention of enzymic and antigenic activity to the same degree is difficult as each criteria compromises an adequate expression of the other criteria. Low concentrations of the commonly used aldehyde fixatives glutaraldehyde and formaldehyde, i.e. 3-4% coupled with low temperature 0-4°C for short periods of fixation 24 hr as a maximum have been used to adequately preserve morphology together with enzyme activities (Burnett, 1982; Alpers & Beckstead, 1984, 1985) and antigen activity (Beckstead, 1985). For these reasons the standard fixation procedure for rat and mouse tissues was immersion for 24 hr in formal calcium fixative (4% v/v formaldehyde, 1% w/v calcium chloride, based on the method from Bancroft & Stevens, 1982). The addition of extra cellular calcium enhances the retention
of enzyme activity. Pig tissues were fixed for longer periods between 48-72 hr because of the increased mass of pig kidneys. This did not seem to have a detrimental effect on the retention of histochemically determined enzymic activity.

3.4.2 Postmortem and Tissue Processing.

1. Animals were sacrificed by: either cervical dislocation or anaesthetised using sodium pentabarbitalone - Sagatal at 60 mg/kg body weight i.p., unless otherwise stated.

2. The abdominal cavity was opened via a midline incision from pubic symphysis to xyphoid process and the peritoneum incised carefully with scissors then retracted, together with the skin flaps to expose the intestines.

3. The intestinal tract was carefully moved to the left hand side of the animal thus exposing the kidneys, ureter and bladder.

4. A loose ligature was placed around the 'neck' of the bladder. The bladder was then gently emptied of urine (if it was still full) by applying manual pressure using forefinger and thumb, or if a sample of urine was require then a 2ml syringe attached to a 23G needle was used to aspirate the bladder of urine.

5. A 2 ml plastic syringe containing cold 4°C formal fixative (see section 3.3.1 for details) was attached to a 23G needle. Introducing the needle close to the neck of the bladder, the fixative is injected thus inflating the bladder with 1.5 - 2 ml of fixative.

6. Once the bladder was inflated, the ligature was tied tightly, the needle and syringe removed and the bladder dissected free from the animal using scissors and immersed immediately in cold 4°C formal:calcium for 24 hr.
7. The kidney(s) and ureter were dissected intact after the surrounding fat and fascia had first been removed by blunt dissection.
8. The kidney(s) were depoled using a single edged razor blade to facilitate the diffusion of fixative to the inner medulla and papilla regions. The depoling cuts were made such that it is possible to distinguish easily between left and right kidneys.
9. After 24-48 hr fixation tissue specimens were obtained as follows
9a. The bladder was sectioned longitudinally in half. The internal surface was examined under a stereomicroscope.
9b. The kidney was sliced in a transverse place through the hilus so as to obtain a 1 - 2 mm thick slice containing the papilla tip. The upper 2 cm of the ureter was dissected free of the kidney.

3.4.3 Buffer Washing removes excess fixative solution from the tissue which could affect the infiltration and embedding of the tissue. Later, once sections have been prepared, excess fixative solution remaining in the tissue may alter the staining properties of the tissue. Buffer washing also maintains the osmolality of the tissues, reducing shrinkage and artefacts.

Buffer - 0.1M Phosphate buffer pH 7.2-7.4
1. Stock A - 0.2 M sodium dihydrogen orthophosphate (MW 156.01)
2. Stock B - 0.2 M disodium hydrogen orthophosphate (MW 141.96)
3. Solution for pH 7.2 14.0 ml - Stock A, 36.0 ml - Stock B and make up to 100 ml with 50.0 ml - Distilled water.
This solution should also be stored and used at 4°C.
Procedure was modified from traditional electron microscope procedures with tissue being rinsed and washed in four changes of 0.1
M. Phosphate buffer of 30 minutes each on a rotatory mixer maintained at 4°C.

3.4.4 Dehydration removes aqueous tissue fluids including buffer and replacing it with an organic solvent which is usually miscible with the embedding media. A graded series of alcohols (ethanol) was used (following the procedure below) to dehydrate the tissue and still maintain enzyme activity. All the dehydrating solutions are used at a temperature of 4°C.

1. Replace buffer, rinse with 50% v/v ethanol and allow tissue to 'rotate' in this solution for 20 - 30 min.
2. 70% v/v ethanol was substituted for 50% ethanol - 20-30 min.
3. 100% v/v ethanol was substituted for two changes of 60 min.

3.4.5 Infiltration.

The choice of glycolmethacrylate as the embedding media was made because of several factors:

1. Plastic embedding preserves tissue structure much more faithfully than does paraffin.
2. The hydrophilic nature of glycolmethacrylate allows convenience in dehydration and versatility in staining sections.
3. The ability to cut 1-2 um semithin sections offers unprecidented light microscope resolution.
4. Glycolmethacrylate resin allows the use of enzyme histochemistry and high resolution autoradiography on the same and serially cut tissue sections.
5. En bloc macroscopic examination and photography is possible, as is low magnification electron microscopy (on formvar coated grids using carbon coated sections).
6. Immunohistochemistry, including the use of colloidal gold labels is also possible.

**Procedure**

* ALL procedures involving glycolmethacrylate should be performed in a fume cupboard.*

A 100 ml volume of JB-4 solution A was catalysed by adding 0.90 g of catalyst (1 spoonful) while stirring until all the solid has dissolved. Approximately 30-45 min at room temperature with a magnetic stirrer. Exclude air by the use of cling film over neck of flask. Catalysed Solution A is then used to infiltrate the alcohol dehydrated tissue. The final 100% ethanol solution was decanted off the tissue specimen and solution A is poured directly onto the specimen. Sufficient solution A was added to allow the tissue specimen to float. Upon becoming properly inflated the tissue became translucent and sink to the bottom of the container. Overnight gentle rotation at 4°C is recommended for most tissue specimens to become infiltrated.

**3.4.6 Embedding.**

1. 1 ml of JB-4 solution B was added to 25 ml of freshly catalysed solution A (which is over ice to slow down polymerisation), stirred well, and used as quickly as possible after mixing.

2. The moulds used for embedding were clean, dry and free of dust (which would otherwise become embedded and scratch the knife-edge during sectioning). Using a disposable pipette (and wearing protective disposable surgical gloves), 2-3 ml of solution A+B was placed into a mould cup. The tissue specimen was placed into the cup and orientated in the middle of the cup leaving some excess resin around the tissue.
3. A prewritten label which has the tissue identification code written in pencil (many inks are soluble in JB-4 resin!) was placed on a nylon embedding stub. The block holder was then placed over the tissue in the mould and the remaining capacity filled with resin.

4. For best polymerisation results, the moulds were placed in a dessicator under vacuum or nitrogen atmosphere, because oxygen inhibits the polymerisation of JB-4 resin. However, as a very small surface area of the block is exposed to the air when a nylon holder is placed in the mould adequate polymerisation can be achieved in normal atmosphere. The polymerisation was allowed to proceed at room temperature for 2-3 hr or alternatively overnight at 4°C. Once polymerised the blocks were removed from the moulds. There was often a liquid film at the air/resin interface which was wiped off.

5. JB-4 resin blocks were found to be easier to section if allowed to stand open in the air for a few hours prior to sectioning. If the blocks soften due to absorption of atmospheric moisture in humid weather, they were dried over silica gel. Conversely if they become too brittle due to drying out they were restored by placing in controlled humidity. The blocks were placed in a dessicator containing a dish of saturated solution of potassium carbonate, allow 2 hr to equilibrate then remove the potassium carbonate and the blocks were left to stand for 1-2 days.

3.4.7 Sectioning.

To obtain the 1-2 um thick semithin sections required to allow high resolution light microscopy it is necessary to cut the JB-4 resin using glass Ralph knives.

3.4.7.1 Making Ralph type glass knives.
1. The glass was cleaned with detergent (Decon 90) or a suitable solvent (absolute alcohol or acetone) to remove all grease and dust. Rinsed thoroughly in water and dried.

2. Ralph knives can be made adequately using the "LKB 2078 Histoknife" maker following the manufacturers recommended procedure.

4. The knives are mounted onto aluminium strips (dimensions: 17 x 2.5 x 0.6 cm) which fit into the knife holder of the Bright 5030/WD/MR-CV microtome.

5. The strips are warmed to 60°C on a hot plate. A single large drop of dental wax is placed on the centre of the strip.

6. With the knife edge uppermost, place the knife onto the drop of dental wax. Using firm downward pressure position the knife so that there is a thin layer of wax covering the underside of the glass knife and the knife edge is parallel to the edge of the aluminium strip.

7. Remove the assembled knife and strip from the hot plate and allow the wax to cool and harden. This method of attachment is quick, economical and quite satisfactory when sectioning soft tissues such as kidney.

3.4.7.2 Cutting sections.

1. Use a dry glass knife with a Bright 5030/WD/MR-CV microtome, this is capable of advancing between cuts in increments to 1 um.

2. Blocks are mounted in the microtome so that its shortest edge is parallel to the knife edge and its block face is vertical relative to the knife edge.

3. Excess resin has to be trimmed off the block prior to sectioning the actual tissue. The microtome setting for this procedure can be at a thickness between 5 um and 12 um.
4. Once tissue is visible in trimmed sections, reduce the advance setting to 2 um and continue trimming until the exact tissue area appears in the section. Orientation of the block may be necessary relative to the knife edge. Once the correct tissue area has been reached, 'lock' the knife holder and platform using the locking bar.

5. A slow cutting speed is used, as fast speeds produce vibration between block and knife edge resulting in 'chattering'.

6. As the section is cut it is often folded on itself. The operator can, with practice, tease and stretch the section to loosen the folds, using watch makers forceps and a small brush. As the section comes off the knife edge lightly breathing on it helps it to flatten out and reduces the folding because the section absorbs some of the warm water vapour.

7. The section is then dropped onto a water bath containing clean distilled water at room temperature. The section will spread out and should be a clean scratch-free and fold-free surface with the tissue portion clearly visible.

8. To mount the section of a glass slide, insert the clean slide at an angle into the water on which the section is floating. The slide is then brought over the section so its edge is attracted to the slide by surface tension. By drawing the slide upwards and vertically out of the water the section will attach.

9. The slide is then placed on a hot plate at a temperature of 60°C and the section is allowed to dry onto the slide.

10. Prior to picking up the section, the glass slide was marked with the block identification number using a diamond pencil, or an ordinary lead pencil when frosted end glass slides were used.

11. The sections were now ready for staining and required no
dehydration or clearing procedures.

3.4.8 Preparation of Tissue for Transmission Electron Microscopy (TEM).

The preparation of tissues for TEM is basically the same as for JB-4 glycolmethacrylate preparation; with stages of fixation, dehydration, infiltration and embedding. The main difference being that the tissue blocks are much smaller (ideally about 1 mm$^3$) which means more care in the initial selection of tissue samples. This procedure has been designed to fit in around our fixation method for light microscopy to maximise the use of time etc.

3.4.8.1 Fixation.

1. Immersion of tissues in 2% (v/v) glutaraldehyde (TAAB) in 0.1 M phosphate buffer pH 7.4 at 4°C for 12-18 hr.

   Our routine fixation method is immersion in formal calcium at 4°C (see section 5.4.1) and frequently TEM is decided as a necessary technique only after examining the histological slides at the light microscope level. Thus if the tissue has been processed through the formaldehyde fixation instead of the usual glutaraldehyde fixation it will still be suitable for ultrastructural morphology, even if not "ideal".

2. Wash tissue in four changes of 0.1 M phosphate buffer pH 7.4, at least 30 min per wash.

3. Post fix tissue in 1% (v/v) osmium tetroxide in 0.1 M phosphate buffer pH 7.4 for 1 hr. Use minimal volume of solution, ie sufficient to cover the tissue blocks, osmium is very toxic and expensive using a minimal volume reduces exposure risk and maximises the use of the osmium.
4. Wash the tissue in distilled water prior to dehydrating the tissue.

3.4.8.2 Dehydration.

A graded series of alcohols (50% (v/v), 75% (v/v), 95% (v/v) and 100% ethanol) is used to dehydrate the tissues, change the solutions every 30 min except for the 100% alcohol step when the time is extended to 2 changes of 60 min. An additional dehydration step with propylene oxide may be necessary (if the resin is not miscible with alcohol e.g. Epon) the final alcohol solution is replaced with a 1:1 propylene oxide:alcohol solution left for 20 min and then this solution is replaced with propylene oxide only, again for 20 min.

3.4.8.3 Infiltration.

3.4.8.3.A Spurr Resin (TAAB)

1. Use a rotating mixer. An equal volume of resin is added to the last dehydration solution to obtain a solution of 50:50 concentration Spurr:alcohol, leave for 30 min.

2. A second equal volume of Spurr (Spurr, 1969) is added to the above solution to now give a solution of 75:25 concentration Spurr:alcohol, leave for 30 min.

3. The 75:25 solution is decanted off and 100% Spurr added, leave for 8 hr, the solution is changed for a fresh volume of 100% Spurr and left overnight.

3.4.8.3.B Epon Resin (TAAB).

The final propylene oxide solution is decanted off and replaced with a 1:1 propylene oxide:Epon solution, leave for a minimum of 30 min preferably longer 1-2 hr.

3.4.8.4 Embedding.
3.4.8.4 A Spurr resin: the tissue blocks were placed in fresh Spurr resin within suitable moulds and polymerised at 70°C for 8 hr.

3.4.8.4 B Epon resin: the tissue blocks were placed in moulds containing fresh resin and polymerised at 60°C for 48 hr.

3.4.9 Preparation of Fixed Frozen Sections.

3.4.9.1 Principle of fixed frozen sectioning is that tissue is placed on freezing stage which is connected to continuously running cold water supply and a thermocouple to control the temperature of the stage. The electrical setup cools the block by the cold water extracting the heat away from the stage and tissue block, thus gradually freezing the tissue such that it becomes hard enough to section.

3.4.9.2 Procedure.

1. Tissue was placed on the stage with a small piece of damp tissue paper between the stage and tissue to act as an adherent surface. The tissue was allowed to freeze, bathing the tissue in with fixative applied with a small brush helps the tissue to freeze rapidly.

2. The position, angle and height of stage was adjusted with respect to the microtome blade so that the upper most surface of the tissue is horizontal.

3. The tissue block was trimmed down with the knife to give the largest area possible of the tissue. Narrow blocks cut easier than wide blocks so it was necessary on occassions to trim the sides of the tissue away with a razor blade.

4. Sections were cut at 15 um although with practice and slight adjustments and a well frozen block it was possible to section at 10 um.
5. Sections were picked up by rolling a wet small brush along the section which adheres to the brush and then placing the section in a container of fixative eg a sterilin.

6. To manouvere the sections on to a slide; the sections plus fixative were placed into a beaker or petri dish, then with a glass rod manipulator (glass pipette with rounded end), a section was moved on to a slide. Holding the section in position with the glass rod the slide was removed from the fixative solution and the section adhered to the slide and was allowed to dry for staining.

3.5 STAINING.

3.5.1 Routine Histological Stains.

All of the staining procedures used with JB-4 resin are modifications of procedures already well described for paraffin wax sections (Bancroft & Stevens, 1982; Pearse, 1972).

3.5.1.1 Toluidine Blue.

Solutions

1% (w/v) Toluidine Blue in a 1% (w/v) Borax solution pH 9-11.0.

Procedure

1. Place sections in 1% (w/v) Toluidine Blue solution for 1 min.
2. Rinse sections in running tap water to differentiate for 5 min.
3. Dry sections in slide dryer at temperature of 40°C.
4. Mount coverclips on section using single drop of D.P.X.

Results

Tissue is blue, but surprisingly good differentiation is possible between nuclei, cytoplasm, intracellular granules. In kidney proximal tubule lysosomal granules are particularly well stained.
Metachromasia occurs in interstitial matrix of papilla and medulla where tissue components cause pinkish staining to occur.

This stain is rapid, simple and easily modified by altering pH, time of staining and differentiation to achieve ideal staining for particular tissue being examined. It allows the quality of fixation and embedding to be assessed, and a general idea of the tissue orientation in the block.

3.5.1.2 Haemotoxylin and Eosin (Modified from standard paraffin procedure, times extended slightly).

Solutions
1. Harris Haematoxylin (freshly filtered).
2. 1% (w/v) Eosin Solution
3. 1% (v/v) acid alcohol (1% (v/v) 1 M HCl in 70% (v/v) alcohol).

Procedure
1. Stain sections in Haematoxylin for 15 - 20 min.
2. Wash well in running tap water (for 5 min) until sections 'blue.'
3. Differentiate sections in 1% (v/v) acid alcohol, dip once or twice for 1-2 seconds.
4. Wash in running tap water for 2-3 min until sections 'blue' again.
5. Stain in 1% (w/v) Eosin for 10 min.
6. Wash in running tap water for 5 min.
7. The dehydration and cleaning stage is usually performed here prior to mounting the sections. This is essential in paraffin sections, but can be omitted at this stage for semithin sections. Directly mounting the sections in D.P.X. after air drying at 40°C in a slide dryer.
Results

This stain differentiates a wide range of tissue structures. When coupled with high resolution microscopy on resin embedded semithin sections many intracellular organelles such as mitochondria and lysosomes are also visible.

Typical results are:
Nuclei - (blue black), Cytoplasm - (varying shades of pink), Muscle fibres - (deep pinky red), collagen - (pale pinky red), red blood cells - (orange/red), Fibrin - (deep pink), Mitochondria - (varying shades of pink/red), Lysosomes - (contrasting against cytoplasm).

Steps 1 - 4 are followed as a counterstain to Periodic Acid Schiff (PAS) stain.

3.5.1.3 Giemsa.

Solutions

1. 10% (v/v) of stock Giemsa and water
2. 95% alcohol

Procedure

1. Stain sections in solution of 10% (v/v) Giemsa to distilled water
2. Sections can be stained at room temperature overnight, or for 1 hr, 30 min at 45°C
3. Rinse briefly in distilled water
4.* Differentiate for 1-2 min in 95% (v/v) alcohol
5. Allow to air dry and mount in D.P.X.

* Omit this step if sections stained at 45°C.

Results

This stain gives superb results with respect to interstitial matrix differentiation and is preferred to the more traditional PAS method to stain the mucopolysaccharides. Basement membrane and interstitium -
deep pink, Nuclei - blue/black, Cytoplasm - pale blue.

5.3.1.4 Periodic Acid Schiff (PAS) stain for carbohydrates.

Modified from (Bancroft & Stevens, 1982)

Procedure

1. Immerse sections in a 1% (w/v) periodic acid solution for 15-20 min.
2. Rinse sections in distilled water for 2-3 min.
3. Immerse sections in Schiffs Reagent for 60 min.
4. Rinse sections in running tap water for 5 min.
5. Counter stain with Haematoxylin (follow steps 1-4 in Haematoxylin and Eosin procedure. section 3.5.1.2).

Results

Tissue areas having carbohydrate containing moieties within them stain pink, e.g. proximal tubule brush borders, basement membranes, lamina propria of urothelium and interstitial cell matrix.

3.5.1.5 Oil red O stain for lipids.

Performed on fixed frozen or frozen sections only.

Solutions

Stock solution: Oil red O saturated in 95% (v/v) isopropanol.

Working solution (prepare 1 hr in advance), 3 volumes of Oil red O stock solution with 2 volumes of distilled water and filter prior to use.

Procedure.

1. Dry sections onto the slides.
2. Rinse sections in 60% (v/v) isopropanol.
3. Stain in Oil red O working solution for 15 min.
4. Differentiate in 60% (v/v) isopropanol until a delipidised
5. Wash in water.
6. Counterstain in Mayer's haemalum.
7. Wash in tap water.
8. Rinse in distilled water, mount in glycerin jelly.

Result

Unsaturated hydrophobic lipids and mineral oils stain red, some phospholipids stain pink.

Lipid extraction for delipidised control sections.

Chloroform-methanol solution (2:1 v/v) for 1hr at room temperature.

3.5.2 Enzyme Histochemical Stains.

The following methods are modified from the methods of Burnett (1982) used in a study of enzyme histochemistry in JB-4 methacrylate sections.

3.5.2.1 Gamma-glutamyl-transpeptidase (GGT) (Modified by Pearse, 1972 from the method of Glenser & Folk, 1961)

Solutions

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. N-(gamma-L-glutamyl-beta-napthylamide) 4 mg/ml acetone.</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>2. Aqueous glycglycine 20 mg/ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>3. TRIS buffer 0.1M pH 7.2</td>
<td>10.0 ml</td>
</tr>
<tr>
<td>4. Fast Garnet Red (G.B.C.) 15 mg/10 ml incubating media.</td>
<td></td>
</tr>
</tbody>
</table>

These volumes were adjusted to fill a coplin jar for routine staining of 10 slides, i.e. 50 ml.

Procedure

1. Incubate sections in medium for 4-6 hr at 37°C, checking sections macroscopically periodically for adequate staining.
2. Rinse throughout with distilled water.
3. Counterstain with Harris Haematoxylin (follow steps 1-4 in Haematoxylin and Eosin method, see section 3.5.1.2)

4. Air dry and mount in glycerin-gelatin mountant, as D.P.X. will 'lift' stain from sections!

Results

A reddish-brown final reaction product indicates positive activity.

3.5.2.2 Alkaline phosphatase (alk. phos.). (After Burnett, 1982)

Gomori calcium method

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 2% (w/v) sodium glycerophosphate</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>2. 2% (w/v) sodium barbitone</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>3. 2% (w/v) calcium nitrate</td>
<td>10.0 ml</td>
</tr>
<tr>
<td>4. 1% (w/v) magnesium chloride</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>5. Distilled water</td>
<td>2.5 ml</td>
</tr>
</tbody>
</table>

The pH of the incubation medium should be between pH 9-9.4, the final volumes adjusted to fill a 50 ml coplin jar.

Procedure

1. Preheat the incubation medium to 37°C

2. Incubate the sections for 21 hr at 37°C

3. Rinse sections in distilled water, at least 3 changes

4. Immerse sections in 2% (w/v) cobalt nitrate for 5 min.

5. Rinse repeatedly in distilled water

6. Immerse in 1% (v/v) ammonium sulphide for 2 min.

* Perform this procedure in a fume cupboard as hydrogen sulphide gas generated is poisonous and smells of rotten eggs!

7. Rinse repeatedly in distilled water.

8. Counterstain in 2% (w/v) methyl green for 5-10 min., or Harris Haematoxylin for 5 min.
9. Rinse in running tap water
10. Air dry and mount in D.P.X.

Results

A black final reaction product indicates positive activity.

3.5.2.3 Adenosine Triphosphatase (ATPase) (Calcium method of Padykula & Herman, modified by Bancroft & Stevens, 1982)

Solutions

1. Veronal buffer pH 4.6: sodium acetate (trihydrate) (1.94 g in 100 ml distilled water) - 5 ml, sodium barbitone (2.94 g in 100 ml distilled water) - 5 ml, 0.1M Hydrochloric acid - 10 ml, distilled water 8 ml.

   Incubating Medium

2. 0.1M sodium barbitone 2.94 g in 100 ml distilled water 6 ml
3. 0.18M calcium chloride 2.34 g in 100 ml distilled water 3 ml
4. Distilled water 21 ml
5. Adenosine Triphosphate 75 mg

   Adjust incubating medium to pH 9.4

Procedure

1. Pre-incubate the sections in the veronal buffer pH 4.6 for 15 min. at 37°C
2. Incubate sections in incubating medium at 37°C for 21 hr.
3. Rinse in 3 changes of 0.18M calcium chloride solution
4. Immerse sections in 2% (w/v) cobalt chloride for 5 min.
5. Rinse in 4 changes of a 1:10 dilution of 0.1M sodium barbitone solution
6. Rinse in tap water
7. Transfer to 1% v/v ammonium sulphide for 2 min.
8. Wash in running tap water
9. Counterstain in 2% (w/v) methyl green for 5-10 min.
10. Wash in running tap water for 5 min.
11. Air dry, and mount in D.P.X.

Results
A black final reaction product indicates positive activity.

3.5.2.4 Acid Phosphatase (Acid Phos.) (Modified from Burnstone, 1958)

Solutions
1. Napthol As-Bl phosphate  5.0 mg in 0.5 ml DMSO
2. 0.2M acetate buffer pH 5.2  25 ml
3. Distilled water  24 ml
4. Red violet LB  30 mg
5. 10% (w/v) manganese chloride  2 drops per coplin jar

Media

Procedure
1. Incubate sections in medium for 21 hr at 37°C
2. Rinse thoroughly in distilled water
3. Counterstain in Haematoxylin for 10 min.
4. Wash in running tap water for 5 min.
5. Air dry, and mount in glycerin-gelatin mountant

Results
Red final reaction product indicates positive activity.

3.6 EXPERIMENTS.

3.6.1 Short-term Acutely Induced RPN in Wistar Rat.

This study was performed to identify the earliest morphological change occurring during the onset of acute RPN induced with BEA. Enzyme histochemical staining was performed to try and elucidate if subtle
cellular changes occurred which could not be detected by routine histological staining and may give an insight into the mechanism of pathogenesis.

Thirty animals were dosed with BEA at 100 mg/kg as described in section 3.2.2. Groups of 3 rats were sacrificed at 2, 4, 6, 8, 12, 18, 24, 48, 72 and 144 hr, and groups of 2 control animals were sacrificed at 12, 48 and 144 hr. Kidney, ureter and bladder tissues were processed for glycolmethacrylate resin embedding (described in section 3.4.2). Semithin sections were stained with routine histological stains (section 3.5.1) and by enzyme histochemistry (section 3.5.2).

3.6.1.1 Monastoral blue B Vascular labelling Study.

This investigation was performed to determine whether leaky vasculature was involved in the initiation of RPN. Monastoral blue B is a copper phthalocyanine used for as a intravascular marker. This colloidal substance has certain advantages over colloidal carbon; water-insolubility, non-toxicity, uniform particle distribution, commercially available and has high contrast in both thick and thin sections (Joris et al, 1982).

Two animals from each group was anaesthetised using Sagatal (see section 3.3.1.1) and the abdominal cavity opened as described earlier (see section 3.4.2). Monastoral blue B (3% (w/v) suspension) was administered (0.1 ml/50g body weight) by introducing a 23G needle (attached to a 1 ml syringe) through the diaphragm directly into the left ventricle of the heart. The Monastoral blue B label was injected slowly, after 1 min. the kidneys were removed, fixed in formal calcium and processed for high resolution light microscopy as described in sections 3.4.3-3.4.6.
3.6.2 Chemically Induced RPN in Mice.

3.6.2.1 Study of different doses of BEA in the Schnider mouse.

Twelve male Schnider mice (University of Surrey strain) were housed as outlined in section 3.2.3. Five groups of 2 mice were treated with different doses of BEA (section 3.3.2) at concentrations ranging from 25-200 mg/kg. Control animals (2) were dosed with vehicle only. Each dose volume was calculated for a mouse of average weight of 25g, this injection volume was adjusted ± 0.1 ml/5g body weight depending on the body weight.

Animals were ear clipped for identification (see section 3.2.6) and allowed free access to food and water for a period of 60 hr when all animals were sacrificed by cervical dislocation.

Tissues were prepared as outlined in section 3.4 and sections stained for Giemsa, H&E, Toluidine blue and PAS stains (section 3.5.1).

3.6.2.2 Time course study of different doses of BEA in Nude (MF1-nu/nu/Ola/Hsd) mice.

In the nude mouse xenograft studies (see section 3.6.6) animals were sacrificed because no tumour had taken based on the lack of any visible growth in the region of transplantation after 9 weeks. Immunological changes have been implicated as both the primary event and secondary consequence of a variety of renal lesions (Druet et al, 1987), but there is no published data on the use of Nude mice in nephrotoxicity studies. There has been some suggestion that immunological changes may have an important role in the development of analgesic nephropathy (Hook et al, 1979) based on the argument that metabolites of phenacetin (eg p-phenetidine) and salicylate were strongly immunogenic. Gault et al, (1971) found no immune deposits in
patients with early analgesic nephropathy, but there is little information on such changes in experimental models. These animals were used for additional studies in BEA-induced RPN to increase the species/strain data; to determine whether immunologically deprived strains responded differently to mouse strains that are immunologically complete.

Twenty two male MF1-nu/nu/Ola/Hsd mice (Harlan Olac) were dosed ip with either 50, 100 or 200 mg/Kg bodyweight dose of BEA, injection volume 0.1 ml per 10 g body weight of a 10 mg/ml (w/v) solution of BEA in physiological saline. Control animals received physiological saline ip at 0.1 ml per 10 g body weight. Animals were observed after treatment for any gross behavioural changes. Periodic sacrifices of animals from each dose group were performed at 24, 48 and 72 hr post BEA treatment. Animals were sacrificed by diethyl ether anaesthesia and then cervical dislocation. Table 3.1 summarises the dosing and sacrifice protocol. Animals were postmortemed and the tissues processed as described in section 3.4.

Table 3.1 showing dose and sacrifice time points for nude mice acute BEA studies.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>0</th>
<th>50</th>
<th>100</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>48</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>72</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Totals</td>
<td>4</td>
<td>4</td>
<td>8</td>
<td>6</td>
</tr>
</tbody>
</table>
3.6.2.3 Time course study of BEA in 3 different mouse strains.

The part of this study reporting data from 3-48 hr was performed as part of an M.Sc Toxicology student project with Ms. J. Scarlett (Scarlett, 1988; Scarlett et al, 1990). This investigation was extended to include additional time points (7, 14 and 21 days) by the author, and is presented with the histopathology of the earlier time points as an integral part of this thesis.

3.6.2.3.A Experimental protocol.

Male Obese mice (University of Surrey strain) body weight: 30±8g; male Balb/c mice (Bantam and Kingman Ltd, Hull, E. Yorkshire, U.K.) body weight 20±1.25g and male C567Bl/6 mice (Bantam and Kingman Ltd.): body weight 22±2g were used in this study. The Obese mice had a greater body weight range as a consequence of the supplier (University of Surrey Animal Unit) breeding up a small colony to provide sufficient numbers of mice, so supplying mice of a greater age/weight range than the commercial suppliers. The Obese mice used in this experiment were not old enough for those destined to develop gross obesity to be identified.

The animals were housed (10-12 animals per cage) in translucent shoe box type cages of dimensions 32 x 25 x 16 cm on sterile soft wood shavings in a controlled environment. Food and water was available ad libitum. One group was dosed with BEA 100 mg/kg i.p., (0.1 ml per 10 g body of a 10 mg/ml (w/v) solution in physiological saline (0.9% (w/v) sodium chloride) and the controls received physiological saline only.

Urine collection: animals (n = 3) were housed in metal metabolism cages (aluminium with a wire mesh floor) immediately after dosing for the 3, 6 and 12 hr groups, and 12 hr prior to sacrifice for the 24 and
48 hr groups. Water was available *ad libitum*, but no food was present. In individual animals any urine present in the bladder was aspirated using a 23G needle and placed in a microcentrifugation tube.

Groups of animals (n=3) from treated and control groups were sacrificed periodically at 3, 6, 12, 24, 48 hr and 7, 14 and 21 day after BEA administration by anaesthesia in diethyl ether, then cervical dislocation. Animals were weighed at sacrifice, kidney weight was recorded. Tissues were fixed in formal calcium at 4°C and embedded in glycolmethacrylate resin. Semithin (2 um) sections were stained with H&E, Giemsa, PAS and enzyme histochemistry performed for acid phosphatase and alkaline phosphatase and gamma-glutamyltranspeptidase (see sections 3.5.1 and 3.5.2).

**Urine samples:** Individual urine samples obtained from post mortem at were immediately analysed for osmolality. Any urine remaining was placed in a microcentrifuge tube and stored at 4°C for urinary urea nitrogen determination at a later time. Pooled urine samples were stored at 4°C for the later determination of osmolality and urea nitrogen.

**Blood samples:** These were centrifuged at 5,000 rpm for 3-4 minutes and serum stored at 4°C until analysed for osmolality and urea nitrogen content.

**Osmolality:** the osmolality of both urine and serum samples was determined in mmol/kg using a Wescor Inc. 5100C Vapour Pressure osmometer using standards from 290-1000 mmol/kg.

**Urea nitrogen:** the urea nitrogen in urine (diluted 100 fold) and serum was determined using a blood urea nitrogen kit No. 535 (Sigma Diagnostics, Poole, Dorset, U.K.).
Calculations: all data are presented as mean ± standard deviation.

3.5.3 BEA induced RPN in Marmosets.

Two animals were used in this experiment and housed as outlined in section 3.2.5. One animal was dosed with BEA at 50 mg/kg, the other received a dose of BEA at 100 mg/kg, prepared as detailed in section 3.3.2. Both animals were then allowed food and water ad libitum for 48 hr after which they were sacrificed by staff at D.H.S.S., Dept of Toxicology, St. Bartholomew's Hospital Medical College, London. Kidney, bladder and ureter tissues were immersion fixed and processed for glycolmethacrylate resin and embedded (section 3.4) routine histological stains of Giemsa, H&E, Toluidine Blue and PAS (section 3.5.1) and alkaline phosphatase (section 3.5.2) were performed.

3.6.4 Chemically Induced RPN in the Pig.

3.6.4.1 Introduction.

The primary choice of rodents for nephrotoxicity studies reflects their low cost, ready availability, ease of handling and the considerable base-line data on renal function and toxicity. However, the extrapolation of nephrotoxicity data from rodents to man is complicated by the marked renal anatomical and functional differences between the two species. In contrast the human and pig kidney are remarkably similar in terms of physiological and anatomical characteristics (Table 3.2). Both are multipapillate, produce urine of similar osmolality, and have GFR values, numbers of glomeruli per kidney and nephron dimensions that are comparable. The pig kidney is thus an attractive model with which to study clinically important nephrotoxicities, particularly RPN. A series of studies were
<table>
<thead>
<tr>
<th>Species</th>
<th>Gross Anatomy</th>
<th>GFR ml/min/1.73 m²</th>
<th>N*Glomeruli</th>
<th>Glomeruli radius mm</th>
<th>Prox. Tubular radius mm</th>
<th>Maximal Osm.</th>
<th>Data from Stolte &amp; At (1982)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Man</td>
<td>MP 75</td>
<td>2 x 10⁶</td>
<td>100</td>
<td>16</td>
<td>1400</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>UP 35</td>
<td>6 x 10⁴</td>
<td>61</td>
<td>12</td>
<td>2610</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pig</td>
<td>MP 72</td>
<td>2 x 10⁶</td>
<td>83</td>
<td>30</td>
<td>1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dog</td>
<td>UP 104</td>
<td>8.8 x 10⁵</td>
<td>90</td>
<td>20</td>
<td>2610</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MP = Multipapillate
UP = Unipapillate
undertaken to determine whether BEA would induce an acute RPN lesion in the pig in a similar manner to that developed in the rat.

3.6.4.2 Study 1

Four female Large White pigs, 6-7 months old (weight 61±9 kg), were given freshly prepared 2-bromoethanamine (BEA) hydrobromide (section 3.3.2) by a slow i.v. infusion (over 2-3 minutes) while anaesthetised with a mixture of 2-3% halothane, ~30% nitrous oxide plus ~70% oxygen. BEA was given at dose levels of 50 or 100 mg/kg (n=2). The animals were allowed access to food and water and monitored daily for any signs of distress.

After 7 days the animals were killed with an overdose of halothane anaesthetisation and postmortemed. The kidneys, ureters and bladder were removed intact. The kidneys were then bisected longitudinally along the lateral border through the hilus. All tissues were fixed in calcium:formaldehyde (4°C) for 72 hr. Control kidneys and bladder were obtained from abattoir material. The cut interior surfaces of the kidneys were examined macroscopically and tissue blocks of renal pyramids were prepared (Figure 3.2) to include the papilla tip as described by Lomax-Smith & Seymour (1980a). Together with pieces of ureter and bladder, the blocks were processed and embedded in glycolmethacrylate resin. Semithin sections (1um) were routinely stained with Haematoxylin and Eosin, Giemsa and Toluidine Blue, (section 3.4.1). Fixed frozen sections (section 3.4.9) were cut from control and treated papillae and stained with Oil red O for the presence of lipids (see section 3.5.1.5).

3.6.4.3 Study 2.

Two female Large White pigs of body weight 70 kg were given BEA at a dose of 100 mg/kg iv together with an anti-emetic (Maxalon: high dose,
Figure 3.2 Schematic diagram illustrating how tissue blocks containing a single papilla were obtained from the bisected kidney A) by cutting in a transverse plane B) to allow all the papillae to be seen and dissected C). From Lomax-Smith & Seymour, (1980a).

One animal died 24 hr later, the remaining animal survived with no further distress until sacrifice at 14 days. Kidneys, ureter and bladder were taken for histological examination as described above (see section 3.6.3.1).

3.6.4.4 Study 3.

Four female pigs, 12 weeks (body weight 25-30 kg) were purchased and maintained at the Churchill Hospital Research Institute for a 2 week acclimatisation period prior to commencing the study. This study was performed to determine whether the degree of RPN could be increased with 2 x 50 mg/kg doses of BEA rather than a single dose of 100 mg/kg BEA (which killed the pigs).
Two groups of 2 animals were treated as follows:

Group 1; animals given a single iv infusion of BEA at a dose of 50 mg/kg. These animals were then given a second dose of BEA (50 mg/kg) 7 days after the first dose, then allowed to survive for a further 7 days before sacrifice.

Group 2; animals were dosed with the sodium salt of N-phenylanthranilic acid (NaNPAA), see section 3.1.1.1. This compound has been found to induce RPN acutely in the Wistar rat (Gregg & Bach, unpublished observation) in a similar manner to that after BEA administration. It is a water soluble compound (unlike NPAA) and as such could be administered by a single ip injection (in the rat) up to dose levels of 1 mmol/kg.

One animal was infused iv (as for BEA) with a 0.5 mmol/kg solution of NaNPAA (118 mg/kg). The animal was to have been left 7 days and then sacrificed, however, this compound was extremely toxic and the pig died within 3 min of receiving the dose. The breathing rate dropped dramatically and the animal died whilst still under anaesthesia.

The second animal received a reduced dose of 0.25 mmol/kg infused over a 30 min period. This animal survived until it was sacrificed 7 days later. Kidneys ureters and bladder were taken for histological examination.

3.6.4.5 Study 4.

Four young female pigs (12 weeks old) were purchased and housed for a 2 week acclimatisation period prior to study commencing, a fifth pig was donated by another group at the hospital for use as a control animal (this animal had been a control animal in their study on effects of low dose radiation on skin).
Group A: 2 animals to receive 2 x 100mg/kg dose of BEA via an iv infusion over 30 min. Second dose given 7 days after first, animals to be sacrificed on day 14 of the study. First animal died 14 hrs after being dosed. It was decided not to dose the second animal in this group with the second dose as it was apparent that 100 mg/kg dose of BEA was a toxic dose in pigs (3/6 pigs dosed died within 24 hrs of dosing with BEA 100 mg/kg). Second animal was sacrificed after 14 days together with control animal and animals from group B.

Group B: animals received NaNPAA at dose levels of 0.5 and 1.0 mmol/kg via iv infusion over 30 min. Animals survived until sacrifice 14 days later.

3.6.4.6 Study 5: Effect of paracetamol on the pathogenesis of BEA induced RPN in the pig.

Increasingly there has been a focus of interest in the possible role of acetaminophen (paracetamol) in contributing to end stage renal disease (Sandler et al, 1989; Bennett & DeBroe, 1989). While paracetamol has been implicated in causing renal papillary necrosis (Krikler, 1967; Masters & Krikler, 1976; Dubach, 1983), this drug appears to be one of the least papillotoxic analgesics for experimental animals (Molland, 1978; Nanra et al, 1978; Eknoyan, 1982; Prescott, 1982; Henry & Tange, 1987). Much of the epidemiological information on its use has been confounded by its coformulation with aspirin and/or caffeine and/or polypharmacy with other analgesics and nonsteroidal anti-inflammatory drugs. The use and sale of paracetamol in mixed analgesic powders and as the single compound has, certainly in Europe, increased dramatically over the last decade or so (Pommer et al, 1986; Wing et al, 1989).
There is a need to establish the sub-acute toxicity of paracetamol on a multipapillate kidney with a pre-existing RPN.

3.6.4.6.A Animals and dosing procedures.

Eight young (12 weeks old) Large white female pigs were housed for a two week acclimatisation period prior to commencing the study and animals had access to water on a demand/feed system throughout this investigation. Four study groups were set up: Group 1 - 2 animals were given an iv infusion of BEA (50 mg/kg dose) as described above (section 3.6.3.1), Group 2 - 2 animals were given paracetamol in their diet so that each animal received a daily dose of 100 mg/kg body weight. This dose was calculated on the assumption that a typical analgesic abuser of bodyweight 60 kg consumes annually 2 kg of analgesics. The appropriate quantity of paracetamol powder was added to water-moistened normal pig diet pellets (Speedipork, BBO Ltd, Oxford, UK) and thoroughly mixed by hand. This mixture was then given to the pigs twice daily and was consumed immediately within 10-20 min. Group 3 - 2 animals were treated with a combination of the treatment regimes for Groups 1 and 2; animals were given BEA (50 mg/kg), then fed on a diet containing paracetamol (100 mg/kg/day). Group 4 - two animals were controls and received only the normal diet.


In an attempt to monitor any changes non-invasively we measured the kidney length, diameter and parenchymal thickness of both kidneys for each animal by ultrasound scan (using a Roche Abdoscan instrument). Renal functional parameters were determined by radioisotopic renography (Robbins, 1985): glomerular filtration rate (GFR) using $^{99m}$Tc-DTPA and effective renal plasma flow rate (ERPF) using $^{131}$I-
hippuran. These parameters were determined prior to commencing treatment and on day 26 of the study. Values were expressed as mean ± standard error, the 95% confidence limit values were calculated according to Bailey, (1981).

3.6.4.6.C Gross and microscopic pathology.

At postmortem kidney length, weight and body weight were noted. Tissues (kidney, ureter and bladder) were then fixed in formal calcium and subsequently processed for embedding in glycolmethacrylate resin (see section 3.4). Semithin (2 μm) sections were stained with H&E, Giemsa and Periodic-acid-Schiff (PAS) (see section 3.5.1) and examined for any histopathological changes at the light microscopical level.

3.6.5 Experimental Carcinogenesis Studies

3.6.5.1 Study 1: N-butyl-(4-hydroxybutyl)-nitrosamine (BBN) initiation and BEA induced RPN in rat.

3.6.5.1.A Study 1, aims:

To study the effect on urothelial carcinogenesis of N-butyl-N(4-hydroxybutyl)nitrosamine (BBN) initiation and 2-bromoethanamine (BEA) induced renal papillary necrosis as a promotion step.

The reasons for separate smaller studies (in terms of animal numbers) initially were three fold; 1) we had no previous experience of carcinogenesis studies, 2) the capacity of room available where a maximum number of 180-200 animals could be accommodated, 3) not wanting to commit too many resources.

3.6.5.1.B Study 1, experimental protocol:

Seventy-five male Wistar rats with an average weight of 110±10g were housed in a room with controlled access and environmental conditions.
maintained as described in section 3.2.2. Groups (each of 25 animals) were treated as follows:

Group A: animals were dosed with BBN according to procedure detailed in section 3.3.3, then after one week's respite each was given a single dose of BEA (100 mg/kg) prepared as detailed in section 3.3.2.

Group B: animals were dosed with BBN as described in section 3.3.3.

Group C: animals were dosed with vehicle alone (20:80, ethanol:water).

After one week's respite the animals were dosed with a single dose of freshly prepared BEA (100 mg/kg) as described in section 3.2.2.

All animals were allowed food and water ad libitum and the bedding was changed twice weekly. Animals were monitored twice daily during the BBN dosing period and daily afterwards. Animals that were moribund were removed, humanely sacrificed and postmortemmed to determine the cause of death. Any animals which died during the dosing periods were discounted from the study. Frequently the animals were found cannibalised and it was not possible to retrieve any tissues for histopathological assessment.

Groups of animals (n=3) were periodically sacrificed at 1, 2, 3, 4, 7 days and 3, 6, weeks post BEA injection. The animals were sacrificed by terminal sodium pentabarbitone anaesthesia. The animals were postmortemmed as described in section 3.4.2 with the following modification:

The left ureter and kidney pelvis was retrogradely perfused by glutaraldehyde via a ureteral cannulation (Ijomah, unpublished data). The right kidney, ureter and bladder tissues were processed (section 3.4.2) and routine and enzyme histochemical staining was performed (sections 3.5.1 and 3.5.2).

The very early time points (1-7 day) were performed to determine
whether BBN pretreatment had any effect on the usual pathological response of the animals to BEA ie did BBN alter the induction of acute RPN by BEA.

3.6.5.2 Study 2: Effect of superimposing additional carcinogen dosing after BBN/BEA initiation/promotion treatment

3.6.5.2.A Study 2, aims:
A small pilot group was set up to determine whether further administration of the carcinogen during a period of intensive proliferation of the target cells; (pelvic and ureteric urothelium as a consequence of BEA-induced RPN), resulted in an increased tumour yield or increased targeting for the urothelial cells of the upper urinary tract.

3.6.5.2.B Study 2: protocol.
Ninety male Wistar rats with an average weight of 110±10g were housed and maintained as described in section 3.2.2. Four groups of animals were treated as described above in section 3.6.5.1.B, with 37 animals in Group A, 20 animals in Group B and 25 animals in Group C. Group D: 8 animals were treated as group A (section 3.6.5.1.B above), then further dosed with BBN receiving a single dose (80 mg/kg in a 0.5 ml volume) on three consecutive days after the BEA dose. Groups of animals (n=3) from Groups A-C were sacrificed at 3 day and 3, 6, 13, 21, 30 and 40 weeks post BEA injection and groups of animals (n=2) from pilot study (Group 4) were sacrificed at 13, 21, 30 and 40 weeks. Tissues were taken for histological examination as described in section 3.6.5.1.B.

3.6.5.3 Study 3: Effect of superimposing analgesic dosing after BBN/BEA initiation/promotion treatment.
3.6.5.3.A Study 3, aims:
To determine if superimposing analgesic dosing after an initiation/promotion treatment would affect either the target region of tumour induction, reduce the latent period of tumour induction and increase the number or degree of severity of the tumours induced.

3.6.5.3.B Study 3: protocol.

One hundred and thirty-five male Wistar rats weight 100±15g were divided into five groups:

Groups A-C were treated as described in section 3.6.5.1.B with Group A (n=28), Group B (n=30) and Group C (n=35).

Group D - consisted of 21 animals which were dosed with BBN as previously described (see section 3.3.3) and after one weeks respite received a single ip dose of BEA (see section 3.3.2) at a modified dose concentration of 75 mg/kg. The 21 animals were then subdivided into three groups of 7 animals. These subgroups then received either aspirin (as described in section 3.3.4), paracetamol (as described in section 3.3.5.1) or NPAA (as described in section 3.3.6).

Group E - these 21 animals were dosed with vehicle alone (20:80 ethanol:water solution) and then BEA only (at the same time as previous group), given at the modified dosage of 75 mg/kg. These 21 animals were subdivided into three groups of 7 animals which were then treated with either aspirin, paracetamol or NPAA as described for Group D animals.

Groups of animals from groups A-C (n=3) were sacrificed at 7, 14, 18, 28, 40 and 48 weeks post BEA injection. Groups of animals from Groups D and E were sacrificed by sodium pentabarbitone anaesthesia (section 3.3.1) at 7 (n=2), 14 (n=2) and 18 (n=3) weeks post BEA injection.

Tissues were taken for histology as described in section 3.4.

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3.6.5.4 Study 4: BBN initiation and RPN promotion and subsequent BBN dosing.

3.6.5.4.A Study 4: aims:

To determine whether results from pilot study from study 2 (section 3.6.5.2) could be reproduced with larger numbers of animals. To investigate if giving the "promotor" (BEA) prior to the initiator (BBN) affected the incidence of tumours and urothelial hyperplasia.

3.6.5.4.B Study 4: protocol.

Male Wistar rats (n=180) average weight 100±15g were divided into six groups (each of 30 animals), housed in cages in a separate room maintained as for previous experiments. The animals were dosed as follows:

Group A - animals dosed with BBN (section 3.3.3) and BEA after one weeks respite (section 3.3.2) at a reduced dosage of 75 mg/Kg. The reduced dosage was an attempt to reduce the number of deaths that had occurred in the earlier studies (studies 1 & 2, sections 3.6.5.1 and 3.6.5.2).

Group B - animals were dosed with BBN only (see section 3.3.2).

Group C - animals were dosed with vehicle alone (20:80 ethanol: water solution) and then BEA as described in section 3.3.2 at the reduced dosage of 75 mg/kg.

Group D - animals were dosed exactly as the Group A animals but in addition received a further four doses of BBN on consecutive days after receiving the dose of BEA.

Group E - animals were dosed as for Group B animals but in addition received four further doses of BBN on the same days as the animals in Group D.
Group F - animals were dosed with BEA and then received four doses of BBN as for Groups D and E.

Note: it has only come to my attention as I wrote this up that Group F does not fulfill the control group role it was intended to. For a true control for promoter before initiator the animals should have received BEA, then BBN (as described in section 3.3.3), i.e. 10 doses over 5 weeks to get a total dose of 800 mg. In fact they only received 4 doses, a total of 320 mg!!

Groups of animals (n=3 or 4) were sacrificed by sodium pentobarbitone anaesthesia (section 3.3.1) at 12, 18, 30, 35, 40, 46-47*, 51-52* and 58 weeks. Weeks denoted with * indicate sacrifice time points when donor animals were used in tumour xenograft studies (see section 3.6.6).

3.6.5.5 Study 5; chronic dosing with paracetamol after BBN/BEA treatment.

3.6.5.5.A Study 5, aims:
To study the effect on urothelial carcinogenesis of chronic dosing with analgesics (paracetamol) after BBN initiation and BEA-induced renal papillary necrosis as a promotion step. The study is to investigate whether pre-neoplastic changes can be induced earlier with paracetamol dosing.

3.6.5.5.B Study 5: protocol.
Male Wistar rats (n=200) weight 106±17 g (*) were divided into eight groups and housed in cages (6 animals to a cage) in a separate room; environment maintained (as described in section 3.2.2). (*) - because of a delay in commencing this study due to the shipping delay of BBN from the USA, the animals weighed 148±30 g when dosing began.

Group A - animals (n=20) were untreated controls, received only normal
diet and water.

Group B - animals (n=30) received BEA only as described in section 3.3.2 at the reduced dose of 75 mg/Kg.

Group C - animals (n=15) were dosed with BBN only as described in section 3.3.3. The total dose was reduced to 400 mg. The animals were given 5 doses of BBN over a 2.5 week period.

Group D - animals (n=30) were dosed with paracetamol as described in section 3.3.5.2. The dosing commenced on the same day as for groups F and G.

Group E - animals (n=30) were dosed with BBN (as for Group C) and BEA (as for Group B).

Group F - animals (n=30) were dosed with BBN and BEA (as for Group E) in addition these animals received paracetamol after the BEA dose (as for Group D).

Group G - animals (n=30) were dosed with BEA (as for Group B) and then paracetamol (as for Group D).

Group H - animals (n=15) were dosed with BBN (as for Group C) and then paracetamol (as for Group D).

Animals were sacrificed at intervals of 3, 6, 9, 12, 18, 24 and 30 weeks, by ether pre-anaesthesia and sagatal anaesthesia. Kidney, ureter and bladder tissues were taken for pathological examination at the light and electron microscopic level (as described section 3.4 and 3.4.8). Other tissues (liver, lung, stomach, intestine, spleen, heart) were initially taken for gross examination with histology performed if deemed necessary.

3.6.6 Tumour Xenograft Studies in Nude Mice.

These studies were concerned with the transplantation of tumour tissue
derived from donor rats (from BBN study 4 (section 3.6.5.4) which had been treated by the BBN/BEA initiation/promotion protocol), described above in section 3.6.5.

3.6.6.1 Gnotobiotics.

As mentioned in Section 2.4, the Nude mouse has an incomplete immune system (Hansen, 1978) and is hence susceptible to bacterial and viral pathogens. It is necessary to maintain and breed these animals in a pathogen-free, controlled environment to ensure their good health and longevity (Ediger & Giovanella, 1978).

Gnotobiotics has been defined as the science involved with maintaining a microbiologically controlled environment and the biological knowledge necessary to obtain and use living specimens in this environment (Trexler, 1987).

All aspects of gnotobiotic research, the appropriate techniques and related problems have been reviewed (Coates, 1968; Coates & Gustaffson, 1984; Trexler, 1987).

3.6.6.2 Isolator Design.

The work space in which a microbiologically controlled environment is maintained is termed an isolator. Isolators may take on several forms such as the; rigid 'stainless steel' isolators (Gustaffson, 1948, 1959), flexible plastic films eg polyvinylchloride (PVC) (Trexler & Reynolds, 1957), or laminar air flow cabinets are all suitable for keeping gnotobiotic animals (see Trexler, (1984) for review on isolators and equipment).

Flexible PVC isolators are by far the most commonly used because of their ease of manufacture, and their relative low cost compared to rigid and laminar air flow cabinets. They also offer five main
advantages which outweigh their main disadvantages, see Table 3.3).

The basic design of an isolator of this type is a plastic bubble into which access is via an entry port and flexible plastic sleeves attached to arm-length rubber gloves provide manipulation access for the experimenter. The entry port is sealed at both ends with a PVC cap which provides an air-lock system into which cages, equipment, diet and water can be passed from the external environment and then into the controlled environment without contamination (Figure 3.3).

Table 3.3

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Great flexibility allows greater ‘in-isolator’ manipulation.</td>
<td>1. Readily damaged-by sharp edges, but easily repaired.</td>
</tr>
<tr>
<td>2. Transparency of PVC allows good observation.</td>
<td>2. Cannot be steam sterilised but can be sterilised chemically and by radiation.</td>
</tr>
<tr>
<td>3. PVC films can be cut and joined easily which permits design modification and running repairs.</td>
<td></td>
</tr>
<tr>
<td>4. Cost of materials are low and in-house design and construction possible.</td>
<td></td>
</tr>
<tr>
<td>5. Flexible walls act as reservoir assisting in keeping air pressure differential which allows low air pressure operation</td>
<td></td>
</tr>
</tbody>
</table>

Sterilisation is required for the whole isolator prior to commencement of the experiment and any equipment subsequently introduced must be sterilised too. Design and planning of experimental procedure in advance is vital. This allows all the necessary cages, surgical instruments to be placed within the isolator prior to sterilisation. The working space is very restricted in the isolator and the
conditions in the isolator (maintaining a germ-free environment) prevents any instant access to non-sterile equipment from outside if it is required.

Figure 3.3 Diagram of a typical flexible film isolator.

3.6.6.3 Sterilisation procedures.

Sterilisation can be achieved 3 ways:

1. Autoclaving at 121°C for 20 min or 180°C for 30 min (dry heat).
2. Gamma irradiation which requires special equipment.

3. Chemical - simple, effective, requires no special equipment. A 2% peracetic acid solution is the most commonly used method of chemically sterilising isolators and items which must be placed in the isolator. Vapour from 2% v/v peracetic acid solution kills spores etc in 20 minutes by its potent oxidative action. However, peracetic acid cannot penetrate hydrophobic materials such as grease etc, so the 2% peracetic acid solution has a small quantity of a detergent solution (Triton X-100) added as a wetting agent to allow the sterilising solution to reach all bacteria. It is for this reason that rubber gloves should be worn when handling any items to be placed in the isolator should be handled. Vapour is much more effective because it disperses to all areas of isolator, so use a household spray to administer the peracetic acid. Personal protection is required when using peracetic acid; a flow helmet (which provides a filtered air supply) should be used (see Methods section 3.1.3) gloves and safety glasses in a well ventilated environment.

3.6.6.4 Experimental procedures.

3.6.6.4.A Isolator.

The isolator was a flexible plastic variety (Figures 3.3, 3.4 and 3.5), constructed "in-house" by Mr S. Tuttlebury, and consisted of a clear flexible polyvinyl chloride (PVC) envelope (dimensions of approximately 125 x 90 x 60 cm) resting on a rigid chipboard base covered by a pad of plastic foam. A supporting frame over the cage area prevented the envelope from collapsing in the event of a decrease in the pressure of the air supply. The envelope walls and sleeves
Figure 3.4: isolator in working situation, showing entry port cap and securing rubber band and tapes, thermometer, humidity gauge, flexible envelope and gloved sleeves.

Figure 3.5: shows the internal environment of isolation with cages in position, glass wool (4 um pore) air filter, water bottles and limited available working space.
consisted of clear PVC, thickness 0.2 mm and the floor of coloured PVC plastic, thickness 0.5 mm. Wrist length household gloves were attached securely to the sleeves by being fitted over rigid plastic rings using rubber bands, rubber "O" rings followed by 2.54 cm white PVC pressure sensitive tape. A sheet of stiff PVC plastic lined the floor of the envelope to prevent the puncturing of the PVC envelope from the inside by for instance dried faeces, diet pellets, surgical instruments. The frame also supported a double capped entry port made of rigid PVC, the inner cap was secured by means of a 2.54 cm rubber band and the outer cap was secured with a 2.54 cm wide rubber band and 2.54 cm white PVC pressure sensitive tape. The frame also supported a small blower fan type 26-BTC blower, mains generated as the air supply and a liquid paraffin outlet trap. Air exits through a paraffin oil trap which prevents backflow of external air when sleeves are used, the trap also had a lid to prevent the entry of flying insects (Ward, 1986). The air supply flow rate was such to maintain a positive pressure on the isolator walls between 2-5 mm water via flexible hosing through a glass wool filter. The filter consisted of a stainless steel wire cylindrical support around which 3 layers of 12 mm thick, 4 um pore diameter glass fibre wool were wrapped. The filter was sited within the isolator near the top of one corner where it was least likely to be damaged when cages or other equipment was moved. Prior to introducing equipment, cages, etc the isolator envelope had to be checked for leaks and holes, by inflating the isolator, sealing the isolator outlet vent and the air input port using rubber bungs. Then a fluorocarbon containing gas mix (Dustoff) was sprayed into the isolator ensuring that the sleeves contained some of the mixture. A "sniffer" device (Halidehound) which detects halide
gases eg fluorocarbons is carefully scanned over all isolator surfaces concentrating particularly on sleeves and any join welds. An alarm indicates a hole where halogen containing gas is escaping. This is marked with a wax pencil, and then may be temporarily repaired with white adhesive tape or a more permanent repair is performed using a radio frequency sealing machine.

Once the isolator is leak-free it can be sterilised. Autoclaved cages and ear punch pliers together with sealed packets of gamma irradiated (50 Gy from a $^{60}\text{Co}$ source at Isotron, Wiltshire, UK) tissues, plastic bags, syringes, needles, plastic ruler, were all placed inside prior to sterilising the whole isolator. A 2% (v/v) peracetic acid:1% (v/v) Triton X-100 detergent solution was liberally sprayed on all inside surfaces of the isolator concentrating on the arm sleeves and port. The isolator was left inflated for 48 hr, which also served to finally check no leaks were present and then the air input supply was attached and the sterilising atmosphere vented outside the lab for 48 hours to remove all traces of peracetic acid.

3.6.6.4.B Animals.

Thirty male isolator-reared MF1-nu/nu/Ola/Hsd mice, age 5 weeks, were obtained from Harlan Olac Ltd, Shaw's Farm, Blackthorn, Bicester, Oxfordshire, UK.

Animals were housed in three groups of 10 in autoclaved sterile mouse boxes 48 x 16 x 15 cm on irradiated-sterile bedding (Irradiated at 2.5 Gy, Harlan Olac Ltd).

The temperature of laboratory (and inside the isolator), was maintained at 21±2°C and humidity was nominally 40±15%. A 10 hr light:14 dark lighting schedule commencing at 0800GMT was maintained.
(However, the isolator was housed in a general laboratory so the light period sometimes extended to 12 hr).

Animals were allowed to acclimatise to the isolator and laboratory environment for 10 days prior to experimentation. They were allowed food and autoclave sterilised tap water ad libitum. Food was an irradiated pellet diet (2.5 Gy) CD₂ mouse diet (Harlan Olac Ltd). Tap water was sterilised tap water (autoclaved at 121°C for 50 minutes). Vitamin supplements were added to the water, after sterilisation in the stock bottles within isolator. Vitamin K supplement (Konatin 10) are supplied sterile in vials diluted in 20ml sterile distilled water to give stock to vitamin K solution, 1 ml of this stock vitamin K solution was added to 1 L of drinking water to give a concentration of 800 ug/L. Vitamin B₁₂ had to be made up and sterilised by autoclaving prior to adding to drinking water. Cyancobalamin 0.0200g, NaH₂PO₄·2H₂O 1.2g, and sodium chloride 53.0g were added to 400 ml distilled water, 2 ml of this autoclaved solution was added to 1L of drinking water to give a concentration of 100 ug/L.

Animals were checked daily and handled regularly to minimise the stress of handling during experimentation. To avoid them biting through the gloves animals were picked up by the base of the tail using rubber tipped forceps.

Cages were cleaned thrice weekly on Monday, Wednesday and Fridays (am). Animals were transferred to a spare sterile cage while cleaning was in progress.

3.6.6.4.C Transplantation protocol.

The transplantation procedure was performed in a sterile working environment within a horizontal flow cabinet.
1. Donor animal (W/A rat (University of Surrey strain) weight approx 600g previously treated with BBN/BEA (see section 3.6.5.4.B) 45 weeks earlier) was killed by ether anaesthetisa followed by cervical dislocation.

2. Animal was then placed abdomen downwards onto double sided tape (which had been attached prior, to a plastic bag 30cm x 40cm). The bag was reversed such that the rat was contained within it with the abdomen sealed to the plastic wall via the double sided tape.

3. Commencing at pubis the skin layer of abdomen was opened using scissors to xiphoid process, and laterally on both sides at the level of iliac crests and lower ribcage to give two skin flaps through the plastic bag to provide a sterile environment. These flaps were retracted by means of surgical clamps.

4. The muscular layer of the peritoneum was swabbed down with 100% ethanol, prior to peritoneum being opened and retracted as in 3.

5. Using a fresh pair of sterile scissors and forceps the bladder, kidneys and ureter were dissected free, whole and placed immediately into a sterile petri dish containing cold (4°C) sterile RPMI-1640 (pH 7.0-7.2) medium, containing sodium hydrogen-carbonate, glutamine 200mM 10x and Antibiotic-antimycotic solution. Media was sterilised by filtering the solution through a Tower filter system.

6. Excess fat and connective tissue were blunt dissected free of kidney and ureter. Viewing through a stereomicroscope (Stereo Nikon SMZ-10), within the flow cabinet the ureters were examined closely for any abnormalities in appearance and then cut away from kidney and replaced into sterile cold RPMI-1640 medium. (Dissection and examination procedures were performed on tissue placed on sheet of sterile filter paper, Whatman No 1 and continually bathed in sterile
cold RPMI-1640 medium.

7. The bladder was bisected longitudinally and examined for any tumours or nodular areas on its interior surface. Such areas were then isolated and dissected using fine forceps and microscissors; any tissue pieces being placed into separate multiwell compartments in cold RPMI-1640 medium.

8. Kidneys were bisected and depoled, the cut surfaces were examined using a stereomicroscope. Any tumours or highly irregular tissue areas were carefully dissected and placed in 24-well multiwell chambers containing cold RPMI-1640 media.

9. The multiwell chamber was closed, then placed inside a sterile specimen bag. This was placed inside the isolator port to sit on a cold aluminium block (also inside a sterile specimen bag). The port was sprayed with 2% peracetic acid vapour. In order to maintain the sterile environment within the isolator there is a minimum transfer time of 15 min (to allow the peracetic acid to sterilise contents in the port). The cold aluminium block serves to keep the tissues at a temperature of 4°C during this transfer time.

10. After 15 min the interior side of port was opened and multiwell plus block washed down with sterile water before being moved into isolator. Using a sterile scalpel blade, tissue pieces were minced up in a few drops of cold media to a fine mince, tissue pieces being approximately 1mm³ or less in volume.

11. Using a 1ml sterile syringe and a 16G cannula tube this minced tissue was picked up to that the minced tissue was enclosed inside the cannula tube. The syringe contained sufficient media to expel the tissue into the animal.
12. Each nude mouse was scruffed by the loose skin at the base of the neck and restrained by holding the base of the tail down with the little finger of left hand whilst the second and third fingers supported the body of the animal from beneath.

13. A small nick was placed in flank of the animal using small scalpel blade no 22. Through this incision, the cannula tube was introduced. The tube was manoeuvred subcutaneously such that the bolus of media (containing the tissue mince) was inserted on the right anterior lateral flank just posterior of right foreleg.

14. This procedure was repeated on several animals, each receiving one or more pieces of tissue either from bladder tumour, ureter or pelvic wall.

15. Animals were earmarked according to our usual system (see Methods, section 3.2.6), and then allowed food and water ad libitum.

16. Any remaining tissue which was not utilised in transplantation procedures was further minced and subdivided among a 24-well multiwell chamber containing complete RPMI-1640 media (containing 10% Foetal calf serum) for cell culture (see Section 3.6.7).

16. Mice were monitored daily and measurements and observations of tumour growth taken weekly. Tumour diameter measured 2 directions placed into following formula:

\[ L \times (B)^2 \times 0.4 = \text{Volume mm}^3 \]  

(Kyriazis et al, 1985).

17. When tumour volume according to formula had reached between 800mm\(^3\) - 1000mm\(^3\) (1 cubic cm) the tissue was harvested.

18. The tumour tissue was then processed according to the diagram below allowing maximal use of tissue for examination procedures (Figure 3.6).
3.6.6.4. Harvesting tumours in nude mice.

1. Animals were sacrificed by cervical dislocation in the isolator. Using sterile forceps and scissors the tumour was dissected out of the animal. The tumour consisted of 2 cystic portions containing pale yellow straw coloured fluid and a larger nodular solid portion, tissue was placed in sterile cold RPMI-1640 media.

1. Approximately one quarter was placed in sterile cold RPMI-1640 media and minced with a scalpel blade, then passaged subcutaneously into another mouse (as described above in section 3.6.6.3).

2. Approximately one quarter (consisting of 2 x 1/8 portions) were snap-frozen by the method of Chayen et al, (1976). Chips of dry ice
were added to methanol until the vigorous bubbling stopped, then a smaller beaker containing n-hexane was placed in this methanol-dry ice slurry and allowed to equilibrate to -70°C.

3. Tissue portions were dropped directly into the hexane and allowed to freeze for approximately one minute. Tissue was removed with cold forceps (having equilibrated on dry ice to -70°C temperature beforehand), and placed in a cryogenic storage tube (NUNC) which was then immediately placed into an insulated box containing dry ice.

4. These tubes were then stored in a -80°C freezer for future micro enzyme- and immuno-cytochemistry.

5. The remaining tissues were fixed for either light microscopy in formal calcium fixative (section 3.4.1), or electron microscopy in 2.5% (v/v) glutaraldehyde fixative in phosphate buffer.

3.6.6.5 Assessing the growth of transplanted tissues.

In practice the index of tumour growth was expressed as a linear dimension; calculated as the mean of the product of two dimensions measured at 90° to each other i.e. \( A \times B / 2 = I \), and not calculated as \( L \times (B)^2 \times 0.4 \) for a number of reasons.

For ease, speed and convenience a short plastic ruler was used to measure the linear dimensions. Although one person had there was some difficulty in measuring the tumour dimensions with this method it was superior to using calipers. It was virtually impossible to measure the tumour dimensions using the calipers as they were not "user friendly" and had no easily readable scale etc. The ruler was easily sterilised with the peracetic acid method, had an "easy to read" scale and was less likely to cause injury to the mouse should he struggle, which they were quite apt to do.

The actual act of handling the mice served to make the measuring
difficult as the skin on the flank was often obscured under a restraining finger, or the skin "bunched up" producing false lumps. As the animals got older the flank skin often became scarred due to the numerous fights which occurred regularly also the presence of lymphatic nodes gave false positive results.

Raw data was recorded and later calculated according to the formula \( L \times (B)^2 \times 0.4 \), where \( L \) was assumed to be the largest dimension.

\[
e.g. 7 \times 10 \text{ was calculated as } 10 \times (7)^2 \times 0.4 = 196 \text{ mm}^3
\]

and not calculated as \( 7 \times (10)^2 \times 0.4 = 280 \text{ mm}^3 \).

3.6.7 In vitro Cell Culture of Urothelial Cells From Ureter, Bladder and Pelvic Epithelia of Wistar Rat.

3.6.7.1 Introduction.

This experiment was performed as part of the nude mice tumour xenograft study (section 3.6.6 described above). The aim was to culture urothelial cells from the ureter, pelvic and bladder epithelial regions and any tumours present. Then to establish cell lines, and characterise these cell lines in terms of morphology, biochemistry and metabolism.

3.6.7.2 Experimental procedures.

1. Kidney, ureters and bladders were removed from BBN/BEA treated animals as described in section 3.6.6.4.B.

2. The methods were a modification of culture procedures described by Masters et al, (1986) and Lawson et al, (1986) where cold (0-4°C) incomplete RPMI-1640 media was used as a maintaining medium until microdissection could be performed (see section 3.6.6.4.B).

3. Tissues were placed in cold RPMI-1640 media in a 24-multiwell plate which stood on a aluminium block placed on ice to maintain the
tissues at 0-4°C. Tissues were taken for nude mouse xenografting, the remainder of the tissue was divided and seeded out into a second plate containing complete RPMI-1640 medium (with 10% fetal calf serum) added. The holding medium in the first plate was replaced with complete medium.

5. These plates were then placed within an incubator maintained at 37°C in a 5% carbon dioxide:95% oxygen atmosphere. These were left undisturbed for 48 hr to allow cells to attach.

6. The cultures were examined every 24 hr after the initial 48 hr period using Nikon Labphase microscope. The cultures were scored according to a simple scheme which recorded a) any adherence and b) if any growth had occurred.

7. The media was changed every 48 hr by aseptic transfer techniques performed within a flow cabinet.

Cell cultures showing significant growth were photographed using Ilford FP4 film on a Nikon FE2 SLR camera, and Nikon phase microscope with objective lens magnifications of x10, x20, x40 and x90.

3.7 ASSESSMENT.

3.7.1 Macroscopic Assessment

During postmortems all major tissues (organs within abdominal and thoracic cavities) were examined for any gross pathological changes. After the tissues had fixed for 24-48 hr a more thorough examination was performed during the tissue slicing stage of tissue processing (see section 3.4.2, part 9), using a stereomicroscope. This was particularly important in the carcinogenesis experiments where the bisected bladder and kidney pelvic cavity were closely examined for the presence of tumours. The macroscopic assessment enabled particular
regions of tissues could be taken for subsequent histological examination. Photographs of tumours and kidney lesions from all experiments (rat and pig) were taken using the stereoscope and Nikon UFX-II camera system.

3.7.2 Microscopic Assessment.

All histological sections were assessed using Leitz Dialux light microscope and any photographs taken using a Nikon UFX-II camera system attached to the microscope.

The RPN lesions were graded using the criteria of Burry (1977), and Henry & Tange (1982) as a basis for my grading system which was modified and upgraded throughout the period of these investigations as I became more familiar with the lesion and the pathological changes present. The assessment of pathological changes was done on an anatomical region basis, with the basic regions examined as shown in Table 3.4. The pelvic, ureteric and bladder urothelia (in an arbitrary order of priority) were assessed for hyperplastic and neoplastic changes according to Kunze (1987), Mostofi et al, (1973) and Helpap et al, (1985).

3.8 Tissues from Patients with Analgesic Nephropathy.

3.8.1 Introduction.

One of the frequent criticisms of the use of animal models of human disease is the difficulty in extrapolating data from these experimental models to the clinical situation. It was therefore of great interest to obtain some tissue samples from analgesic abusing patients so that a comparison of the pathological changes between the clinical and the experimental specimens could be performed.
Table 3.4: Pathological changes assessed in grading of RPN.

<table>
<thead>
<tr>
<th>Anatomical Region</th>
<th>Nephron segment/region</th>
<th>Change assessed</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Papilla</strong></td>
<td>interstitial cell</td>
<td>nuclei changes: pyknosis, basophilia, enlargement</td>
</tr>
<tr>
<td></td>
<td>interstitial cell</td>
<td>increase/decrease stain -ing, granularity, PMN influx, fibrosis</td>
</tr>
<tr>
<td></td>
<td>matrix</td>
<td>adherence of platelets, erythrocytes</td>
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<tr>
<td></td>
<td>capillaries</td>
<td>nuclei enlargement, mitoses, necrosis, casts</td>
</tr>
<tr>
<td></td>
<td>collecting duct</td>
<td>cytoplasmic swelling, PAS positive cytoplasmic granules, lipidosis</td>
</tr>
<tr>
<td></td>
<td>covering epithelium</td>
<td>hyperplasia, lipidosis, necrosis, denudation</td>
</tr>
<tr>
<td><strong>Medulla</strong></td>
<td>collecting ducts</td>
<td>as for papilla, also PAS casts early in loops</td>
</tr>
<tr>
<td></td>
<td>loops Henle</td>
<td>as for papilla</td>
</tr>
<tr>
<td></td>
<td>interstitial cells</td>
<td>endothelial nuclei basophilic, PAS casts, cellular debris</td>
</tr>
<tr>
<td></td>
<td>vasa rectae</td>
<td>dilatation of tubular elements, sclerosis &amp; fibrosis (late stages only), inflammatory cell influx</td>
</tr>
<tr>
<td></td>
<td>proximal tubule</td>
<td>disruption of brush border, loss of associated enzymes, atrophy in late stages</td>
</tr>
<tr>
<td><strong>Cortex</strong></td>
<td>general</td>
<td>hyperplasia, mitoses, loss of glycocalyx, increase in cytoplasmic PAS granules</td>
</tr>
<tr>
<td></td>
<td>capillaries</td>
<td>histochemical, microangiopathy</td>
</tr>
<tr>
<td><strong>Pelvis &amp; Ureter</strong></td>
<td>urothelium</td>
<td>PMN: Polymorphonuclear leukocytes.</td>
</tr>
</tbody>
</table>

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A collaborative study with Dr Anke Schwarz (Freie Universitat, Berlin) was initiated because of her mutual interest in the pathogenesis of analgesic nephropathy. Dr Schwarz works as the consultant nephrologist in a urology department which has a large number of dialysis patients who are known to abuse analgesics, she therefore kindly agreed to send tissue specimens when they were available.

The tissue specimens had been fixed in routine 10% (v/v) neutral buffered formalin for unspecified time periods (a minimum of one week), this compromised the enzyme histochemistry staining because of the extended periods in high concentrations of fixative.

This did have the benefit of ensuring that all the specimens were adequately fixed including any potential biohazardous pathogens that were in the specimen.

3.8.2 Experimental Procedure.

The specimens were examined macroscopically, renal pyramids (if identifiable as such) or a representative slice through cortex and medulla together with any pelvic or ureteric urothelia were taken (according to the method of Lomax-Smith & Seymour, 1980a, see Figure 3.2) and processed for embedding in glycolmethacrylate resin as described in section 3.4.

3.8.3 Clinical Histories.

Specimen 1: a shrunken kidney (80 x 40 x 30 mm, 60g weight) was obtained from a 68 year old woman who had undergone a nephrectomy because of a small uroepithelial carcinoma (15 x 3 x 5 mm) at the uretero-pelvic junction. The patient had consumed several kinds of compound analgesics for a number of years, at least 1 tablet for at least 8 years (probably more and for longer - the patient gave several
contradictory statements regarding her analgesic intake. Ultrasound scan showed shrunken kidneys with calcification (papillary necrosis) and a renal insufficiency with a serum creatinine of 500 umol/l before nephrectomy. The patient was now on hemodialysis.

Specimen 2: shrunken kidneys (Left weight 70g, Right weight 80g) from a 58 year old woman who had died (1988) from pulmonary metastasis of breast carcinoma (mastectomy performed in 1982). The patient had consumed compound analgesics containing phenacetin for at least 10 years, about 10 tablets daily (cumulative amount of phenacetin 5kg). Renal insufficiency was detected in 1969, patient presumed to have continued to abuse analgesics, had complicating urinary tract infections, urinary obstructions by calcified papillae and haemodialysis was commenced in 1978. Prolonged hemodialysis treatment has resulted in secondary polycystic degeneration in both kidneys. Also secondary arteriosclerotic stenosis of kidney arteries is present, which is present in 60% of analgesic kidneys. Renal tuberculosis (which patient had in one kidney) is commonly associated with renal interstitial disease, mostly analgesic nephropathy (Dr A. Schwarz, personnel communication).

Specimen 3: kidney was obtained from a 60 year old woman who had died of acute cardiac failure (induced by coronary heart disease, heart insufficiency, hypokalaemia by tubular loss of potassium resulted in ventricular fibrillation). The patient had completely denied any form of analgesic intake, however clinical and pathological symptoms suggested she had analgesic nephropathy; shrunken irregular kidneys with papillary cavities, a history of recurrent renal concrements and had already undergone unilateral nephrectomy some years previously because of pyelonephritis. Hemodialysis had been commenced just 4
weeks prior to death.

**Specimen 4**: one kidney from a 63 year old woman who had undergone a nephrectomy because of renal pelvis carcinoma. Patient had consumed analgesic compounds 3-4 tablets daily for 30 years (because of headaches!). Compounds consumed were **Togal** (which contains acetylsalicylic acid) and **Spalt** (which contained phenacetin up to 1965 and since then has contained salicylamide and phenazonsalicylate). The patient had advanced renal insufficiency with a serum creatinine of 350 umol/l (upper normal limit of 110 umol/l), and now is receiving haemodialysis.
CHAPTER 4

CHEMICALLY INDUCED RENAL PAPILLARY NECROSIS: RESULTS AND DISCUSSION.

RESULTS.

4.1 2-Bromoethanamine (BEA) Induced RPN in Rats.

4.1.1 Short-term Time Course Study.

The reasons and methods for this investigation are described in Chapter 2, section 2.5, Methods section 3.6.1 respectively.

Tables 4.1A and 4.1B summarise the morphological and histochemical changes that occurred over 144 hours after a single ip dose of BEA (100 mg/kg) see Figures 4.1-4.15.

4.1.1.1 Monastoral blue B assessment of microvascular filling and integrity.

See Methods section 3.6.1.1 for reasons for and methods used in this investigation. The macroscopic distribution of Monastral blue B in the control kidneys showed marked labelling of glomeruli in the cortex, medullary rays and to a lesser extent the papilla. In treated kidneys there was a progressive increase in medullary rays and papillary labelling in the viable zone, but not in the necrotic tip. The labelling of glomeruli remained virtually constant, with a slight decrease during the 24-48 hr period, coinciding with more Monastral blue B in the medullary rays and papilla. At 144 hr the cortico-medullary labelling had reverted to that observed in control kidneys.

Neutral red counter-stained 1 um sections showed capillary labelling in the capillaries within the papilla, glomeruli and pelvic urothelium. No label was, however, seen to have extravasated into the interstitial matrix prior to the initial frank necrotic changes observed at 8-12 hr.
**Table 4.1A:** Early (0-144 hrs) morphological changes in BEA-induced RPN in male Wistar rats.

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Morphological Changes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>Normal with papilla interstitial matrix increasing in volume towards tip (Figure 4.1). Urothelium 2-4 cells thick, paler superficial layer which is PAS positive (Figure 4.2).</td>
</tr>
<tr>
<td><strong>2 hours</strong></td>
<td>No changes discernible</td>
</tr>
<tr>
<td><strong>4 hours</strong></td>
<td>Medullary interstitial cell nuclei becoming acutely irregular and pyknotic (Figure 4.3).</td>
</tr>
<tr>
<td><strong>6 hours</strong></td>
<td>Mild hyperplasia of papilla covering epithelium to 2-3 cells thick</td>
</tr>
<tr>
<td><strong>8 hours</strong></td>
<td>Marked pyknosis of interstitial cell nuclei, sloughing of hyperplastic covering epithelium. Platelets adhering to endothelium dilated distal tubular profiles.</td>
</tr>
<tr>
<td><strong>12 hours</strong></td>
<td>Leading edge of sloughing covering epithelium showing mild 2-3 cell thick hyperplasia. Degenerative changes in tubular elements in papilla. Hyperplasia in upper ureteric urothelium.</td>
</tr>
<tr>
<td><strong>18 hours</strong></td>
<td>50% of papilla affected by RPN. Cytoplasmic granules in necrotic collecting duct cells (Figure 4.4), mitotic figures present. Hyperplasia of pelvic urothelium opposite leading edge of covering epithelium (Figure 4.5).</td>
</tr>
<tr>
<td><strong>24 hours</strong></td>
<td>Medulla affected by necrosis, loss of tissue integrity with cellular debris and proteinaceous casts in papilla. Casts in dilated distal tubules in cortex. Fornix area of urothelium, hyperplastic, mitotic figures present</td>
</tr>
<tr>
<td><strong>48 hours</strong></td>
<td>Covering epithelium leading edge 5 cells thick, cells very irregular nuclei, cytoplasmic granules present (Figure 4.6) General hyperplasia of pelvic and ureteric urothelium.</td>
</tr>
<tr>
<td><strong>72 hours</strong></td>
<td>75% denudation of papilla covering epithelium. Tubular necrosis extending to outer medulla. Marked dilatation of proximal and distal tubules (Figure 4.7).</td>
</tr>
<tr>
<td><strong>144 hours</strong></td>
<td>75-100% loss of covering epithelium, papilla is a mass of necrotic tubules, (Figures 4.8) extensive adhesion of platelets in capillares and total loss of tissue integrity (Figure 4.9) lymphocytic infiltration. hyperplasia of pelvic and ureteric urothelium (Figure 4.10), bladder urothelial superficial layer becoming increasingly basophilic. Numerous granules in proximal tubule segments.</td>
</tr>
</tbody>
</table>
### Table 4.1B: Early (0-144 hrs) histochemical changes in BEA-induced RPN in male Wistar rats.

<table>
<thead>
<tr>
<th>Time point</th>
<th>Histochemical changes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>Occasion PAS positive granules in urothelium Alk Phos and ATPase enzyme staining in proximal tubule brush borders (also GGT), intermediate and basal layer of urothelium with superficial layer devoid of stain (Figure 4.11).</td>
</tr>
<tr>
<td><strong>2 hours</strong></td>
<td>No changes discernible</td>
</tr>
<tr>
<td><strong>4 hours</strong></td>
<td>Increase in interstitial matrix staining with Giemsa. Slight increase in ATPase staining around pyknotic nuclei</td>
</tr>
<tr>
<td><strong>6 hours</strong></td>
<td>Slight decrease in Alk Phos and GGT proximal tubule brush border staining</td>
</tr>
<tr>
<td><strong>8 hours</strong></td>
<td>Increase in Alk Phos staining in pelvic urothelium opposite denuded covering epithelium (Figure 4.12).</td>
</tr>
<tr>
<td><strong>12 hours</strong></td>
<td>Increase interstitial matrix staining at papilla tip. Hyperplastic urothelium has increased apical Alk Phos staining and increased ATPase staining of endothium sub-urothelial capillaries</td>
</tr>
<tr>
<td><strong>18 hours</strong></td>
<td>Mosaic pattern of Alk Phos and ATPase staining in hyperplastic urothelium (Figure 4.13). ATPase endothelial staining increase, moderate Acid Phos staining of interstitial cells in medulla</td>
</tr>
<tr>
<td><strong>24 hours</strong></td>
<td>Decreased interstitial matrix staining, loss of proximal tubular brush border enzymes Alk Phos, ATPase and GGT coincide with an increase of these enzymes in proteinaceous casts in the necrotic papilla (Figure 4.14). Loss of Acid Phos staining from S2 proximal tubule segment</td>
</tr>
<tr>
<td><strong>48 hours</strong></td>
<td>PAS positive staining material in proteinaceous casts. Continued loss of Alk Phos, ATPase and GGT from proximal tubule brush borders, also loss of Alk Phos and ATPase to slight degree from pelvic and ureteric urothelium</td>
</tr>
<tr>
<td><strong>72 hours</strong></td>
<td>Variable enzyme staining in proteinaceous casts, GGT most pronounced. Urothelial staining in ureter reduced to few Alk Phos positive cells, no bladder staining at all. Sub urothelial capillary ATPase staining increased (Figure 4.15).</td>
</tr>
<tr>
<td><strong>144 hours</strong></td>
<td>Granular Alk Phos and GGT staining in interstitium adjacent to hyperplastic fornix urothelium. Increased Acid Phos lysosomal staining in proximal tubule segments. Sub-urothelial capillary ATPase endothelial staining increase in quantity to occlude lumen.</td>
</tr>
</tbody>
</table>
Figure 4.1. Papilla tip from kidney of control animal showing characteristic pink (Giemsa) staining of interstitial matrix and single cuboidal covering epithelium (arrowhead). Giemsa x 89.

Figure 4.2. Control urothelium from Wistar rat showing three distinct layers; a) epithelium, b) lamina propria, c) lamina muscularis. Giemsa x 357.
Figure 4.3. Pyknotic interstitial cell nuclei in the papilla tip, 4 hr after BEA (100 mg/Kg), Giemsa x 178.

Figure 4.4. Collecting duct (CD) epithelial cells undergoing simultaneous repair and necrosis with basophilic staining cytoplasm granules and sloughing cells, 48 hr after BEA (100 mg/Kg), Toluidine blue x 223.
Figure 4.5. Hyperplastic pelvic urothelium (PE) opposite denuded papilla covering epithelium (CE) 72 hr after BEA (100 mg/Kg), Toluidine blue x 178.

Figure 4.6. Hyperplasia of leading edge of remaining covering epithelium (CE), blebbing, sloughing cells have atypical nuclei and numerous eosinophilic cytoplasmic granule, 72 hr after BEA (100 mg/kg), H&E x 893.
Figure 4.7. Dilatation of distal and proximal tubular lumen in cortex, 72 hr after BEA (100 mg/Kg), Toluidine blue x 89.

Figure 4.8 Low magnification micrograph showing typical RPN lesion 72 hr after BEA, note denudation of covering epithelium (arrowheads). Giemsa x 36.
Figure 4.9 Loss of tissue integrity at papilla tip with extensive platelet adherence in capillaries (arrowheads), 72 after BEA. Giemsa x 893.

Figure 4.10. Hyperplastic ureteric urothelium, 72 hours after BEA (100 mg/kg), Giemsa x 223.
Figure 4.11. Alkaline phosphatase (Alk. Phos.) staining in control bladder urothelium (arrowheads). Superficial "umbrella" cell layer devoid of stain. Alkaline phosphatase x 357.

Figure 4.12. Increased Alk. Phos. staining in pelvic urothelium (PE) opposite denuded covering epithelium (arrowheads) 8 hr after BEA, Alkaline phosphatase x 178.
Figure 4.13. Mosaic pattern of Alk. Phos. staining in hyperplastic urothelium, 18 hr after BEA, Alkaline phosphatase x 357.

Figure 4.14. Heterogenous ATPase staining in proteinaceous casts in necrotic tubules in papilla, 72 hr after BEA, ATPase x 36.
4.1.2 Intermediate and Long-term Time Course Study.

4.1.2.1 Three weeks time point.

The result presented here are from animals used in these studies were actually the BEA only treated control group of the series of carcinogenesis studies (see section 3.6.5). It was felt that to present them separately from the results of those studies (see chapter 5) was justified in establishing the time course description of the pathological changes occurring at extended time points after BEA-induced RPN was important for comparison with the carcinogenesis results and with the human tissue from analgesic abusers.

All animals displayed morphological changes associated with total RPN. There was complete denudation of the covering epithelium, with the totally necrotic papilla beginning to slough off from the medulla along a line joining the two fornices of pelvis (Figure 4.16).
Figure 4.16. Necrotic "ghost" papilla (Gh) sloughing of medulla, along crescentic line denoting extent of lesion (arrowheads), 3 weeks after a single ip dose of BEA (100 mg/kg), Toluidine blue x 43.

Figure 4.17. Increased deposition of mucopolysaccharides in the interstitial matrix and basement membranes (arrow) of juxtamedullary glomeruli and adjacent distal tubules, Giemsa x 357.
This "ghost" papilla had proteinaceous casts (which stained faintly for alk. phos. and GGT and more intensely for PAS) in the degenerative loops of Henle and collecting ducts. Numerous platelets were adherent to the capillary endothelium together with erythrocytes. There was papillary hyperplasia of the pelvic and ureteric urothelium. The bladder urothelium showed no morphological changes, however, the sub-urothelial capillaries continued to stain intensely for ATPase, whereas the urothelium had variable staining for alk. phos. and ATPase.

There were areas of marked necrosis radiating out from the medulla to the cortex along the vasa rectae affecting both the proximal and distal tubules. In these areas of necrosis there was an increase in and mucopolysaccharide deposition in the interstitial matrix and in the basement membranes around distal tubules and juxtamedullary glomeruli (Figure 4.17). The necrotic proximal tubules had variable brush border staining for ATPase, alk. phos. and GGT with the most intense staining in the \( P_3 \) proximal tubule segment. Interstitial cell nuclei adjacent to the vasa recta (medullary rays) were enlarged and basophilic. At the junction between 'ghost' papilla and medulla there were aggregates of polymorphonuclear leucocytes.

4.1.2.2. Six weeks time point.

The major morphological change at this time point was the re-epithelisation of the covering epithelium of the papilla/urothelium, where the 'ghost' papilla had sloughed off completely (Figure 4.18). This new epithelium was mildly hyperplastic with the occasional cell blebbing into pelvic space, nuclei were of variable shape and there was an occasional mitotic figure present. The restoration of the
Figure 4.18. Papilla stump showing re-epithelialisation of the covering epithelium where the ”ghost” papilla sloughed off, 6 weeks after a single ip dose of BEA (100 mg/kg), Giemsa x 177.

Figure 4.19 Sclerosis and basement membrane thickening of glomeruli (G) and distal tubules together with monocytic leukocyte infiltration (arrowhead), 6 weeks after BEA (100 mg/kg), Giemsa x 223.
covering epithelium increased the deposition of interstitial matrix components whereas at earlier time points the pink (Giemsa) staining had decreased. This interstitial matrix staining was not as intense as control medullary interstitial matrix and there was no increase in the number of interstitial cell nuclei either. There was some granular alk. phos. and ATPase staining in this restored matrix areas, basal to the re-epithelised covering urothelium.

The pelvic urothelium was mildly hyperplastic with cell being of variable size, shape and basophilic staining. Severe hyperplasia of the ureteric urothelium occurred which was papillary in some regions with a few areas of enlarged nuclei and increased dysplasia together with sloughing of the superficial cells into the ureteric lumen. The bladder urothelium was either normal or very mildly hyperplastic. The urothelial changes usually consisting of a ruffled superficial cell surface with an occasional basophilic staining nuclei. Within the urothelium of all areas the following distribution of enzyme staining was noted: pelvic urothelium had reduced alk. phos. and ATPase staining with only trace to faintly positive staining in a mosaic pattern, this pattern was also found in the bladder urothelium.

The ureteric urothelium was severely hyperplastic which was nodular/papillary in some areas. Variable alk. phos. and ATPase staining was observed throughout the ureteric urothelium. This staining was negative in some areas, whereas other regions had a mosaic pattern with an apical distribution in those areas of positive staining. Sub-urothelial capillaries in all areas stained positively and intensely for alk. phos. and ATPase with some occlusion of the lumen in some vessels. PAS staining of pelvic and bladder urothelia was usually confined to the glycocalyx on the superficial cell layer.
with occasional intensely staining granules present.

The necrotic changes in cortex described in section 4.2.1 were more pronounced after 6 weeks. The radiating pattern of atrophying tubules and increasingly sclerotic distal tubules and glomeruli (which were intensely PAS-positive stained), was accentuated by interstitial cell necrosis together with monocytic inflammatory cell infiltrates. These changes are indicative of a progressive interstitial nephritis. Enzyme staining for alk. phos. and GGT was variable and evident only in the proximal tubule brush borders where no necrosis had occurred. PAS staining of brush borders followed a similar pattern with the P3 segment being stained most intensely. Some sclerotic glomeruli had increased ATPase staining within the glomerular tuft.

4.1.2.3 Thirteen weeks time point.

The morphological changes at 13 weeks were a continuation and development of the degenerative cascade described at 6 weeks (see section 4.1.2.2). The papilla, if present, remained as a stump less than a quarter of its original length. Often it was just a re-epithelialised ridge from which the necrotic "ghost" papilla had broken away. The covering epithelium was mildly hyperplastic, with condensed basophilic nuclei and the occasional blebbing superficial cells. An increased interstitial matrix deposition occurred in the medulla/papilla stump. This was often devoid of tubular elements and any nuclei present were usually basophilic and pyknotic. The matrix appeared fibrous in nature, often bright pink and extended throughout medulla and cortex into areas undergoing atrophy. Granular intensely positive staining areas of interstitial matrix for alk. phos.
continued to stain in areas adjacent to the re-epithelialised covering epithelium.

In general the pelvic urothelium was mildly hyperplastic, but had disorganised cellular layers with large basophilic nuclei, together with large cells with eosinophilic granular staining cytoplasm (macrophages) scattered throughout. In the ureteric urothelium the lamina propria was very prominently stained for mucopolysaccharides with Giemsa, in some areas this extended up into the hyperplastic urothelium where it was papillary in nature. Throughout the ureteric and bladder urothelium there were patches of sloughing cells which were often very basophilic and vacuolated. The bladder urothelium was very ruffled on the luminal surface of the superficial layer.

In the ureteric urothelium there was an extremely intense mosaic pattern of cytoplasmic staining for alk. phos., whereas the pelvic and bladder urothelia were devoid of such staining.

The cortical interstitial nephritis and necrotic tubules was more advanced with prominent sclerotic and thickened basement membranes especially of the glomerulus (Figure 4.19), Bowman’s capsule and distal tubules. Any enzyme staining was restricted to viable proximal tubule brush borders which were often extremely disrupted, extruding into the lumen.

4.1.2.4 Twenty-one weeks time point.

An exacerbation of the morphological changes already described at 13 weeks was apparent at this time point. In particular the re-epithelialised covering epithelium was hyperplastic up to 4 cell layers thick in one area. The central portion of the cortex opposite the papilla/hilum had total interstitial nephritis with very few tubules present, many were sclerotic, also there was marked numbers of
inflammatory cell infiltrates (Figure 4.20), cortical scarring and collapse of the underlying parenchyma. Only proximal tubules which were intact gave positive brush border staining with PAS and the alk. phos.

The pelvic urothelium was generally of normal appearance, but some areas had basophilic nuclei in basally located cells and the occasional vacuolated, sloughing cell with pyknotic nuclei in the superficial layer. There were intensely PAS staining granules present in the superficial layer of the pelvic urothelium. The bladder urothelium was normal with a few hyperplastic areas up to 5 cells thick, whose superficial layers were extremely ruffled, occasional PAS positive inclusion bodies were present in this urothelium too. Variable hyperplasia (from simple to papillary) occurred in the ureter with many basophilic enlarged nuclei in the superficial layer together with numerous intensely PAS positive staining inclusion bodies which were more obvious in hyperplastic and dysplastic regions (Figure 4.21).

4.1.2.5 Thirty weeks time point.

The following changes were apparent at this time point (being a progression of the RPN and chronic severe interstitial nephritis. Opposite the papilla stump, complete collapse of cortical parenchymal tissue had occurred with no identifiable tubular elements present only very sclerotic and fibrotic glomeruli (Figures 4.22a,b). Medullary collecting ducts and distal tubules were extremely dilated had basophilic casts together with mineralised deposits in the necrotic loops of Henle. The hyperplastic covering epithelium was 4-5 cells thick with extreme basophilic nuclei and positive cytoplasmic alk.
Figure 4.20 Interstitial nephritis with tubular atrophy and sclerosis inflammatory cell infiltrations (arrow) in the cortex of kidney, 21 weeks after BEA (100 mg/kg). Giemsa x 108.

Figure 4.21 Numerous PAS positive staining granules and inclusion bodies in superficial layer of hyperplastic ureteric urothelium, 21 weeks after BEA (100 mg/kg). PAS x 893.
Figure 4.22a. Collapse of cortex (Cx) due to interstitial nephritis and atrophy of tubules producing surface scarring and decrease in thickness of cortex relative to medulla (M), 30 weeks after BEA. Giemsa x 43.

Figure 4.22b Higher magnification of above figure showing extensive tubular atrophy, inflammatory cell infiltrates and pronounced sclerosis of glomeruli (arrow). Giemsa x 434.
phos. staining. There was severe hyperplasia, dysplasia and loss of polarity in the ureteric urothelium with papillary hyperplastic areas which had intensely PAS-positive inclusion bodies within the superficial layer, but no alk. phos. staining. The bladder urothelium was 2-3 cells thick and extremely ruffled in the superficial layer. It had a few PAS-positive inclusion bodies (less than ureter) and some areas of urothelium alk. phos. positive staining was present.

![Figure 4.23](image)

**Figure 4.23** Papillary and nodular hyperplasia of ureter urothelium showing faint mosaic pattern of alkaline phosphatase staining, 40 weeks after a single ip dose of BEA (100 mg/kg). Alkaline phosphatase x 270.

### 4.1.2.4 Forty weeks time point.

The main changes were an increase in mineralised deposits within and adjacent to the lumen and basement membrane of the loops of Henle in the papilla stump. Distal tubules and collecting duct casts were more
extensive and contained PAS-positive material and exfoliated cells. Solid nodular hyperplasia occurred in one animal in the pelvic urothelium at the mouth of the ureter. The ureteric urothelium had variety of hyperplastic changes (papillary and nodular) (Figure 4.23) with dysplasia throughout and variable basophilia of nuclei. Cells with eosinophilic granular cytoplasm (macrophages) were prominent throughout all urothelia; pelvic, ureteric and bladder. At a gross level calcified "ghost" papillae were often present in pelvic space producing caliculi. This occurred in a few animals through the study, but increasingly from 13 weeks onwards.

4.2 BROMOETHANAMINE INDUCED RPN IN MICE.

4.2.1 Results From Schnider Mouse Strain Treated With a Range of BEA Doses (25-200 mg/kg).

Methods for this study are described in section 3.6.2.1. Total necrosis of the papilla similar to that observed in Wistar rat (section 4.1) extending into the medulla was only evident in mice dosed with 200 mg/kg BEA. The necrosis evident in the 100 mg/kg and 150 mg/kg dosed animals had morphological changes concomittant with intermediate RPN in Wistar rats dosed with 50 mg/kg BEA. The loss of covering epithelium does not appear to be a primary morphological change in Schnider mice, but changes in the interstitial matrix staining are the major necrotic change together with vascular wall damages in the capillaries. The dose levels of 25 mg/kg and 50 mg/kg produce early RPN with swollen collecting duct cells, casts in the loops of Henle and platelets adhering to the endothelium. This vascular wall damage precedes the interstitial changes that are concomittant with early RPN.
in the Wistar rat. Interstitial cell nuclei and matrix changes are initiated in the mid-papilla region and not at the tip as in the rat. No hyperplasia of pelvic urothelium occurred, but sloughing appeared to happen and detachment of the covering epithelium from papilla occurred.

The morphological changes described above are after 60 hr, whereas similar changes have been described in the rat much earlier in the time course see Table 4.1A.

4.2.2 Time Course Study of BEA in Three Different Mouse Strains.

4.2.2.1 Functional results.

The pooled urine results did not produce consistent results for osmolality, the Balb/c strain did not produce sufficient urine to collect and analyse at any time point. It was for these reasons that the individual samples collected direct from the bladder were used to produce mean values for each time point.

Mean control values for urine and serum osmolality, urea nitrogen and urine serum ratios are shown in Table 4.2.

4.2.2.2 Gross pathological results.

Table 4.3 shows the mean control kidney weight/body weight ratio values for each strain.

Balb/c mice had a higher mean control kidney/body weight ratio value (0.0177±0.0004), than either Obese (0.0158±0.0008) or C57B1/6 (0.0148±0.0004), n = 15 for all strains.

4.2.2.3 Histopathological results.

4.2.2.3.1 Controls.

There were no differences in morphology or histochemistry in any of the strains.
Table 4.2: Mean Urine and Serum Control values for Urea Nitrogen and Osmolality.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Urea Nitrogen</th>
<th>Osmolality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Urine (U) Serum (S) U/S ratio</td>
<td>Urine (mmol/kg) Serum (mg/dl) U/S ratio</td>
</tr>
<tr>
<td></td>
<td>(mmol/kg) (mg/dl)</td>
<td>(mmol/kg) (mg/dl)</td>
</tr>
<tr>
<td>Obese</td>
<td>1496 18.28 81.84 1234 317.29 3.89</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>675 6.39 397 87.0</td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>5 14</td>
<td></td>
</tr>
<tr>
<td>Balb/c</td>
<td>1304 295 4.42</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>ND ND ND 387 82</td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>3 14</td>
<td></td>
</tr>
<tr>
<td>C57B1/6</td>
<td>1069 16.92 63.18 991 292 3.39</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>491 5.98 249 82</td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>9 15</td>
<td></td>
</tr>
</tbody>
</table>

M = mean value, SD = standard deviation, n = number samples, ND = no data.

Table 4.3: Mean Control values of Kidney/Body Weight ratio.

<table>
<thead>
<tr>
<th>Strain</th>
<th>KW/BW ratio (g/kg) SD SE (x10^-4) 95% cf (x10^-4) range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balb/c</td>
<td>0.0177 0.0004 1.03 ±2.018 0.0175-0.0179</td>
</tr>
<tr>
<td>Obese</td>
<td>0.0158 0.0008 2.065 ±4.047 0.0154-0.0162</td>
</tr>
<tr>
<td>C57B1/6</td>
<td>0.0148 0.0004 1.03 ±2.018 0.0146-0.0150</td>
</tr>
</tbody>
</table>

KW/BW ratio - kidney weight/body weight ratio, SD - standard deviation SE - standard error, 95% cf - 95 % confidence limits, n = 15 for all groups.

In all the strains the proximal tubule segments in the outer cortex (predominantly P1 and P2) stained preferentially for alk. phos. where as the P3 segment stained most intensely for GGT.
4.2.2.3.B Three hr time point
The initial changes in all strains were pyknotic interstitial cell nuclei (Figure 4.24) in the upper papilla region and areas of intensely staining interstitial cell matrix in the papilla with PAS and Giemsa. The C57B1/6 mice also had hydropic cytoplasmic changes in the P₃ segment with some brush border disruption.

4.2.2.3.C Six hr time point
All strains had adherent platelets and erythrocytes in the capillaries in the papilla. There was an increase in the number of pyknotic interstitial cell nuclei in Obese mice (upper papilla region) and C57BL/4 mice (papilla tip). Obese and C57B1/6 mice had collecting duct cells which contained PAS positive staining granules within the cytoplasm. C57B1/6 mice also had casts in the loop of Henle and intensely eosinophilic staining granules/droplets in the cytoplasm of loop of Henle, collecting duct and proximal tubules. Some P₃ segments had lost alk. phos. brush border staining.

4.2.2.3.D Twelve hr time point
Obese mice had enlarged vacuolated covering epithelium and pelvic epithelium cells in the fornices. All tubular lumen in the papilla were dilated with occasional alk. phos. staining casts in the loop of Henle. Cortical changes were evident as PAS positive staining casts in the collecting duct. Balb/c mice had an increasing number of enlarged collecting duct cells with decreased interstitial cell matrix staining in the mid-papilla region. C57B1/6 mice had enlarged collecting duct cells in papilla with more PAS positive, basophilic cytoplasmic granules (Figure 4.25), and cortical collecting duct cells exfoliating into the lumen. Proximal tubule brush border
Figure 4.24. Pyknotic interstitial cell nuclei (arrow) in papilla of C57Bl/6 mouse 3 hr after 100 mg/kg BEA, Giemsa x 543.

Figure 4.25. Enlarged collecting duct (CD) epithelial cells containing basophilic cytoplasmic granules (arrow), C57Bl/6 mouse 12 hr after 100 mg/kg BEA, Giemsa x 868.
disruption and blebbing into the lumen was observed together with loss of alk. phos. staining in both Obese and C57B1/6 mice (P2 segment).

4.2.2.3.E Twenty-four hr time point.

Obese and C57B1/6 mice showed the following changes; prominent interstitial cell matrix staining in at the papilla tip, an influx of polymorphonuclear leucocytes in capillaries and interstitial cell matrix of papilla, covering epithelium cells in fornices were swollen and blebbing containing basophilic cytoplasmic granules (Figure 4.26) which were also PAS positive (C57B1/6 > Obese). Basophilic staining casts with exfoliated cells were present in loops of Henle, collecting duct, in all regions of the kidney. Balb/c mice had a total loss of interstitial cell matrix staining in the upper papilla, but the collecting duct and loop of Henle were still intact with no necrosis evident. A few basophilic casts present in the cortical collecting duct and occasional alk. phos. staining casts in the papilla.

Figure 4.26. Hyperplastic, blebbing covering epithelial (CE) cells with vacuoles and basophilic cytoplasmic granules, C57B1/6 mouse 24 hr after 100 mg/kg BEA, Giemsa x 868.
4.2.2.3. F Forty-eight hr time point
C57Bl/6 mice had dilated tubular lumen in the papilla with many collecting duct cells containing PAS positive staining cytoplasmic granules, increased interstitial cell matrix staining at papilla tip, mild hyperplasia (2-3 cells thick) of the pelvic epithelium in the fornices with lobulated basophilic staining nuclei. Balb/c mice had pyknotic nuclei in loop of Henle and collecting duct in upper papilla, enlarged basophilic interstitial cell nuclei adjacent to the vasa rectae, PAS positive staining casts in the cortical collecting duct. There was no noticeable progression in intensity of lesion at 48 hr in the Obese mice.

4.2.2.3. G Seven day time point
Obese mice had necrotic collecting duct and loop of Henle with exfoliating cells, some proximal tubules which had basophilic staining nuclei and cytoplasm in a radiating pattern. Some glomeruli and Bowman's capsule had thickened basement membranes and sclerosis particularly the juxtamedullary glomeruli. C57Bl/6 mice had intact collecting duct and loop of Henle in the papilla the overall appearance of the lesion was worse with the changes described previously being more intense. Many casts throughout the kidney and more adherent platelets, variable alk. phos. and PAS staining of the proximal tubule brush border and displacement of collecting duct nuclei to luminal surface of cells.

4.2.2.3. H Fourteen day time point
C57Bl/6 mice had alk. phos. staining casts in the papilla, in the cortex there were some proximal tubule segments which had flattened epithelial cells with basophilic staining cytoplasm. In Obese mice there was no noticeable difference in the appearance of kidneys from
BEA treated animals from the kidneys from control animals. Balb/c did not show any differences from control kidneys.

4.2.2.3.1 Twenty-four day time point

The C57B1/6 mice had casts within the papilla (Figure 4.27) but no gross total necrosis of the loop of Henle, collecting duct or covering epithelium usually found in the Wistar rat. There was focal interstitial nephritis with aggregates of monocytic leucocytes and basophilic staining atrophying tubules adjacent to juxtamedullary glomeruli (Figure 4.28), very prominent basement membrane staining (with PAS). Balb/c mice showed no gross necrosis in the papilla but prominent PAS staining casts were present in the vasa rectae vessels which also had thickened basement membranes, and some focal interstitial nephritis in the cortex but not to the same degree as C57B1/6 mice. No slides from Obese animals were available for examination at the 21 day timepoint.

4.2.3 Results From Dose and Time Course Studies in Nude Mice.

The reasons for having Nude mice available and the basis for using them in acute RPN studies have been presented in Methods section 3.6.2.2.

4.2.3.1 Controls.

No abnormal morphology was observed in any of the control kidneys examined (n=4) and structural and histochemical features were the same as seen in other mouse strains. The brush border of all proximal tubular segments stained for PAS positive material. The P₁ and P₂ segments stain positively for alk. phos. while the P₃ segment showed
Figure 4.27. Casts and numerous platelets in capillaries and intact covering (CE) and collecting duct (CD) epithelia, C57Bl/6 mouse 21 days after 100 mg/kg BEA, Giemsa x 543.

Figure 4.28. Thickened basement membranes and atrophying tubules in focal interstitial nephritis, C57Bl/6 mouse 21 days after 100 mg/kg, Giemsa x 543.
the preferential localisation of GGT.

Whereas rat papilla stains prominently for the interstitial cell matrix Nude mice (in common with other mouse strains, section 4.2.2) did not. The interstitial mucopolysaccharide matrix in the mouse stained much more uniformly and did not increase in intensity towards the papilla tip as it does in the rat (Gregg et al, 1990a,b).

4.2.3.2 Twenty-four hour time point.

4.2.3.2.A 50 mg dose.

The interstitial matrix showed no changes in PAS, but intense pink Giemsa staining was observed throughout the papilla. In the papilla tip the collecting duct epithelial cells had slightly swollen cytoplasm which contained small PAS positive granules. The P$_3$ proximal tubule brush border had a decreased staining intensity for PAS. There were no changes in the other histochemical stains.

4.2.3.2.B 100 mg dose.

The interstitial matrix was disrupted at the papilla tip as shown by decreased staining with both PAS and Giemsa. Interstitial cell nuclei were pyknotic and associated with polymorphonuclear leukocyte infiltration (Figure 4.29). Capillaries in the papilla tip had adherent platelet and numerous erythrocyte thrombi. Swollen collecting duct cells contained basophilic and eosinophilic staining granules which were PAS positive (Figure 4.30). Loops of Henle in the mid-papilla region contained proteinaceous casts which were also PAS positive. The P$_2$ brush border was necrotic with variable alk. phos. staining.

4.2.3.2.C 200 mg dose.

All of the changes described for the 100 mg dose were observed with
Figure 4.29. Pyknotic interstitial cell nuclei and polymorphonuclear leukocytes in interstitial matrix (arrow), 24 hr after 100 mg/kg BEA. Giemsa x 543.

Figure 4.30. Swollen collecting duct epithelial cells contain PAS positive staining granules in cytoplasm (arrow), 24 hr after 100 mg/kg BEA. PAS x 868.
the following additions. The luminal membrane of collecting duct epithelial cells was irregular and blebbing into the lumen, there was also adherent cellular debris within the lumen. There was vacuolation of the P3 cytoplasm and a decrease in alk. phos. brush border staining in all segments of the proximal tubule.

4.2.3.3 Forty-eight hour time point.

4.2.3.3.A 100 mg dose.

There was a loss of interstitial matrix staining at the papilla tip together with an increase in pyknotic interstitial cell nuclei throughout the papilla and the medulla. Numerous adherent platelets and erythrocytes were present in the capillaries (Figure 4.31). The collecting duct (medullary and cortical) contained numerous proteinaceous casts in the lumen, which stained positively for PAS, and similar casts were observed in the loops of Henle. The P2 proximal tubule segment showed a loss of alk. phos and GGT staining from the brush border. Necrosis of the P3 proximal tubule segment was evident with cytoplasmic vacuolation, detachment of the brush border and extrusion of nuclei into the lumen (Figure 4.32).

4.2.3.3.B 200 mg dose.

There was progressive necrosis of many nephrons with extensive cast formation (Figure 4.33) in the papilla, medulla and cortex. Many of the changes for the 100 mg dose at 48 hr were also observed. Additional changes included dilatation of the collecting ducts in the mid-papilla region, which contained alk. phos. positive casts, as did the loops of Henle. The epithelium covering the papilla, together with the upper pelvic urothelium, was hyperplastic in the fornix region. Proximal tubular necrosis was more marked than at the 100 mg dose,
Figure 4.31 Adherent platelets and erythrocytes in capillaries (arrow), 48 hr after 100 mg/kg BEA. Giemsa x 868.

Figure 4.32 P3 proximal tubular segment cytoplasmic vacuolation and disrupted brush border (arrow), 48 hr after 100 mg/kg BEA. Giemsa x 543.
Figure 4.33. Extensive cast formation in papilla and medulla, 48 hr after 200 mg/kg BEA. Giemsa x 89.

Figure 4.34. Necrotic F3 with PAS positive cytoplasmic droplets and large basophilic droplets in lumen (arrow), 48 hr after 200 mg/kg BEA. PAS x 868.
particularly in the P₃ segment where PAS positive staining cytoplasmic granules were numerous (Figure 4.34). There was a loss of GGT brush border staining and large basophilic droplets within the lumen of P₃, collecting duct and loops of Henle in the medulla. The P₂ segment showed extensive necrosis, but some evidence of re-epithelialisation was also present with flattened basophilic cells. These cells exhibited increased alk. phos. staining intensity along the apical (brush border) membrane (Figure 4.35). In addition focal severe interstitial nephritis with thickened glomerular membrane and capsular membrane was observed. Adjacent tubules were atrophyic and surrounded by invasive monocytic cells.

4.2.3.4 Seventy-two hour time point

4.2.3.4.A 50 mg dose.

The interstitial cell matrix was granular where loss had occurred. There were numerous polymorphonuclear leukocytes within the matrix space. Interstitial cell nuclei were pyknotic at the papilla tip. There was hyperplasia (up to 3 cells thick) of the epithelium covering the papilla tip, many of these cells contained PAS positive staining granules. The changes in the proximal tubule were restricted to variable alk. phos. and GGT staining along the brush border of the P₂ and P₃ segments.

4.2.3.4.B 100 mg dose.

The changes at this dose are almost identical to those described for 200 mg at 48 hr, this description will suffice.

4.2.3.4.C 200 mg dose.

Evidence of frank RPN was observed with the total loss of the interstitial cell nuclei and matrix (Figure 4.36), denudation of the
Figure 4.35. Re-epithelialisation of P₂ segment with intense alkaline phosphatase staining of apical membrane (arrow), 48 hr after 200 mg/kg BEA. Alkaline phosphatase x 543.

Figure 4.36. Denudation of covering epithelium (arrow), total necrosis of tubular and interstitial elements of papilla, collecting duct and loop of Henle "ghosts" contain exfoliated cellular debris (arrowheads), 72 hr after 200 mg/kg BEA. Giemsa x 217.
covering epithelium at the papilla tip (Figure 4.36), hyperplasia of the leading edge and numerous PAS positive granules in the cytoplasm. There was hyperplasia of the pelvic urothelium in the fornices with mitotic figures present in the pelvic urothelium opposite the papilla tip. All tubular elements of the cortex were necrotic with extensive cast formation (Figure 4.37) which stained positively for alk. phos., GGT and PAS. Some proximal tubular segments were re-epithelialising having a flattened epithelial cell (with intensely luminal staining for alk. phos. see Figure 4.38) the majority of proximal tubular segments were, however, necrotic with extensive cast formation exhibiting positive staining for alk. phos. and GGT. The cortical collecting duct cells had swollen pale staining cytoplasm, these tubules were extremely dilated with cellular debris, and PAS staining casts within their lumen (Figure 4.39).

Figure 4.37. Extensive necrosis and cast formation of cortical tubules, 72 hr after 200 mg/kg BEA. Giemsa x 543.

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Figure 4.38 Re-epithelialised proximal tubule segment has flattened cells with intense alkaline phosphatase staining on luminal membrane, 72 hr after 200 mg/kg BEA. Alkaline phosphatase stain x 868.

Figure 4.39 Hyperplasia of collecting duct (CD) in cortex, lumen contains intensely PAS positive staining casts. PAS x 217.
4.3 MORPHOLOGICAL CHANGES IN MARMOSET KIDNEYS TREATED WITH BEA.

The reasons for using marmosets are briefly discussed in section 1.4.4 and the methods in section 3.6.3.

4.3.1 Animal Dosed with BEA 50 mg/kg.

Marmosets do not have a true papilla as found in the rat but have a rounded papilla ridge. In this kidney after BEA (50 mg/kg) the papilla was intact with no gross morphological changes. The morphology of cortex and medulla was "normal" with no gross necrotic changes. In the papilla a few interstitial cell nuclei were pyknotic in the papilla tip. The intensity of the interstitial cell matrix staining intensity was darker in the mid-medulla area, but was a paler, pink towards the papilla tip (as the volume of this matrix increased relative to the volume of tubular elements). The collecting duct cells were cuboidal in medulla becoming columnar in papilla tip, a few cells had swollen nuclei and cell cytoplasm. A few loops of Henle had basophilic casts which had some mineralised deposits, together with crystalline deposits (shown by darkfield microscopy) in the lumen. The covering epithelium of the papilla was between 1-3 cells thick with variable cytoplasmic and nuclear staining, the occasional cytoplasmic vacuolation and sloughing cell. Superficial cells were covered with a prominent pink glycocalyx layer in several areas which was blebbing into the pelvic space.

The morphology of pelvic urothelium was very similar to the papilla covering epithelium increasing to 4 cells thick with variable basophilic and eosinophilic cellular staining with Giemsa stain.

4.3.2 Animal Dosed with 100 mg/kg.

As with results described in section 4.3.2 the morphology of cortex was "normal" with no gross necrotic change. The papilla ridge was
intact with areas of interstitial cell matrix disruption and nuclei pyknosis. The most prominent change was a hyperplasia of the covering epithelium of the papilla (to 7 cells thick, see Figure 4.40) some cells were vacuolated with extremely basophilic nuclei and others had eosinophilic cytoplasmic staining with a loss of glycocalyx from cell surface (Figure 4.41). The pelvic urothelium was also hyperplastic with eosinophilic staining granules in large vacuolated cells. Basophilic casts were present in the loop of Henle (Figure 4.41), together with large numbers of erythrocytes and platelets in capillaries. All these changes suggest the presence of an early RPN due to BEA similar to that in the rat, but far less severe considering the duration of the experiment was 48 hr.

Figure 4.40. Papilla of Marmoset 48 hr after a single ip dose of BEA (100 mg/kg), note hyperplastic covering epithelium (CE). Giemsa x 43.
4.4 CHEMICALLY INDUCED RPN IN THE PIG.

A number of the investigations (section 3.6.4) were performed on pigs were repeated due to the limitation of only being able to 4 animals in any single experiment because of a lack of pen space. It thus made sense to combine results from studies where the experimental protocol was similar.

4.4.1 Results from Studies 1 and 2: BEA-induced RPN in Pigs (6-7 Months Old.

4.4.1.1 General observations

In study 1 neither animal given 50 mg/kg BEA showed any signs of distress, but those receiving 100 mg/kg had an emetic response 15 min after recovering from the anaesthetic. This lasted for several hours,
and 1 animal died (from unknown causes) approximately 24 hr later. In
study 2 despite the administration of an anti-emetic both animals
still vomited a considerable number of times on recovering from the
anaesthetisia. One animal died within 24 hr (from unknown causes) in
the early hours of the morning and thus was not found until the next
morning and the tissues had undergone too much autolytic changes for
useful assessment of histopathological changes. The remaining animal
survived up to sacrifice (14 days later) with no apparent clinical
effects as a consequence of the BEA.

4.4.1.2. Macroscopic changes
There were prominent casts in the medullary rays, and dark areas at
the papilla tips from which the epithelium covering had exfoliated in
pigs given the high dose (Figure 4.42). There were no apparent
pronounced changes in the pigs given 50 mg/kg BEA. In study 2 there
was, however, no gross exfoliation of the covering epithelium.

4.4.1.3 Microscopic changes
In both studies morphological changes that were associated with the
onset of early RPN were present after dosing with 50 and 100 mg/kg
BEA. These included disruption of interstitial matrix (Figure 4.43),
focal interstitial cell necrosis (Figure 4.44) exfoliation of the
papilla covering epithelium and necrosis of collecting duct epithelial
cells (Figure 4.45). These changes were more pronounced in the
animals given 100 mg/kg BEA. In the single animal which was
sacrificed at 14 days after dosing with 100 mg/kg BEA had some changes
which were not apparent in the animal killed at 7 days. There was
hyperplasia of the pelvic/calyceal urothelium which had a vacuolated
superficial layer with amorphous material erupting from the distended

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Figure 4.42. Papilla tip, 7 days after single iv dose BEA 100 mg/kg showing exfoliated covering epithelium exposing underlying capillary bed and interstitium. x 5 approximately.

Figure 4.43. Papilla tip from pig kidney 7 days after single iv dose BEA (100 mg/kg) showing disruption of interstitial matrix (arrow) with a decrease in 'pink' Giemsa staining. Giemsa x 223.
Figure 4.44. Focal interstitial cell necrosis (arrowheads) together with disrupted interstitial matrix (*), 7 days after single iv dose of BEA (100 mg/kg), Giemsa x 714.

Figure 4.45 Focal interstitial cell necrosis (arrow) and necrotic collecting duct epithelial cells (CD) in papilla, 7 days after single iv dose BEA (100 mg/kg), Giemsa x 714.
cells. Many collecting duct profiles were hyperplastic with basophilic cells having nuclei of variable shape and size together with mitotic figures present. There were also areas of microhaemorrhaging within the interstitial matrix. Fixed frozen sections showed an intense deposit of lipid material in the papilla covering epithelium in control tissues, but lipid droplets were absent in the renal medullary interstitial cells with Oil red O. The early RPN diagnosis was confirmed by Oil red O staining showing the presence of lipid droplets in the collecting duct epithelium in the 100 mg/kg treated group (Figure 4.46).

**Figure 4.46** Fixed frozen section of papilla 7 days after single iv dose of BEA 100 mg/kg showing Oil red O stained lipid in collecting duct (arrow) and covering epithelium cells. Oil red O x 223.

### 4.4.2. Results from studies 3 and 4: BEA and NaNPA-induced RPN in young pigs.

The justification in not combining the results with those of studies 1 and 2 is that the animals used in this study were younger (14 weeks old compared to 28-32 weeks) for the animals reported above.
4.4.2.1 General observations.

Four of the animals in the studies were dosed with BEA; two received two doses of 50 mg/kg and two received a single dose of 100 mg/kg. Again one of the high dose BEA animals died within 24 hrs of receiving the dose from unknown causes after a period of sustained vomiting. Four animals were dosed with NaNPAA; two received 1.0 mmol/kg dose and two received 0.5 mmol/kg dose. One of the animals which received the high dose of NaNPAA died almost immediately. The animals which died had too much autolytic changes to consider worthwhile assessing because they died during the night and were not found until morning. The animals which were given NaNPAA over an extended infusion period of 30 min survived with no observable ill effects for the duration of the experiment.

4.4.2.2 Microscopic changes.

4.4.2.2.A Control results

In the single control animal available in these studies there were no obvious abnormalities with regard to tissue and cellular morphology. Giemsa staining of the interstitial matrix was uniform and not disrupted although there was the occasional aggregation of interstitial nuclei (Figure 4.47).

Collecting duct cells were usually columnar with basally located rounded nuclei and at the papilla tip contained apical and basally located PAS positive staining granules. These granules were also present in pelvic calyceal and ureteric urothelia and in the covering epithelium. Occassionally vacuoles were observed within the apical region of the superficial layer of pelvic calyceal cells. These vacuoles contained PAS positive staining material.
Figure 4.47. Control papilla showing uniform staining interstitial matrix and occasional aggregate of interstitial cell nuclei (arrowhead), Giemsa x 89.

4.4.2.2.B Treated group results.

Overall changes supported a RPN lesion or interstitial cell nephritis, particularly because of interstitial cell proliferation, collecting duct epithelial cell hyperplasia, thick ascending limb necrosis and hyperplasia of the pelvic/calyceal and ureteric urothelium with vacuolation and mitotic figures present throughout many of the tubular cells in the papilla and medulla. Changes observed were very similar to previous experiments using BEA reported above section 4.4.1.3. There appears to be little difference between the morphological changes in the lesions produced by the BEA or NaFPA compounds. Any inter-section differences were not due to the different populations of papillae in kidney ie polar or mid zonal. The most prominent changes occurred in collecting duct epithelial
cells usually in region midway down length of the papilla where hyperplasia and dysplasia of these cells was often evident. The nuclei exhibited variable shapes and frequently intense basophilic staining and shrunken or undergoing mitosis (Figure 4.48). The hyperplasia was of a low papilloma type pattern with small clumps of cells protruding into the lumen which was often entirely occluded (Figure 4.49). Frequently there were aggregates of proliferating interstitial cells (or monocytic leukocytes) adjacent to the hyperplastic collecting ducts cells, often to the extent that it was difficult to differentiate between these aggregates and the collecting duct epithelial cells.

In addition to these prominent changes other morphological abnormalities included enlarged, lobulated nuclei with basophilic cytoplasmic staining in urothelia covering papilla and calyceal which also frequently had blebbing or vacuolated superficial cells which were PAS positive staining (Figure 4.50).
Figure 4.48. Hyperplasia and dysplasia of collecting duct (CD) epithelial cells with mitotic figures present (arrowheads) 7 days after BEA or NaNPAA. H&E x 446.

Figure 4.49. Hyperplasia in collecting ducts (CD) cells form a low papilloma clump pattern (arrowheads), H&E x 89.
Figure 4.50. Pelvic calyceal epithelium (PE) with vacuolated blebbing, superficial layer cells which are also PAS positive (arrowheads). PAS x 357.

PAS positive granules evident in distal papilla collecting duct epithelial cells. In the loops of Henle (thick ascending portion) the basophilic staining, condensed cell nuclei were frequently displaced to the luminal apical region of the cell and/or exfoliating into the lumen.

4.4.3 Study 5: Effects of Superimposed Paracetamol Dosing on an Already Existing RPN.

Since renal size, weight and function increase with age in the pig up to approximately 1 year of age, the experimental results were also compared with those obtained for age-matched historical controls.

4.4.3.1 Renal function.

4.4.3.1.A Glomerular filtration rate (GFR).

The percentage changes in GFR for each experimental group, compared with the pooled mean pretreatment value, as shown in Figure 4.51. All
groups showed an increase in GFR after 24 days, the largest increase occurring in the control pigs.

4.4.3.1.B Effective renal plasma flow (ERPF).

These data were plotted for trend with individual values at day 0 (prior to treatment) and at day 26 of the study (Figure 4.52). In both the BEA/paracetamol and BEA only treated groups there is a trend for an increase in ERPF value with the final values falling outside the 95% confidence limits for historical control data.

4.4.3.1.C Ultrasound assessment.

There was no obvious significant differences in any of the data (not shown) obtained from the ultrasound scan measurements in any of the groups.

4.4.3.2 Gross pathology: kidney weight and body weight data.

Figure 4.53a illustrates the mean body weight values for all groups. Although the mean values for treated groups were significantly greater than that observed in controls, all values were within the 95% confidence limits obtained from historical control data. Thus there was no significant difference in the extent of the increase in body weight between the groups.

The mean kidney weight values are shown in Figure 4.53b. Only the paracetamol treated group was significantly different from the experimental control value; it also lay outside the 95% confidence limits for historical control data. When expressed as a fraction of the body weight (Figure 4.53c) this value fell within the 95% confidence limits for historical control data and thus there was no significant difference between any of the mean group values.
Figure 4.51. Mean GFR values at day 26 expressed as a percentage compared to the pooled mean GFR pretreatment values at day 0. BEA = 2-bromoethanamine, APAP = paracetamol.
Figure 4.52. Individual ERPF values plotted for trend at day 0 and day 26. BEA = 2-bromoethanamine, APAP = paracetamol.
Figures 4.53a-c. illustrate: a) the mean body weight values, b) the mean kidney weight values and c) the mean kidney weight/body weight values. BEA = 2-bromoethanamine, PARA = paracetamol.
4.4.3.3 Histopathological results.

The papillae from the kidneys of BEA and BEA/paracetamol treated animals showed similar changes: hyperplasia with blebbing of covering epithelium, some pyknotic interstitial cell nuclei and slightly disrupted interstitial cell matrix (Figure 4.54) compared to control papillae (Figure 4.55). There were also pyknotic, exfoliating nuclei within the thick ascending loop of Henle (Figure 4.56). The most obvious morphological change in the papillae from BEA and BEA/paracetamol treated animals was an extensive hyperplasia of the pelvic calyceal and ureteric urothelium with vacuolation and blebbing of the superficial cells (Figure 4.57), compare with control pelvic calyceal urothelium (Figure 4.58). Both Giemsa and PAS stains highlight the carbohydrate nature of the substance within these vacuoles and cells. Similar but less severe changes were also observed in the bladder urothelium. There were no necrotic changes in the paracetamol only treated animals, but some dilatation of the kidney tubules were seen (Figure 4.59), the urothelia was no different from that in controls.

4.5 HUMAN ANALGESIC ABUSERS.

4.5.1 General Comments.

The information obtained from the clinical histories told us that the few human tissue samples provided by Dr Anke Schwarz came from typical analgesic abusers. All the patients were women aged between 50 and 63 yr (mean 62.25 yr) who had each had a history of analgesic abuse with the period of consumption (of mixed analgesic compounds) ranging from 8-30 yr. Typically patients denied abuse and continued even when previous medical treatment had been necessary (unilateral
Figure 4.54. Papilla tip from BEA only treated animal with hyperplastic, blebbing, covering epithelium (arrow), pyknotic interstitial cell nuclei and disrupted interstitial matrix. Giemsa x 178.

Figure 4.55. Papilla tip from control animal shows normal appearance of covering epithelium (CE) and interstitial matrix (*), Giemsa x 178.
Figure 4.56. Pyknotic, exfoliating nuclei (arrow) in thick ascending limb of Henle from BEA treated animal. Giemsa x 446.
**Figure 4.57.** Extensive hyperplasia of pelvic calyceal urothelium with vacuoles containing granular or amorphous mucopolysaccharide material, from a BEA/paracetamol treated animal. Giemsa x 357.

**Figure 4.58** Pelvic calyceal urothelium from control animal, Giemsa x 357.
nephrectomy) because of the condition. All patients displayed typical symptoms of analgesic nephropathy: shrunken kidneys, calcified papillae, recurrent urinary tract infection, and clinical evidence of progressive renal insufficiency. Hemodialysis had been required by all the patients (one for a period of 10 yr), 2 patients were still receiving hemodialysis, illustrating the long-term economic cost to health services of analgesic nephropathy. 2 patients had urothelial carcinoma of the renal pelvis and ureter.

4.5.2 Histopathological Changes.

Histologically the tissues showed many changes associated with advanced analgesic nephropathy as described by Burry et al, (1977). Tissues were often so shrunken and covered in fat that it was hard to believe that the tissue did actually represent a kidney. Papillary necrosis was evident with total loss of tissue integrity in the papilla (when present) with many casts, adherent platelets in the
capillaries (Figure 4.60). There was a lot of fibrosis and sclerosis of the medullary and cortical tissue and atrophy which is associated with a chronic interstitial nephritis. There were thickened basement membranes around the distal tubules and collecting ducts in medulla, and atypical basophilic nuclei in these nephron segments too (Figure 4.61). In the cortex there were numerous sclerotic glomeruli and aggregates of monocytic leukocytes (Figure 4.62). Although no urothelial carcinoma of the pelvis or ureter was observed in these samples this may have been because the pathologists in Germany removed most of these lesions for their own histopathological samples (Dr Anke Schwarz, personal communication). However, hyperplastic blebbing covering epithelium of the papilla was observed (Figure 4.63) which was atypical, frequently sloughing, though this may have been an autolytic artefact.
Figure 4.60 showing adherent platelets in capillaries within papilla from the kidney of an analgesic abuser, Giemsa x 357.

Figure 4.61 Thickened basement membrane (arrowhead) around distal tubule and atypical basophilic nuclei in collecting duct (CD) in medulla from kidney of an analgesic abuser. Giemsa x 357.
Figure 4.62. Interstitial nephritis in the cortex of kidney from an analgesic abuser. Note sclerotic glomeruli (arrowheads), tubular atrophy and aggregates of monocytic leukocytes (arrow), Giemsa x 357.

Figure 4.63. Hyperplastic, blebbing, covering epithelium of papilla from kidney of an analgesic abuser, Giemsa x 357.
4.6 DISCUSSION OF CHEMICALLY INDUCED RPN INVESTIGATIONS.

4.6.1 Rat: Morphology and Enzyme Histochemistry.

In this study, the detailed sequence of morphological changes associated with a BEA-induced RPN in male Wistar rats has been assessed. High resolution microscopy using lum glycolmethacrylate embedded tissue (sections 4.1.1-4.1.1.6) identified the earliest cells to show degenerative changes, the cascade of injury during the development of RPN, the role of microvascular integrity, and changes in the carbohydrate containing components in different parts of the kidney.

Histochemistry provides a sensitive means of detecting subtle changes in cells following chemical insult. It is therefore a powerful tool to study the progression of a model lesion in the kidney and to develop a more rational understanding of the sequence of pathophysiological changes that lead from a primary (and often focal) injury to the final advanced (and possibly end stage) nephropathy. Relatively little is known about the histochemical changes that are associated with the genesis of analgesic-related renal papillary necrosis in man (Bach & Bridges, 1985).

The data reported in this study (section 4.1.1) clearly illustrate that the renal medullary interstitial cells undergo the earliest degenerative changes in rats. They are also the first cell type in which necrosis was clearly established. This confirms earlier studies using BEA (Hill et al., 1972; Bach et al., 1983), and supports similar changes for RPN induced by aspirin (Molland, 1976, 1978), and N-phenylanthranilic acid (Hardy & Bach, 1984). This suggests that the basis of papillotoxicity caused by three different classes of chemicals resides in the biochemical characteristics of the medullary
interstitial cells. It does not, however, explain the failure of Hill et al, (1972) and Murray et al, (1972) to observe similar changes. Other differences in morphological changes following BEA have been reported between female Hooded rats (Hill et al, 1972; Murray et al, 1972) and male Wistar rats (Bach et al, 1983; Gregg et al, 1990a,b). Renal medullary interstitial cell necrosis and medullary matrix changes were early targets in the development of the lesion in male Wistar rats (Bach et al, 1983; Gregg et al, 1990a). Most of the changes in female Hooded rats were reported in terms of simultaneous tubule and blood vessel degeneration at the light (Murray et al, 1972) and ultrastructural levels (Hill et al, 1972). These authors did not report matrix changes.

The data reported in this thesis (section 4.1.1) confirm earlier investigations (Bach et al, 1983; Mattingley et al, 1985) in which the mucopolysaccharide matrix first showed an increased, followed by a loss of, staining intensity in those areas where necrosis had occurred. Similar matrix changes have been reported in animals following aspirin-induced RPN (Molland, 1976, 1978) and treatment with BEA (Bach et al, 1983; Gregg et al, 1989a). The present study made use of a calcium containing fixative, which helps to prevent the leaching of extracellular mucopolysaccharide matrix (Horobin, 1982), and used both Giemsa and PAS staining, each of which interact with carbohydrate moieties, rather than binding or interacting with highly charged groups (-SO₄⁻ or -COO⁻) previously shown by colloidal iron and Toluidine blue (Bach et al, 1983). Thus the medullary mucopolysaccharide undergoes an early perturbation, although the exact chemical changes (loss of matrix or binding sites), conformation
or structural changes of the proteoglycan/glycosaminoglycan material still require full evaluation. Recent studies (Bach & Lu, unpublished observation) have suggested that the matrix undergoes desulphation and depolymerisation following BEA administration. Uronic acid-containing substances are excreted in the urine of rats treated with BEA (Ding et al, 1989). The time course of this excretion parallels the histochemical changes observed in this study (section 4.1.1). These substances could represent degradation products derived from the glycosaminoglycan; hyaluronic acid. This has been reported to be the dominant glycosaminoglycans present in the interstitial matrix of the rat (Pitcock et al, 1988), with an important physiological role in the maintenance of the anatomical integrity of the tubular elements in the papilla and water reabsorption (Pitcock et al, 1988). Such changes in the hyaluronic acid component of the interstitial matrix could drastically alter the micro-environment of the medullary nephron and lead to changes in renal function, with cell degeneration and/or death. It was not possible, from light microscopic data, to be certain if the matrix changes are a consequence or the cause of interstitial cell degeneration, or if a chemical effect on the collecting duct cells plays a role in these mucopolysaccharide changes. Some studies have shown an increase (Burry, 1978; Burry et al, 1977) or a decrease (Gloor, 1978) in the medullary mucopolysaccharide matrix staining in analgesic abusers. But it is not possible to say this represents early and late changes as is the case in animals.

It has been suggested that RPN might be a consequence of ischaemic infarction or that microvascular degeneration could be integral component in the pathogenesis of the lesion (Rosner, 1976; Shelley,
High resolution microscopy (section 4.1.1) in this study showed that platelet adhesion did not occur before 12 hr, at which time the medullary interstitial cell necrosis was advanced. Hill et al (1972) also reported that microvascular occlusion developed late in the genesis of RPN. Previous studies in the acutely induced lesion (Bach et al, 1983) showed that colloidal carbon filled the medullary microvascular regions in which necrosis had already occurred. Colloidal carbon only demonstrates the pattern of vascular filling and cannot provide data on endothelial changes that would result in the leakage of material into the matrix. Monastral blue B has been used by Joris et al, (1982) to show leaky capillaries that were associated with inflammatory changes. Monastral blue filled medullary capillaries in all areas where BEA-induced necrosis had occurred and it was also compartmentalised to the medullary microvasculature with no evidence of extravasation before necrosis was extensive. This established the absence of early microvascular occlusion and/or leakage of plasma material during the development of the papillary lesion. Taken together these data serve to confirm that the endothelial changes in an acutely induced RPN represent a consequence of papillary necrosis, and do not play a preliminary role in the development of the lesion.

The use of fixed, glycolmethacrylate embedded sections to study a series of renal enzyme markers allowed a comparison of the results from the investigations reported here (section 4.1.1) with those of Hill et al, (1972) and obtain other information on the development of the lesion, and changes in the proximal tubule.

Hill et al, (1972) studied both lactate dehydrogenase and ATPase in
frozen, freeze-dried 4um section from rats treated with BEA. They reported a transient loss of ATPase from the vasa recta and the loss of lactate dehydrogenase from the papilla tip. Whereas an increase in ATPase staining in the interstitial cells at the papilla tip was observed. These apparent differences in observations may be due to the different methodologies used. However none of these changes could be related to the pathogenesis of the primary lesion or to its secondary consequences because there were too few time points. There are differences in the morphological course of the BEA-induced lesion described by in this thesis compared to that reported by Heptinstall and co-workers (Hill et al, 1972; Murray et al, 1972; Wyllie et al, 1972). Early cortical proximal tubular hydropic changes are transient (Bach et al, 1983; Gregg et al, 1990a), but the deposition of tubular casts, first in the ducts of Bellini and later in the medullary collecting ducts, appears to play a prominent role in the development of distal tubule dilatation and, later, the dilatation of the proximal tubule (Gregg et al, 1990a). Repair between the junction of the necrosed and still viable tissue was very active at 18 hr, and the presence of substantial macrophage invasion was expected on the basis of their participation in both the inflammatory response and repair processes initiated by cellular damage (Vernon-Booth, 1972).

The loss of proximal tubular brush border marker enzymes at 12 hr (section 4.1.1) shows that these changes occur relatively late in the development of the lesion, considering that the matrix changes occurred as early as 2-4 hr after BEA (Bach et al, 1983; Gregg et al, 1990a,b) and the interstitial cells showed frank degeneration at 8 hr (Gregg et al, 1990a). Thus it is unlikely that proximal tubular
changes play any role in the primary development of RPN. More importantly, routine morphology (eg. H&E staining) of the proximal tubule using 5 um wax embedded sections only shows mild and transient hydropic changes (Bach et al, 1983). The data from this study described above do not make it possible to be certain if the loss of proximal tubular marker enzymes represents subtle BEA-associated cellular injury to this part of the nephron, damage to the glomeruli leading to a protein overloading of the proximal tubular cells, or if the brush border changes resulted from the diuresis that follows early papillary injury (Fuwa & Waugh, 1968; Sabatini et al, 1984; Wilks et al, 1986). It does, however, establish that the presence of enzyme activity in casts from 12 hr represents a consequence of the loss of the brush border from the proximal tubule. An enzymuria has been shown to be sustained for up to 7 days after an acute BEA-induced RPN (Saw, 1988; Delacruz et al, 1989), but the loss of brush border enzymes (alkaline phosphatase and GGT, see section 4.1.1) seemed occur as a consequence of RPN. The casts also contain cellular debris. The sequential dilatation of the distal and then proximal tubules suggests that material in the collecting ducts did, in fact, contribute to the cystic changes that occurred in the nephron, but other factors may be involved. A transient albinuria has been reported after BEA-induced RPN (Moret et al, 1989), it has been suggested that this represents an effect on the glomerular permselectivity by BEA (possibly by the cationic BEA neutralising polyanions), which could exacerbate the loss of brush border enzymes and cast formation.

The development of upper urothelial hyperplasia shortly after the
first definite signs of papillary necrosis suggested that these proliferative changes occurred subsequent to the development of the medullary lesion. Repair of the damaged papillary urothelium appears to be a marked and prolonged process. More importantly, there is also an increase in urothelial proliferation in the pelvis opposite the region of necrosis and also in the ureter, as assessed by the increase in cell layer thickness. There was a distinct pattern of proliferative change in the upper urothelium following BEA administration. The epithelium covering the papilla tip showed a mild proliferation before it was lost. Subsequently, there were marked epithelial cell hyperplasias in the pelvis, the fornix and especially the ureter. It was only at 144 hr that mild proliferative changes were first seen in the bladder.

Using continuous infusion of $^3$H-thymidine and autoradiography, Mattingley and co-workers have investigated the proliferative response in the different regions of the pelvis and ureter after a BEA-induced RPN (Mattingley et al, 1990). Compared to the baseline cell turnover rate there was a 2-3 fold increase in all the areas of the urothelium 144 hr after BEA, except for the collecting duct (8-fold) and the pelvic fornix (16-fold). The most active regions of cell proliferation after BEA were the collecting duct, pelvic fornix, pelvic urothelia (opposite the papilla tip and margin of the papillary injury) and the origin of the ureter. These data show that the urothelia of the upper urinary tract is very responsive to an acutely induced RPN, particularly as assessed by this autoradiographic technique. The delayed development of urothelial changes for the first 24 hr after the necrosis of the papilla suggests that this urothelial hyperplasia may be a consequence of RPN. Bach (1981)
reported a transient excretion of crystals (magnesium ammonium phosphate) in the urine early after administration of BEA to male Wistar rats. These crystals could cause a mechanical injury to the urothelium resulting the hyperplastic response. It is known that mechanical injury by foreign bodies including crystals can stimulate hyperplasia in the urothelium (Mobley et al, 1966).

It is generally agreed that there is a progressive inter-relationship between hyperplasia, dysplasia and malignancy (Farber & Sporn, 1976; Cohen, 1983). The study reported in this thesis appears to be the first that has examined the changes in the renal pelvis and upper ureter following an acutely induced RPN. The very marked pelvic and upper ureteric response to an acute RPN in the absence of a major bladder change highlights the focal nature of the injury and the urothelial response.

More importantly, the hyperplastic cells show marked changes in enzyme histochemistry, with increased alkaline phosphatase staining (section 4.1.1). These changes were maintained, especially in those areas where the hyperplasia was most marked. There is a very high incidence of UUC in human analgesic abusers (Bach & Bridges, 1985) and hyperplasia is also common in the upper urothelial tract (Lomax-Smith & Seymour, 1980a,b; Blohme & Johansson, 1981). Although there is no proven "cause-and-effect" between RPN and upper urothelial carcinoma, the development of bladder carcinoma has been well described in the experimental situation, where hyperplasia may be a prelude to malignancy (Hicks & Choweniac, 1978; Cohen, 1985; Ito & Fukushima, 1986). Hyperplastic bladder cells also show changes in histochemical profiles which include an increased loss of alkaline phosphatase and
an increased staining for GGT (Kunze, 1979; Ozono et al, 1985). The absence of these two features from the hyperplastic urothelia reported in these investigations suggests that the proliferative changes that follow the BEA-induced lesion probably represents a repair of injured tissue (Shimamura & Bonk, 1976; Bach et al, 1983) rather than a pre-malignant change per se. This difference could also be due to differences in methodology used to determine histochemical staining.

It is difficult to explain the difference between the renal ATPase changes recorded by Hill et al, (1972), and those reported in this investigation, but the time-course differences in the morphological changes between the two studies have already been highlighted (Gregg et al, 1990b) and may be due to strain differences in the rodents used. The changes in the staining of ATPase and alkaline phosphatase in the pelvic, ureter and bladder sub-urothelial capillaries in the present investigation were most marked. Similar changes in glomerular or proximal tubular injuries caused by other chemicals have not been observed (Kirby, Gregg and Bach, unpublished observation). The thickening of the ATPase stained areas, to the point where the lumen of the vessel was occluded, suggests that a capillary sclerosis had occurred. A microangiopathy with a progressive narrowing of the capillaries due to basement membrane thickening and the deposit of lipid material, has been described in humans with RPN (Gloor, 1978; Mihatsch et al, 1979, 1984; Palvio et al, 1985). These changes are thought to be pathognomonic in human analgesic abusers, but have not been reported previously in animal models of RPN and therefore warrant further investigation.

In conclusion, the sequence of enzyme histochemical changes reported
above supports the suggestion that the renal medullary interstitial cells and the mucopolysaccharide matrix are the earliest anatomical regions of the kidney to be affected by BEA. The fine elements of the medulla such as the loops of Henle and the endothelia also eventually undergo necrosis. Subsequently, the covering epithelium of the papilla and the collecting ducts are damaged. Once necrosis has developed there is a progressive accumulation of proteinaceous casts (that contain enzyme markers from the proximal tubule) within the loops of Henle and collecting ducts, that may lead to distal and then proximal tubular dilatation. The upper urothelium adjacent to the necrosed papilla undergoes changes from 24 hr. These include hyperplasia and an increased alkaline phosphatase staining, which was maintained for 144 hr. From 18 hr the sub-urothelial microvasculature of the pelvis, ureter and bladder show increased final reaction products for ATPase and alkaline phosphatase changes that may be indicative of a microangiopathy.

In view of the high incidence of upper urothelial carcinoma in human analgesic abusers (Bach & Bridges, 1985), the finding of hyperplasia following an acute lesion suggests that the upper urothelial tract has a high proliferative activity. This needs to be more fully evaluated, but suggests that papillary necrosis may, in itself, be a process that could promote an already initiated urothelium. This concept has been addressed by a series of studies reported below - see chapter 5.

The intermediate and long-term changes reported (sections 4.1.2.1-4.1.2.6) are similar to those reported by Axelsen (1978b) with re-epithelialisation of the papilla stump at 14-21 days. The time course
of the re-epithelialisation and restoration of the interstitial matrix in the papilla stump parallels a functional return of concentrating ability (Saw, 1988). The progressive changes resulting in the development of a severe chronic interstitial nephritis with scarring of the cortical surface, infiltration of monocytic leukocytes, collapse of renal parenchymal tissue, atrophy of tubules, fibrosis, sclerosis and mineralisation of basement membranes (see sections 4.1.2.1-4.1.2.6) mimics closely the long-term changes reported to occur in the clinical situation as a consequence of analgesic-associated RPN (Burry, 1968; Burry et al, 1977; Gloor, 1978) see section 2.1.2.3. These changes were also observed in the human renal tissue specimens obtained from analgesic abusers, provided by Dr A. Schwarz, see section 4.5.2. This provides further evidence for the suggestion that the BEA-induced acute RPN is a valid model for the chronic analgesic-associated RPN in that not only are the early pathological changes similar, but the long-term changes are almost identical.

4.6.2 Mouse: Different Strains.

A pilot study was performed prior to the main study to determine a suitable dose of BEA which caused a renal lesion at 24 hr. Four strains of mice were used; Schnider, Balb/c, C57B1/6 and CD1. Each group of mice (n=2) received either a 50 or 100 mg/Kg ip dose of BEA. The animals were sacrificed after 24 hr and the kidneys processed for histopathology (see section 3.4). The kidney sections were then graded for pathological changes indicative of RPN (see section 3.7, Table 3.4).

The order of sensitivity to BEA was Schnider > Balb/c > C57B1/6 with
the CD1 mice having no RPN. Since Schneider mice had been used in previous studies with BEA (see section 3.6.2.1) it was decided to use the Balb/c and C57B1/6 strains in order to expand the data base on mouse strain response to BEA-induced nephrotoxicity. The absence of RPN in CD1 mice is very interesting but has not been considered further and could form the basis of future studies. The Balb/c and C57B1/6 mice are both inbred strains which originated from stock established at the Jackson Laboratory (Bar Harbor, USA.) around 1947. The choice of Obese mice as the third strain was really due to their availability within the University and their susceptibility to obesity and diabetes. The Obese mice are a random bred strain originating from C57B1/6 mice found to carry a gene for obesity, ob/ob. In 1957 some of these ob/ob or ob/+ C57B1/6 mice were imported to Edinburgh where they were crossed with 2 non-inbred strains maintained as closed colonies; JH and CRL, to obtain faster growth and larger litters (Flatt & Bailey, 1981). As this strain was random-bred not all individuals will carry the gene for obesity (eg ob/+ or ob/ob). This can only be determined when the animals are mature and grossly obese individuals can be differentiated from their litter mates.

Diabetics are prone to a number of nephropathies in the clinical situation including RPN. Therefore it was of interest to see whether this strain of mice with a genetic susceptibility for developing diabetes was more sensitive to the nephrotoxic effects of BEA. Also as the Obese mice are derived from a C57B1/6 strain originally, but have since been crossed with two other strains it was also interesting to determine if hybrid vigour made the Obese mouse less susceptible than the in-bred C57B1/6 mouse.

It is difficult to identify one strain which is more sensitive on the
criteria of responding first as the earliest histological changes occurring in all strains were very subtle and difficult to quantify. However, in terms of continuing lesion development, cortical interaction and actual severity of the appearance of lesion then the C57B1/6 strain are the most sensitive (sections 4.2.2.4.B-4.2.2.4.I). This strain showed greater intensity of necrotic changes than the Obese strain which showed a very similar pattern of lesion development up to the 24 hr timepoint. C57B1/6 mice showed the earliest cortical changes with hydropic P3 segment and disruption of the brush border. The time point when earliest definite morphological changes occurred was 6 hr (section 4.2.2.4.C) with adherent platelets in the papilla, PAS positive staining casts in the loop of Henle (possibly leakage of mucopolysaccharides from disrupted P3 segment brush border), pyknotic nuclei and increased interstitial cell matrix staining. The lesion in the Obese mice seemed to reach a plateau at 24/48 hr (section 4.2.2.4.E) whereas the C57B1/6 mice continued to progress to give focal interstitial nephritis in the region of the juxtamedullary glomeruli and tubules (section 4.2.2.4.I) similar to those observed in the rat (see section 4.1.2.3-4.1.2.5). By 14 days (section 4.2.2.4.I) both Obese mice and Balb/c mice showed little difference from the control sections except the latter had focal interstitial nephritis which was not as pronounced as in the C57B1/6 strain. Mice do not appear to be affected to the same degree as rats by BEA. Schnider mice showed changes associated with an intermediate RPN following doses of 150-200 mg/kg, but only 60 hr after the administration of BEA. The Wistar rat, in comparison, shows changes associated with intermediate RPN (see section 2.1.2.3) 8-18 hr after
BEA at 100 mg/kg. Mice also have a definite cortical necrosis which is more pronounced than the rat and occurs prior to any gross necrosis of collecting duct, loops of Henle, covering epithelium etc which is the opposite of what is observed in the rat. The earliest changes are an increase in interstitial cell matrix staining and interstitial cell nuclei necrosis but usually in the upper papilla region rather than the papilla tip (C57Bl/6 mice were an exception). Adherent platelets and erythrocytes to capillary endothelium appear before necrosis of other tubular elements in papilla which is different to the sequence of events in the rat. The BEA induced lesion progresses more generally in the mouse with no total necrosis of the collecting duct or denudation of the covering epithelium. There was no evidence of hyperplasia of the urothelium of the pelvis or ureter based on the criteria (Table 3.4) used for rats.

The most prominent early changes occurred at 24-48 hr with PAS positive granules present in collecting duct cells. These cells did not become necrotic which suggests the mouse has a better capacity to respond to metabolic changes than the rat, if indeed that is what these histological changes represent. There was a pronounced drop in urine osmolality within the first 6 hr after BEA, similar functional changes have been described in the rat as an early consequence of BEA (Wilks et al, 1986).

Mice seem to be able to recover from doses of BEA up to 200 mg/kg perhaps because there does not seem to be the same degree of targeting for the papilla as in the rat. If BEA has a general nephrotoxic effect in the mouse kidney including the proximal tubule, then because of the greater concentration of xenobiotic metabolising enzymes (eg P-450) in the cortex the mouse is able to inactivate these toxins.
Mice may have differing extrarenal or renal metabolism to the rat which could affect the response of the mouse to BEA. The favoured hypothesis of BEA-induced RPN is via a prostaglandin hydroperoxidase mediated peroxidation within the interstitial cells (Bach & Bridges, 1984, 1985 and see Chapter 6-General Discussion). It could be that differences in the medullary interstitial cells and the prostaglandin metabolism system between the mouse and rat may explain the differences observed.

Functional differences may also explain why the lack of a frank RPN lesion in mice. The C57B1/6 mice had the lowest mean control urine osmolality value of all the mouse strains (see Table 4.2). Their increased sensitivity to BEA would seem to provide evidence that the concentration of toxins in the papilla is not the primary mechanism for the initiation of RPN pathogenesis. This is in contrast to the findings of Sabatini et al, (1981) who found that concentrating capacity was essential to produce BEA toxicity. The low osmolality may represent a diuresis which has been reported to be a factor which can provide some protection against BEA toxicity (Sabatini, 1984). If concentrating capacity was an initiating factor for BEA toxicity in mice, one would have expected the Balb/c mice to be more sensitive to BEA since this strain had the highest osmolality and produced very little urine. This provides additional evidence that differences in the metabolism of rats and mice is a likely explanation for the observed differences or other factors may be involved.

C57B1/6 mice had the lowest kidney weight/body weight ratio value in control animals (Table 4.3). As dose is administered based on body weight, then C57B1/6 received a larger renal dose relative to their
body weight than did the Balb/c or Obese mice (see Table 4.4).

Table 4.4: Renal dose* for each strain calculated for 100 mg/kg BW in
a mouse of body weight 20 g.

<table>
<thead>
<tr>
<th>Strain</th>
<th>KW/BW ratio</th>
<th>KW</th>
<th>Renal dose</th>
<th>Adjusted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(g/kg)</td>
<td>(g)</td>
<td>(mg/g)</td>
<td>(mg/kg)</td>
</tr>
<tr>
<td>Balb/c</td>
<td>0.0177</td>
<td>0.354</td>
<td>282.48</td>
<td>100</td>
</tr>
<tr>
<td>Obese</td>
<td>0.0158</td>
<td>0.316</td>
<td>316.45</td>
<td>89</td>
</tr>
<tr>
<td>C57B1/6</td>
<td>0.0148</td>
<td>0.296</td>
<td>337.83</td>
<td>83</td>
</tr>
</tbody>
</table>

Renal dose* - dose of compound affecting kidney assuming 100% dose reaches kidney, KW/BW - kidney weight/body weight ratio.

Adjusted dose - dose required administered as a mg/kg BW dose to give equal renal dose.

Table 4.4, shows the order of renal dose received is C57B1/6 > Obese > Balb/c, which is also the order of sensitivity to BEA. Thus, effective renal dose may be a critical factor in strain sensitivity coupled with metabolic differences which account for the strain sensitivity order observed.

In future strain comparison experiments it may be appropriate to use historical control data for organ/body weight ratios to ensure that each strain receives the same effective organ dose (mg/g). This effective organ dose parameter assumes that 100% of administered dose reaches the target organ.

4.6.3 Nude Mouse.

The morphological changes in the medulla of the nude mouse closely parallel the development of RPN observed in the rat (Gregg et al, 1990a) at 24 hr after 100 and 200 mg/kg doses of BEA. These included
the earliest changes in the interstitial matrix, as shown by slight increase in staining and the presence of PAS-positive inclusion bodies in the collecting duct epithelial cell cytoplasm. During the 48-72 hr period a spectrum of necrotic changes were observed in the tubular elements of the nude mouse papilla similar to these seen in rats dosed with BEA. The nude mouse is more sensitive to the toxicity of BEA than other murine strains (Scarlett et al, 1990). The necrotic changes in the medulla occur earlier and are more prominent than in the Obese, Balb/c or C57B1/6 mice. The nude mice develop frank necrosis within 48-72 hr, whereas the other strains we have studied do not develop a frank RPN and recover most signs of injury within 7-14 days after BEA (Scarlett et al, 1990). Although there was no kidney weight/body weight data generated from this study, the effective renal dose argument discussed above (section 4.6.2) could be relevant to the greater sensitivity of the nude mouse to BEA than other mouse strains. If the kidney weight/body weight ratio for nude mice is less than those reported in other strains in this thesis (see Table 4.3) (implying that the nude mouse kidneys are small relative to body weight), then since dose is administered according to body weight these mice may have actually received a larger effective renal dose that the other strains.

The hyperplasia of the covering epithelium of the papilla and pelvic urothelium which developed was similar to that observed in rats treated with BEA (Gregg et al, 1990a) and N-phenylanthranilic acid (Hardy & Bach, 1984). No such hyperplasia has been observed in other mouse strains after BEA (Scarlett et al, 1990).

Gross necrosis similar to that reported in the rat, occurred primarily in the medulla/papilla followed by the cortex with the extreme papilla
tip affected at a much later stage. Changes in the cortex included total P₂ segment necrosis followed by re-epithelialisation. The proximal tubular necrosis and re-epithelialisation are similar to the pattern of necrosis observed after p-aminophenol (Davis et al, 1983) and hexachloro-1,3-butadiene administration to rats and mice (Ishmael et al, 1982; Lock et al, 1984). The cortical necrosis preceded the loss of covering epithelium and collecting duct epithelial cells in the papilla. All previous studies using several strains of rat (Bach & Bridges, 1985), conventional mouse strains (Bach, P H & Gregg, N J, unpublished data; Scarlett et al, 1990), the Hamster (Carlton & Englehardt, 1989) and pig (Gregg et al, 1989a) BEA caused a highly selective RPN and minimal early, transient cortical changes (Bach et al, 1983; Gregg et al, 1990a,b). However, high doses of BEA may cause secondary cortical degeneration which develop as an interstitial nephritis affecting the cortex 21 days or more after the initial insult (Axelsen, 1978b; Bach & Bridges, 1985; see section 4.1.2.3-4.1.2.6). By far the most interesting aspect of this investigation was the novel observation that BEA causes cortical necrosis in nude mice. BEA has previously been considered to be totally target selective for the medulla. Cortical changes in the rat (Bach et al, 1983; Gregg et al, 1990a) now appear to be a transient hydropic degeneration associated with a proteinuria (largely albumin) (Moret et al, 1989) that could be due to BEA affecting the glomerular permselectivity (discussed above, section 4.6.1).

The cortical necrosis in the nude mouse were evident prior to the onset of frank RPN (when loss of covering epithelium and collecting duct epithelial cell necrosis occurs). This alteration of a generally
accepted cascade of degenerative changes from papilla tip > medulla > cortex in the rat given BEA may be related to a unique characteristic of this mutant strain. The response of germ-free animals to chemicals is known to vary from conventional animals (Love et al, 1977; Sumi & Miyakawa, 1983). This raises the possibility that gut-flora pay a role in the target selectivity of BEA. This does not appear to be the case in rats where BEA causes a target selective medullary injury in both germ-free and conventional animals (Gregg, NJ, Ward, FW, Coates, ME and Bach, PH, unpublished observation). There is no rational basis to suggest that the athymic nature and therefore incomplete immune system of the nude mouse is implicated in the development of this BEA-induced P2 and P3 proximal tubular necrosis. At present there is no data on the sensitivity of the parent MF1 mouse (from which this nude mouse strain was derived) to BEA. At present the atypical response of the nude mouse to BEA cannot be explained. It may, however, be due to differences in proximal tubule cell biochemistry, an understanding of which could help elucidate the mechanism of BEA toxicity and therefore the understanding the pathogenesis of RPN in general.

In conclusion, this is believed to be the first nephrotoxicity study in the nude mouse, where the need to keep them germ-free has probably precluded their widespread use. The data shows that BEA is not the specific papillotoxin reported in other species and strains investigated so far. This remarkable difference in the response of the nude mouse to BEA warrants further investigation to assess the mechanistic basis of the change, and the role of the incomplete or depressed immune system. Alternatively the germ-free nature of these mice may be central to the atypical lesion. The nude mouse may prove
to be a novel species for investigating nephrotoxicity in those circumstances where the immune system is depressed or has been suppressed in the clinical situation by the use of immunosuppressant drugs such as Cyclosporin A.

4.6.4 Marmoset Results.
The small number of animals used in this study meant there was no control tissue available with which to compare the morphological changes observed. However the initial results would seem to suggest that (if these changes are real and reproducible) BEA does not induce a frank RPN in the marmoset as is the case in the rat at these doses. The pronounced hyperplasia of the covering epithelium of the papilla that was induced may be due to a direct toxic effect of BEA on the covering epithelial cells. The limited number of animals used does, however, make it presumptious to extrapolate this data. In a review on naturally occurring renal disease in non-human primates, Skelton-Stroud & Glaister, (1987) do not report any spontaneous hyperplasia of the covering epithelium or pelvic urothelium. Hyperplasia of these epithelia have been reported to occur in baboons with NSAID-induced RPN (Skelton-Stroud, 1987). In baboons the urothelium has been reported to have a pronounced capacity for proliferation which occurs in response to focal or widespread damage (Skelton-Stroud & Glaister, 1987). If marmosets have a similar capacity and the hyperplasia which was observed after BEA treatment (section 4.3.2) is real then perhaps this is relevant to man. If a nephrotoxin which can induce an acute renal lesion can also induce a pronounced hyperplasia (as reported here in marmoset, rat and pig (sections 4.4.1.-4.4.3) in the urothelium lining the upper urinary tract, then this may provide the
basis for subsequent carcinogenesis. This concept is discussed in
detail below - see Chapters 5 and 6.

4.6.5 RPN Induced in the Pig.

4.6.5.1 BEA-induced RPN.

These studies showed that BEA is also papillotoxic in the pig, but it
has less effect at low doses and is more systemically toxic than in
the rat. The lesion in the pig is not as pronounced as a comparable
lesion in Wistar rat 7 days after treatment with 100 mg/kg BEA.
However, the changes which were most pronounced in both groups were
foci of necrotic interstitial cells together with disruption of the
interstitial matrix (section 4.4.1.3). Similar changes were found
with NaNPAA (section 4.4.2.2.B) in the pig, this compound has also
been observed to induce an acute RPN in the Wistar rat within 24 hr
the morphology (or pathology) of which is essentially identical to
that induced by BEA (Gregg N.J., 1987 unpublished observation). In
the rat the 'fine' anatomical elements are the earliest to show
changes, which are apparent within 4-6 hr (section 4.1.1), and
precede a cascade of necrotic changes which result in an acute RPN
lesion. Since the same anatomical elements are primarily affected in
the pig papilla after BEA, this suggests a similar cascade of necrosis
occurs.

There were differences in the response of the pigs to BEA when
comparing the older animals used in studies 1 & 2 (section 4.4.1) to
the younger animals used in studies 3 & 4 (4.4.2). The older animals
had more pronounced changes than the younger animals. The renal
function of Large White pigs is not fully mature until approximately
12 months of age (Robbins, 1984). If the renal function is still
developing then one can also presume that the renal functional reserve is also developing. The young pigs may be able to adjust better to a toxic insult because the development of new functioning nephrons, and is therefore able to compensate for any nephrons which are affected functionally by nephrotoxins. Since age is known to be a risk factor in developing nephropathies (Porter, 1989) then future studies should use older functionally mature animals in order to extrapolate better the results to the clinical situation.

In the normal rat kidney the medullary interstitial cells stain for lipid (Bojesen, 1974), but once RPN has been induced there are marked lipid staining of endothelia, collecting duct and covering epithelial cells of the papilla (Bach & Gregg, 1988). By contrast the medullary interstitial cells of the control pig did not stain for lipid, but the covering epithelium did. Following BEA there was a substantially increased deposition of lipid material in the collecting duct epithelium (section 4.4.1.3). Since other nephrotoxins that do not affect the medulla (eg hexachlorobutadiene, aminoglycosides, cisplatin, Bach et al, 1987) do not produce this lipidosis in the collecting duct epithelium it has been suggested that this lipid deposition could be pathognomonic for RPN (Bach & Gregg, 1988). The decreased effects of BEA on the pig medulla may be due to a number of causes. The multipapillate anatomy of the pig kidney may be less prone to the papillotoxic effects because BEA is not "concentrated" in a single papillae as it is in the rat, or because the urine concentrating capacity of the pig is less than that of the rat (Stolte & Alt, 1982). Alternatively, both the hepatic and renal metabolism of several chemicals differs in rats and pigs (Litterst et al, 1975;
Shimada et al, 1986; Fujimori et al, 1986). Thus more hepatic metabolism or biliary excretion of BEA by pigs could reduce its papillotoxicity. Interestingly, whereas lipid droplets predominate in the rat medullary interstitial cells they were not apparent in the pigs. Pigs are reported to have less long-chain poly-unsaturated fatty acids (to act as substrates for lipid peroxidation) within the interstitial cell lipid droplets (Bojesen, 1980). Thus if peroxidative metabolism and lipid peroxidation of poly-unsaturated lipid droplets is important in the pathogenesis of RPN (Bach & Bridges, 1984; Bach & Gregg, 1988), pigs would be less sensitive. These factors may account for the reduced severity of the renal lesion in the pig and serves to illustrate marked species differences in the renal handling of nephrotoxic compounds in the multipapillate kidney.

4.6.5.2 Paracetamol Study.

The results presented here indicate that treatment with BEA, paracetamol or BEA followed by paracetamol had no marked effect on the maturational increase in body weight, renal weight and size seen in immature pigs (section 4.4.3.3). These animals exhibit marked increases in renal size and function in their first year of life, by which time stable adult levels are reached (Robbins, 1984). Similarly drug treatment did not prevent the increase in glomerular filtration rate seen in age-matched controls (section 4.4.3.1.A). However, in those pigs treated with BEA or BEA followed by paracetamol, a significantly greater increase in effective renal plasma flow compared with that noted in age-matched controls was found (section 4.4.3.1.B). The addition of paracetamol to the BEA treatment did not increase the effective renal plasma flow over BEA alone (Figure 4.52).

Prostaglandins are believed to be important vasoactive compounds in
the control of renal haemodynamics, particularly when the kidney is
traumatised (Clive & Stoff, 1984; Schlondorff, 1986). The increase in
effective renal plasma flow in the BEA and BEA/paracetamol treated
animals may represent a response to a persisting injury of the kidney.
Previous studies in the rat have shown an increased medullary blood
flow early after BEA (Solez et al, 1974), if a similar early change
occurs in the pig, then this study shows that these haemodynamic
changes are sustained. The major source of renal prostaglandin
synthesis are the interstitial cells (Schlondorff, 1986), which have
indeed been postulated to be the target cells for BEA-induced
nephrotoxicity (Bach & Bridges, 1984, 1985). A subtle injury to the
interstitial cells by BEA (since no widespread frank necrosis was
observed in this study, section 4.4.3.3), may produce a perturbation
of prostaglandin metabolism. This could be an increased synthesis of
prostaglandins or synthesis of a vasodilatory prostaglandin reflecting
the sustained effective renal plasma flow observed.

The lack of any significant changes with the ultrasound technique
could suggest the absence of pathological changes, but also
illustrates the inherent weakness in this non-invasive technique. It
is difficult to identify focal changes in renal morphology at an early
stage in the development of a lesion by most non-invasive techniques.
Hence, the need for histopathological examination which allows one to
identify such focal changes and determine whether the changes are
significant in terms of renal function and/or a potential indicator of
progressive changes which may result in a severe lesion developing.

The most obvious histopathological changes occurred in the BEA and
BEA/paracetamol treated groups: urothelial hyperplasia of the pelvis
and ureter interstitial matrix changes, pyknotic interstitial cell nuclei and necrosis of the thick ascending limb.

It is clear that these, albeit preliminary, observations that the functional and histological changes were only present in the BEA and BEA/paracetamol treated groups. No such changes were observed with paracetamol alone, indicating that the above changes were solely due to BEA. Paracetamol, at this dose and duration of exposure does not enhance BEA-induced RPN in the pig. This is certainly at odds with what is seen in the rat; where treatment with paracetamol prior to and after BEA exacerbates the morphological changes within the RPN lesion (Bach & Gregg, 1988).

The prospective epidemiological study of Sandler et al, (1989) indicates an increased risk of developing chronic renal disease with long-term daily use of paracetamol. These findings contrast the recent retrospective study of McCredie & Stewart (1988) who were unable to show an increased risk for developing analgesic nephropathy due to paracetamol consumption.

There has, however, been an impressive decline in end-stage-renal disease associated with analgesic abuse in Denmark, Finland, Sweden and Australia since legislation restricted access to phenacetin and paracetamol, see Table 4.5 (Schwarz, 1987; Kincaid-Smith, 1988). This decline probably provides the best indirect evidence for the implication of paracetamol in exacerbating role in the development of chronic renal disease. These data cannot differentiate between paracetamol and its consumption co-formulated with other analgesics and/or caffeine.

The results reported in this thesis show that the multipapillate pig kidney is not affected by paracetamol in the dose regimen used.
<table>
<thead>
<tr>
<th>Country</th>
<th>Legislation and sign of decline of analgesic-associated nephropathy.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denmark</td>
<td>Prescription required for phenacetin and acetaminophen in 1961</td>
</tr>
<tr>
<td></td>
<td>9% decline in deaths due to chronic non-obstructive pyelonephritis at autopsy (1956-60 to 1961-65)</td>
</tr>
<tr>
<td></td>
<td>50% decline in RPN at autopsy (1959-1967)</td>
</tr>
<tr>
<td></td>
<td>26% decline in diagnosis of pyelonephritis in women at autopsy; men unchanged (1961-1967)</td>
</tr>
<tr>
<td></td>
<td>61% decline in RPN at autopsy (1961-65 to 1971-75)</td>
</tr>
<tr>
<td></td>
<td>71% decline (women) and 60% (men) in diagnosis of chronic interstitial nephritis (1961-1975)</td>
</tr>
<tr>
<td>Sweden</td>
<td>Warning on drug packages with phenacetin and acetylsalicylic acid in 1957</td>
</tr>
<tr>
<td></td>
<td>Prescription required for phenacetin and acetaminophen in 1961</td>
</tr>
<tr>
<td></td>
<td>75% decline in uremia as cause of death among analgesic users (1962-1970)</td>
</tr>
<tr>
<td></td>
<td>75% decline of analgesic-associated nephropathy in renal transplant series (1965-69 to 1976-1979)</td>
</tr>
<tr>
<td>Finland</td>
<td>Prescription required for phenacetin in 1962</td>
</tr>
<tr>
<td></td>
<td>Phenacetin prohibited in 1965</td>
</tr>
<tr>
<td></td>
<td>94% decline of RPN and 54% decline of death due to kidney disease at autopsy (1962-1977)</td>
</tr>
<tr>
<td>Australia</td>
<td>Gradual removal of phenacetin in pain medication between 1967 and 1975</td>
</tr>
<tr>
<td></td>
<td>33% decline of death due to kidney disease and 50% decline of death due to analgesic-associated nephropathy at autopsy (1964-1971)</td>
</tr>
<tr>
<td></td>
<td>Prescription required for mixtures of analgesics and for large packets of analgesics in 1979</td>
</tr>
<tr>
<td>Canada</td>
<td>Phenacetin combined with acetylsalicylic acid and its derivates prohibited in 1973</td>
</tr>
<tr>
<td></td>
<td>50% decline of analgesic-associated nephropathy in surveys of nephrologists (1971-1980)</td>
</tr>
<tr>
<td>Great Britain</td>
<td>Phenacetin prohibited in 1980</td>
</tr>
<tr>
<td>Switzerland</td>
<td>Prescription required for phenacetin and for acetaminophen in large packages in 1981</td>
</tr>
<tr>
<td>FR Germany</td>
<td>Warning on packages of phenacetin in 1981</td>
</tr>
<tr>
<td></td>
<td>Phenacetin prohibited in 1986</td>
</tr>
<tr>
<td>Netherlands</td>
<td>Phenacetin prohibited in 1984</td>
</tr>
</tbody>
</table>

**Table 4.5** Decline of kidney disease and signs of analgesic-associated nephropathy after phenacetin (and acetaminophen) restriction in several countries. From Schwarz, (1987).
The pig kidney appears less sensitive to papillotoxic chemicals than the rat based on this and earlier studies (Gregg et al, 1989a). It has been suggested that paracetamol toxicity occurs via a mechanism which involves increased biotransformation via cytochrome-P450 oxidation; together with a glutathione depletion this produces toxic metabolites which bind to cellular macromolecules (Siper & Gandolfi, 1986). Pigs are, however, known to have a deficiency in the sulphation pathway (Jackoby, 1980) thus acetaminophen should be more toxic in this species. One possible explanation is that there is more extra-renal metabolism of the paracetamol in the pig which reduces the effect of paracetamol in the kidney. Although there is no information with regard to metabolism of paracetamol in the Large White pig, Peggins et al, (1987) showed that the minipig kidney has large levels of glutathione and glutathione-S-transferase activity in the kidney compared with the liver. The medulla possesses 60-70% of the glutathione compared to the cortex. The apparent lack of paracetamol nephrotoxicity suggested in this study could reflect a protective effect of glutathione conjugation of all potential toxic metabolites thus preventing any toxic effects (Peggins et al, 1987). It is likely that the renal metabolism of paracetamol is similar in the Large White pig.

The similarity of the pig in terms of renal function and structure with that of man raises the important question of how relevant is the data obtained from those rodent studies when applied to papillary nephropathies. These findings highlight the need for further studies to ascertain the applicability of the pig as a suitable experimental model in which to study the pathogenesis of acutely and chronically induced RPN and extrapolate the data to the clinical situation.
CHAPTER 5

RESULTS FROM CARCINOGENESIS STUDIES USING BBN INITIATION FOLLOWED BY BEA-INDUCED RPN

In studies 1-4 where the basic experimental protocol was the same (BBN initiation followed by BEA induction of an acute RPN), the results from the main experimental groups have been combined to produce a more detailed time course of pathological changes with larger numbers of animals. The justification for combining the results was judged on inter-study variation of results that were no greater than expected from studies on RPN and necessitated by the low numbers of animals used at the different time points in each of the four studies. The results for the BEA only control groups for the relevant time points have already been described above - see section 4.2.

The results for various pilot studies performed to try and enhance the severity and frequency of urothelial changes are presented separately (sections 5.3, 5.4.1).

5.1 UROTHELIAL MORPHOLOGY CHANGES UP TO SIX WEEKS

5.1.1 Up to One Week Time Point.

In the first study (see section 3.5.5) animals were sacrificed at 1, 2, 3 and 4 days post-BEA dosing to determine the effects, if any, of BBN on the known urothelial hyperplastic and degenerative changes due to RPN (see section 4.1). The pathology changes in the kidney, bladder and ureter tissues was assessed and it was concluded that no changes other than an acute RPN had occurred in the BBN initiated/BEA treated animals. These were the same as in BEA only treated animals (section 4.1.1). The appearance of kidneys from BBN only treated animals did not differ significantly from the kidneys of control
animals.

After 1 week (5 days) there was little difference in the morphological changes in BBN/BEA treated animals other than the RPN lesion described in section 4.1. As before the kidneys from animals treated with BBN alone were similar in appearance to normal control kidneys.

5.1.2 Three Weeks Time Point.

The BEA treated group had foci marked hyperplasia in the ureteric urothelium some of which was papillary in pattern (see section 4.2). The BBN only treated animals had normal morphology with no hyperplasia present in any of the urothelia.

Animals in the BBN/BEA treated group had (in addition to the morphological changes associated with the RPN lesion, (see section 4.1.2.1) mild hyperplasia of the pelvic and ureteric urothelium, but no urothelial hyperplasia in the bladder which was the same as in the BEA only treated animals (section 4.1.2.1). Alk. phos. staining was positive in bladder urothelium and concentrated towards the apex of intermediate and superficial cell layers. Little or no staining was observed in the pelvic and ureteric urothelium with alk. phos., however dispersed foci of ATP positive staining occurred in both regions.

5.1.3 Six Weeks Time Point.

In the BBN only treated group a number of interstitial cell changes were noted in papilla, but not in significant quantities. Glomeruli were seen to be enlarged having a reduced Bowman’s space with a slight degree of basement membrane sclerosis occurring around the Bowman’s capsule. Animals in the BBN/BEA treated group had total RPN as in the BEA only treated (see 4.1.2.2) with the papilla having sloughed with a
re-epithelialisation of the covering epithelium. Epithelial cells had nuclei of variable size and shape, with mitotic figures present in the fornix together with a slightly reduced alk. phos. staining compared to the remaining pelvic urothelium. Restoration of the interstitial matrix occurred with extremely intense staining granules around the loops of Henle basement membrane. There was focal severe papillary/nodular hyperplasia in the ureteric urothelium (Figure 5.1), with sloughing, dysplastic, basophilic cells in the superficial layer. Mast cells (with intensely basophilic granular cytoplasm) were aggregated below and adjacent to this nodular "invasive" urothelium (Figure 5.2). There was no hyperplasia of the bladder urothelium only a severe "ruffling" of the glycocalyx of the superficial cells. The basal sub-urothelial capillaries showed a progressive microangiopathy similar to that seen in BEA only treated rats (section 4.1.2.2).

5.2 UROTHELIAL MORPHOLOGICAL CHANGES AND TUMOUR INCIDENCE UP TO FORTY WEEKS TIME POINT.

5.2.1 Thirteen Weeks Time Point.

The BBN only treated group had similar changes to those described at the 6 week time point. The urothelium of pelvis and ureter tended to have a more intensely staining lamina propria for mucopolysaccharides with the Giemsa stain, with a "ruffled" superficial cell layer. The animals in the BBN/BEA treated group had total RPN as described at 6 weeks and in BEA only treated group (see section 4.1.2.3) with re-epithelialised stump with a deposition of interstitial matrix together with calcified deposits around the tubular basement membrane. The papilla covering epithelium was mildly hyperplastic, blebbing and had an occasional mitotic figure present. There was a general
Figure 5.1  Nodular and papillary hyperplasia in the ureter, 6 weeks after BBN initiation and BEA promotion (BBN/BEA). Many areas are dysplastic nuclei (arrowhead) and are sloughing into lumen. Giemsa x 177.

Figure 5.2  Higher magnification of above figure showing cellular detail of nodular hyperplasia, note aggregates of mast cells in lamina propria (arrow). Giemsa x 357.
hyperplasia throughout the urothelium including the bladder varying from mild simple hyperplasia to severe focal papillary and nodular hyperplasia with potentially 'invasive' areas (Figure 5.3) One animal in particular had basophilic/eosinophilic staining superficial 'umbrella' cells whose nuclei were enlarged, variable shaped and multi-nucleated (Figure 5.4) Focal papillary hyperplasia with "invasive" nodular hyperplasia (Figure 5.5), of grade P1/P2, (Helpap et al, 1985), penetrating through the lamina propria (Figure 5.6) and aggregates of mast cells with basophilic granular cytoplasm adjacent to the invasive area (Figure 5.7). These "invasive" regions of urothelium were stained negatively for alk. phos. whereas the papillary hyperplastic regions of urothelium had a positive staining mosaic pattern for alk. phos. (Figure 5.8).

5.2.2 Twenty-one Weeks Time Point.

At this time point there were no noteworthy or additional morphological changes compared to those described above (section 5.2.1) at 13 weeks in the BBN only treated group.

The BBN/BEA treated group of animals all showed the greatest numbers of abnormalities within the urothelium particularly in the ureter, with two thirds of the ureters examined having papillary hyperplasia together with foci of nodular hyperplasia or invasive areas. One animal had a large papillary hyperplasia/papilloma located at the mouth of the ureter which completely filled the lumen of the ureter. A papilloma (4.0 mm in diameter) was observed in the bladder (Figure 5.9). It was well differentiated with a well developed interstitial fibrous core and vasculature, together with irregularly staining "nests" of cells and many mitotic figures throughout. Mast cells
Figure 5.3 Dysplastic, hyperplastic urothelium in ureter, 13 weeks after BBN/BEA treatment. Giemsa x 36.

Figure 5.4 Higher magnification of above figure showing nodular "invasive" area and large multinucleated cell in superficial layer. Giemsa x 434.
Figure 5.5 Papillary and nodular hyperplasia in ureter 13 weeks after BBN/BEA treatment. Toluidine blue x 223.

Figure 5.6 Higher magnification of an "invasive" area shown above (arrow) projecting down through lamina propria (LP) and beginning to invade lamina muscularis (Mu). Toluidine blue x 1085.
Figure 5.7 A second "invasive" nodular hyperplastic area (arrow) in ureter 13 weeks after BBN/BEA, invading through lamina muscularis, mast cells are aggregated adjacent to "invasive" area (arrowheads). Toluidine blue x 223.

Figure 5.8 Alkaline phosphatase stained section of ureter shown above. Mosaic distribution of this enzyme occurs throughout hyperplastic urothelium but nodular "invasive" area show a decrease or total lack of staining (arrow). Alkaline Phosphatase x 270.
similar to those observed at earlier time points (section 5.1.3) were seen throughout the epithelial layers of the papilloma and in the interstitial lamina propria core of the tumour adjacent to capillaries. The papilloma showed little staining for alk. phos. except in occasional superficial cells and in the endothelium lining the capillaries of the interstitial core. ATPase staining of capillary endothelial cells in the sub-basal and urothelial coated capillaries showed that the intensity and area of staining for the microangiopathic endothelial cell layer increased towards the lumen of ureter. The cortical changes present were those of the BEA only treated group, due to the progressive RPN and interstitial nephritis (see section 4.1.2.4).

5.2.3 Thirty Weeks Time Point.

Macroscopically all the animals treated with BEA, including the BBN/BEA group had severe cortical scarring indicative of the
<table>
<thead>
<tr>
<th>Animal No</th>
<th>Group</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>69</td>
<td>BEA</td>
<td>both kidneys scarred (Right &gt;&gt; Left), upper ureter 4 mm in width, no abnormalities inside, papilla stump, no tumours in bladder.</td>
</tr>
<tr>
<td>78</td>
<td>BEA</td>
<td>both kidneys scarred and cystic, papilla ghost in pelvis, no tumours in bladder.</td>
</tr>
<tr>
<td>23</td>
<td>BBN</td>
<td>kidneys normal appearance, papilla intact, pelvic space full of blood!, no tumours in pelvis or bladder.</td>
</tr>
<tr>
<td>22</td>
<td>BBN</td>
<td>kidneys normal appearance, papilla intact, pelvis and bladder full of blood, several small microtumours in bladder, 1 tumour papilloma 3 mm diam.</td>
</tr>
<tr>
<td>23</td>
<td>BBN</td>
<td>kidneys normal appearance, dark colour to papilla, 2 papilloma tumours in bladder 3-4 mm diam.</td>
</tr>
<tr>
<td>11</td>
<td>BBN/BEA</td>
<td>both kidneys scarred, focal RPN lesion at papilla tip, blood in bladder, 2 microtumours in bladder &lt; 1-2 mm diam.</td>
</tr>
<tr>
<td>8</td>
<td>BBN/BEA</td>
<td>both kidneys scarred, papilla stump mineralized, blood in pelvic space and in bladder, 1 papilloma in bladder 3 mm diam.</td>
</tr>
<tr>
<td>9</td>
<td>BBN/BEA</td>
<td>both kidneys scarred, papilla stump mineralized, blood in pelvic space, no tumours in bladder.</td>
</tr>
<tr>
<td>53</td>
<td>BBN/BEA/BBN</td>
<td>both kidneys pronounced scarring, right kidney atrophied, papilla stump, blood in bladder, 2-3 papilloma tumours 2-4 mm diam.</td>
</tr>
</tbody>
</table>

*Table 5.1 Macroscopic assessment results from BBN study 2, at thirty weeks time point.*
underlying interstitial nephritis, a secondary change in the degenerative pathogenesis of RPN. Table 5.1 shows a representative example of macroscopic results observed at this time point.

In the BBN/BEA group the animals in general had a greater proportion of the papilla remaining intact after necrosis due to the RPN compared to the BEA only treated animals. Microscopic examination revealed that no tumours were observed in the BEA treated group. One BEA only treated animal did have a severe nodular hyperplastic pelvic/ureteric urothelium with some irregular atypia in the pelvis.

All the BBN only treated animals exhibited visible haematuria in the bladder at post mortem. For the first time bladder papillomas were observed in the bladders of the BBN only treated animals, being of a typical "cauliflower" appearance. The histological appearance of these BBN induced papillomas were similar to those of the BBN/BEA papillomas described above found at 21 weeks (Figure 5.9). One animal had 3 papillomas (2-3 mm in diameter) which had severe cellular atypia and there was also papillary and nodular hyperplasia in the ureteric urothelium (Figure 5.10).

The BBN/BEA treated animals had hyperplasia and dysplastic urothelium in the pelvis and ureter together with some areas of flat nodular "invasive" hyperplasia and small papillomas in the bladder. Cellular atypia in these hyperplastic urothelia comprised of enlarged lobed nuclei and occasional mitotic figures plus multinucleated cells. "Umbrella" cells in the superficial layer of bladder were often basophilic with prominent aggregations of eosinophilic staining macrophage cells throughout the superficial and intermediate layers.
Nodular and papillary hyperplasia of ureteric urothelium from BBN only treated animal, 30 weeks after dosing. Giemsa x 434.

with acid phos. staining in and around these cells. Mast cells were also prominent in and around invasive and severely hyperplastic areas of the urothelium at 21 weeks. There was also some evidence of microangiopathy in the sub-urothelial capillaries where there was occlusion of the lumen and thickening of the endothelial basement membrane.

In the ureteric urothelium there was nodular and papillary hyperplasia with loss of polarity and atypical nuclei, mitotic figures and also possibly areas of carcinoma in situ where the urothelium is of normal thickness or exhibits some mild hyperplasia but the nuclei show severe atypia: enlargement, basophilia, loss of polarity etc. A considerable number of animals had a papilla stump with a severely hyperplastic covering epithelium (Figure 5.11). There was frequently an associated nodular "invasive" hyperplasia of the urothelium in the pelvic fornices (Figure 5.12). In 25-30% of the animals transitional cell
Figure 5.11 Papilla stump (Pap) with severe hyperplastic covering epithelium and nodular hyperplasia of adjacent pelvic urothelium (arrow), 30 weeks after BBN/BEA treatment. Giemsa x 89.

Figure 5.12 Higher magnification of nodular hyperplastic pelvic urothelium (from above figure) invading renal parenchymal tissue (arrow), note collecting duct (CD) with mitotic figure present. Giemsa x 357.
tumours were found in the pelvic/ureter junction, these tumours were typically nodular/papillary (Figure 5.13) with mitotic figures present, did not stain for alk. phos. and also had mast cells present in the lamina propria adjacent to the nodular regions of the tumour (Figure 5.14).

5.2.4 Forty Weeks Time Point.

Table 5.2 shows a representative example of macroscopic results observed at this time point.

The BBN only treated group had normal kidney morphology, no RPN, tubular atrophy or necrosis. Approximately 80% of the BBN only treated animals had bladder tumours. Typically these tumours were 2-6 mm in diameter, well differentiated, with a papillary or papilloma structure with occasional areas of superficial necrosis and haemorrhaging. Many areas of bladder urothelium were devoid of any epithelial cells. The denuded basement membrane was stained prominently for mucopolysaccharides with PAS and Giemsa. The pelvic urothelium was of normal thickness (2-4 cells). There were, however, many variable shaped lobular nuclei present. There was also papillary hyperplasia in some ureters similar to that observed at 30 weeks (Figure 5.10).

Occasionally BBN/BEA treated animal did not exhibit RPN and had an intact papilla (in approximately 2-3 animals, > 2% of total number of animals dosed with this protocol). Morphological changes occurred similar to those described in BEA only animals (see section 4.2.6) due to the underlying RPN and cortical interstitial nephritis lesions. These changes varied among individual animals but generally followed a similar pattern. The papilla remained as a re-epithelialised stump.
Figure 5.13  Typical solid nodular "invasive" tumour found at the pelvic/ureter junction, 30 weeks after BBN/BEA treatment. Giemsa x 108.

Figure 5.14  Higher magnification of invasive region of tumour in above figure showing atypical cell nuclei and aggregates of mast cells adjacent to invading tumour cells (arrowheads). Giemsa x 434.
<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Group</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>83</td>
<td>BEA</td>
<td>both kidneys enlarged, scarred, ghost papillae in pelvis, enlarged ureters, small bladder, no tumours.</td>
</tr>
<tr>
<td>80</td>
<td>BEA</td>
<td>left kidney hypertrophic, scarred, cystic, ureter enlarged, right kidney atrophied, no tumours.</td>
</tr>
<tr>
<td>27</td>
<td>BBN</td>
<td>kidneys normal appearance, bladder enlarged, papilloma tumour 3-4 mm diam.</td>
</tr>
<tr>
<td>26</td>
<td>BBN</td>
<td>kidneys normal appearance, bladder contains 3 tumours (papillomas), 3-5 mm diam.</td>
</tr>
<tr>
<td>37</td>
<td>BBN</td>
<td>kidneys normal appearance, no tumours in bladder.</td>
</tr>
<tr>
<td>45</td>
<td>BBN/BEA</td>
<td>kidneys cystic and scarred surface, bladder full of blood stained urine, large papilloma tumour 5-6 mm diam., necrotic and haemorrhaging on surface.</td>
</tr>
<tr>
<td>7</td>
<td>BBN/BEA</td>
<td>kidneys scarred and cystic, papilla stump, bladder contains 2 tumours 2-3 mm diam.</td>
</tr>
<tr>
<td>21</td>
<td>BBN/BEA</td>
<td>kidneys cystic and scarred surface, papilla stump, bladder contains tumour broken from urothelium 4-5 mm diam.</td>
</tr>
<tr>
<td>58</td>
<td>BBN/BEA/BBN</td>
<td>kidneys slightly scarred and cystic, papilla intact!, bladder contains 1 tumour broken from urothelium 2-3 mm diam.</td>
</tr>
<tr>
<td>57</td>
<td>BBN/BEA/BBN</td>
<td>kidneys scarred, pelvis and upper ureter full of papilloma tumour 8-10 mm diam., bladder contains 1 papilloma tumour 5-6 mm diam.</td>
</tr>
</tbody>
</table>

Table 5.2 Macroscopic assessment results from BBN study 2, at forty weeks time point.
with intact (if somewhat abnormal) tubular elements within a pink staining interstitial matrix. This was covered by an extremely hyperplastic epithelium which was blebbing and papillary in areas. The interstitial matrix of the papilla was fibrotic in nature and also granular in some areas, it did not extend all the way to the tip of the papilla. At this time point all the BBN/BEA treated animals had bladder tumours which were visible macroscopically. They were transitional cell papillomas varying in size from small papilloma (2 mm diameter) to large well differentiated tumours filling the entire bladder. One such tumour (Figure 5.15) exhibited numerous tumour types: nodular invasive (Figure 5.16), keratinisation of papilloma (Figure 5.17), and papillary transitional cell carcinoma. Within this tumour there was variable staining for both PAS and alk. phos., with the keratinised regions being particularly prominently stained for PAS (Figure 5.18).

Urothelial changes were very variable, the most prominent changes occurring in the pelvic/ureter mouth junction where nodular, solid, severe hyperplastic lesions were located (possibly carcinoma) which had cellular atypia, loss of polarity and capillaries within the epithelial cell layers. The pelvic urothelium often had vacuolated, disrupted, superficial layers with numerous macrophages (cells with eosinophilic staining granular cytoplasm) present. The ureteric urothelium was hyperplastic; papillary in areas, nodular "invasive" at the mouth of the ureter and pelvic junction with aggregates of mast cells located in basal region.

The kidneys from one animal at 40 weeks had a tumourous mass filling the entire upper ureter (Figure 5.19) and extending into the pelvis.
Figure 5.15  Large well differentiated tumour filling entire bladder, 40 weeks after BBN/BEA treatment. Giemsa, x 89.

Figure 5.16  Solid nodular transitional cell tumour found in part of large bladder tumour in figure 5.14, 40 weeks after BBN/BEA treatment. Giemsa x 108.
Figure 5.17 Keratinisation and mineralisation in papillary transitional cell carcinoma found in part of large bladder tumour in figure 5.14, 40 weeks after BBN/BEA. Giemsa x 434.

Figure 5.18 Prominent PAS staining (arrowhead) of keratinised areas in tumour shown above. PAS x 270.
Figure 5.19 Macroscopic photograph of enlarged upper ureter portion (arrowheads) containing tumour (shown below) found 40 weeks after BBN/BEA treatment. x 3 approximately.

Figure 5.20 Papillary transitional cell tumour found in upper ureter, with nodular basal region, 40 weeks after BBN/BEA treatment. Giemsa x 36.
The tumour microscopically was a transitional cell tumour, papillary in nature (Figure 5.20). The basal layers were extremely nodular, with loss of polarity and much cellular atypia (enlarged, variable shaped nuclei), basophilic deposits within the tumour tissue (Figure 5.21). It was difficult to differentiate between the lamina propria and lamina muscularis and the epithelial cells suggesting an invasive stage in tumour development (Figure 5.21). The nodular and possibly "invasive" regions of this tumour did not stain for alk. phos. but the superficial papillary regions of this tumour did stain positively for this enzyme (Figure 5.22).

There was no positive staining observed for the marker enzyme GGT in any urothelial tissues at any time point during all of these studies.

5.3 BBN STUDY 3: SUPERIMPOSITION OF SINGLE ANALGESICS.

In general many of the histological changes observed in all groups were similar and due to the BEA insult progressing from an RPN into severe interstitial nephritis affecting many of the nephrons within the cortex (see sections 4.1.2.2-4.1.2.4). These changes were variable between individual animals and between experimental groups.

5.3.1 Morphological Changes at Seven Weeks Time Point.

Superimposing aspirin and NPAA on the BEA-induced RPN did not produce any significant changes from the general changes described above. However, the superimposing of paracetamol did produce two interesting results:

1) One animal in the BEA/PARA group had a severe hyperplastic covering urothelium on the papilla stump which was 7-9 cells thick with a well
Figure 5.21 Higher magnification of nodular region of tumour in upper ureter, showing nuclear pleomorphism, mineralisation (arrow) highlights difficulty in differentiating urothelial cells from basement membrane (*), lamina propria and lamina muscularis suggesting an invasive stage of development. Giemsa x 434.

Figure 5.22 Nodular regions of tumour shown above do not stain for alkaline phosphatase (arrowheads) whereas papillary regions of the tumour stain positively (arrow). Alkaline phosphatase x 223.
developed vascular supply via a stalk of interstitium from the papilla stump (Figure 5.23). The urothelium had a mosaic pattern of cells with basophilic staining nuclei. The superficial cells had eosinophilic granular staining cytoplasm, these cells appeared to be undergoing necrotic changes. A mosaic pattern of alk. phos. staining was evident within 2/3 of the hyperplastic cells the remainder were not stained. The interstitial cells and matrix adjacent and basal to the hyperplastic urothelium also stained positively for alk. phos.

2) One animal in the BBN/BEA/PARA group had a well defined transitional cell carcinoma in the ureter (Figure 5.24) with extremely variable cell size, loss of polarity, good vascular development within the tumour mass (Figure 5.25). There was diverse cytoplasmic staining with PAS and alk. phos. (Figure 5.26) and the PAS granules were particularly pronounced throughout the urothelium of this tumour.

5.3.2 Morphological Changes at the Fourteen Week Time Point.
The BEA/PARA control group had a bladder papilloma which had many areas of dysplasia, mitotic figures present throughout and necrotic areas within the tumour mass (Figure 5.27). Within the ureter there were some extended areas of diffuse nodular hyperplasia, with cellular atypia and necrotic superficial cell layers.

5.3.3 Morphological Changes at the Eighteen Week Time Point.
Mosaic alk. phos. staining was present throughout the hyperplastic urothelium of all groups.

5.3.3.1 In the BBN/BEA/ASP treated group there was nodular hyperplasia at the pelvis/ureter junction with mitotic figures present. There were nests of hyperplastic urothelium in the bladder together with one transitional cell papilloma (Figure 5.28).
Figure 5.23  Severe hyperplastic covering epithelium on papilla stump, 7 weeks after BEA/PARA treatment. Giemsa x 177.

Figure 5.24  Well differentiated transitional cell carcinoma in ureter, 7 weeks after BBN/BEA/PARA treatment. Giemsa x 177.
Figure 5.25  Higher magnification of above figure showing nuclear pleomorphism, loss of cellular polarity and well developed microvasculature within tumour mass (arrowhead). Giemsa x 357.

Figure 5.26  Mosaic staining of alkaline phosphatase within ureter tumour shown in Figures 5.24 and 5.25. Alkaline phosphatase x 177.
Figure 5.27  Papilloma in bladder of animal 14 weeks after BEA/PARA treatment with much vacuolation and necrosis of superficial tumour tissue evident. Giemsa x 36.

Figure 5.28  Bladder papilloma found 18 weeks after BBN/BEA/ASP treatment. Giemsa x 89.
5.3.3.2 There was marked increase in the number of macrophages present in the superficial layers of hyperplastic urothelium in the BEA/PARA group.

5.3.3.3 The BBN/BEA/PARA treated group had papillomas in 2/3 animals, one was extremely large (9 mm) in diameter with well developed interstitial cords throughout the tumour mass (Figure 5.29). There was also severe focal and simple hyperplasia in the bladder and ureter with numerous atypical nuclei and mitotic figures present. Both the papillomas were negatively staining with respect to alk. phos. enzyme, no GGT staining of any hyperplastic or carcinoma areas was observed.

![Figure 5.29 Extremely large bladder papilloma (9mm diameter) found 18 weeks after BBN/BEA/PARA treatment. Giemsa x 36.]

5.4 SUBSEQUENT BBN DOSING FOLLOWING BBN INITIATION AND BEA INDUCED RPN.

The rational for this experimental procedure is described in Methods sections 3.6.5.2 and 3.6.5.4.
5.4.1 Pilot Study

This pilot study (section 3.6.5.2) group showed little difference between BBN/BEA results at the earlier time points.

At 30 weeks, however, the BBN/BEA/BBN treated group had a greater number of urothelial abnormalities with a greater degree of severity than BBN/BEA group. Foci of papillary hyperplasia were present in the ureter with cellular atypia, nodular "invasive" hyperplasia (possible P1/P2 grading) and/or in situ carcinoma was present. Bladder papillomas were very disorganised in structure away from central stalk and fibrous interstitial core with numerous mitotic figures present in cell layers adjacent to fibrous core.

At 40 weeks at the macroscopic level one BBN/BEA/BBN treated animal had a large tumour in the pelvis of the kidney which it completely filled (Fig. 5.30) and extended into the upper ureter, which was enlarged and distended.

At the microscopic level this tumour developed out of the pelvic wall with mitotic figures evident in this area (Figure 5.31). It was a typical well differentiated transitional cell carcinoma with numerous macrophages throughout. Superficial areas of the tumour were undergoing necrosis, with many leucocytes present together with necrotic epithelia cells. One extremity either arose from the side of the ureter or was a downgrowth of the pelvic tumour into the mouth of the ureter. The ureter was extremely dilated with nodular and papillary hyperplasia present. The bladder urothelium was very dysplastic having extremely variable cell nuclei shape and size. One large bladder papilloma was present, which was well differentiated. Prior to the stalk of papilloma the bladder urothelium became severely
Figure 5.30  Macroscopic photograph of tumour found in pelvis 40 weeks after BBN/BEA treatment, followed by subsequent further dosing with BBN. x 5 approximately.

Figure 5.31  Origin of pelvic tumour shown above, tumour arises from pelvic urothelium (arrow), note mitotic figures (arrowheads). H&E x 357.
hyperplastic becoming 8-10 cells thick. Throughout the pelvic and ureteric urothelium there was a noticeable increase in PAS-positive granular inclusion bodies in the superficial layer of hyperplastic areas.

5.4.2 Main Study:
The only differences between the results observed in BBN/BEA and BBN/BEA/BBN treated groups occurred at 18 weeks when keratinization of some bladder tumours was observed in the BBN/BEA/BBN group. This pathological change had only been observed at much later time points in previous studies (40 weeks after BBN/BEA treatment).

At 30 weeks time point the tumour incidence observed macroscopically in all groups was as follows: BEA (0/5), BEA/BBN (0/5), BBN (1/5), BBN/BBN (2/5), BBN/BEA (5/5) and BBN/BEA/BBN (5/5). Overall the tumours in the BBN/BEA/BBN group were larger in size than those found in the BBN/BEA group. The histological changes in the tumours were, however, no different to those already described above in section 5.2.3. for bladder tumours. There were, however, no pelvic or ureteric tumours.

The changes for the tumours found in the animals from this study which acted as donor animals in the Nude mouse tumour xenograft study are described in section 5.6.2.1.

Animals sacrificed at 58 weeks in this study were a terminal sacrifice ending the experiment. Only 5 animals in total (2 BBN, 3 BBN/BEA) were sacrificed and 1 of these was moribund. No tumours were found in the pelvis, ureter or bladder of these animals. One of the BBN/BEA animals did not appear to have any indication of having had a RPN lesion; the papilla was intact and the kidney cortex surface was
smooth and normal in appearance. Due to a lack of time these specimens have not been examined histologically, specimens from the fifth BBN study took priority over these late time points.

5.5 STUDY 5: SUPERIMPOSITION OF PARACETAMOL AFTER BBN/BEA TREATMENT.

The rational for this study is described in Methods section 3.6.5.5. At the first sacrifice point (6 weeks) there was only a 20% gross incidence of RPN (necrosis of papilla tip, leaving a residual stump) in BEA-dosed animals. By the third sacrifice point (18 weeks) the gross incidence of RPN was still only 25% in the animals dosed with BEA. In some of the treated animals with an intact papilla there were histological changes which are indicative of an early RPN - loss of interstitial matrix staining, adherent platelets in capillaries, casts in the loops of Henle. This was surprising as this compound has been used in our laboratory for at least 10 years and has always produced a RPN lesion in 100% of the animals treated at doses greater than the threshold dose of 25–35 mg/kg. One would therefore have expected all the animals to have RPN lesions following the dose of 75 mg/kg used in this study and the histological changes would have been more pronounced than those exhibited by 50% of the animals in this study. These changes were similar to changes expected to occur over the 12-24 hr time point, see section 4.1.

5.6 NUDE MOUSE XENOGRAFT TUMOUR RESULTS.

5.6.1 Transplanted Tumour Growth.

Twenty-one mice were used in xenograft procedures, 19 received xenografted rat tissues, the remaining 2 received passaged tumour tissues from 2 of the original 19 mice. Nineteen mice were originally xenografted with rat tissue: 7 mice received donor tissue from the
renal pelvis region, 7 mice received donor tissue from the bladder, 3 mice received donor tissue from the ureter and 2 mice received donor tissue from the papilla tip. Fifteen of the donor animals had been treated with the BBN/BEA regimen and the remaining 4 animals had been treated with the BBN/BEA/BBN regimen.

Only the xenografted bladder tissues consistently "took" in the nude mice hosts: 5/7 grew to a measurable size by 4-5 weeks after xenografting. Only 2 tumours (from mice numbers 7 and 13) grew to a size such that they were visible macroscopically as gross distended lumps on the flank and had an average size indices of 36 and 35 respectively \( (L_1 \times L_2 / 2) \) see Methods section 3.6.6.5. However, when these values are converted by the formula \( (L \times (B)^2) \times 0.4 \) to give calculated tumour volumes, then the volumes became 230 and 216 mm\(^3\) by week 11 and 13 post transplantation respectively (Table 5.3).

Figure 5.32 shows the growth curve of tumour growth (plotted as calculated volume against time). In both these animals tumour growth was initially slow, then at week 8/9 there was a sudden increase in the growth rate of both tumours with the tumour volume approximately trebling in the subsequent 3/4 weeks. These tumours were passaged into two more mice (numbers 20 and 21), however only one of these tumours "took" but failed to grow well and after 5 weeks it began to diminish in size so the mouse was sacrificed. Also during the moving of the isolator to a different room one of the plastic sleeves became holed, this was quickly repaired. A microbiological analysis of mice faeces was performed (by Mrs T. Amor, Microbiology Dept, University of Surrey) several days later to determine if any contamination had occurred. Several of the remaining mice were becoming thin and losing
Table 5.3: Volumes (mm$^3$) of xenografted tumours.

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M. - mouse number; T. - donor tissue type (P: pelvis, B: bladder, U: ureter, Pt: papilla tip, Bpl: first bladder tumour passage); 6# - animal terminated early because injured in fight; 7* - xenograft tumour passaged into mouse 20; 13** - xenograft tumour passaged into mouse 21; X - terminal sacrifice; nr - not recorded; - - no presence of tumour.
Figure 5.32 shows the growth curve of xenografted tumour growth (plotted as calculated volume against time).
weight including one of the subpassaged mice. The test proved positive with *Proteus sp.*, *Sporing Baccilus sp.* and *Coccus sp.* being identified. Although *Proteus* species of bacteria are normal gut flora in this strain of nude mice the presence of *Baccilus sp.* is often indicative of environmental contamination and the *Coccus sp.* could be any strain of a frequently pathogenic bacteria species (Dr M. Coates, personnel communication). The loss of weight may be attributable to a bacterial infection by any of the above species or one which was not identified (analysis for viral infection was not possible). This loss of weight together with the lack of successful growth of the passaged tumours were the main reasons for terminating this experiment.

5.6.2 Histological results.

5.6.2.1 Donor tissue.

The original bladder tumour from a donor BBN/BEA/BBN animal was a transitional cell carcinoma (Figure 5.33) with well developed interstitial connective tissue cords containing many mast cells. Many of the transitional cells showed pleomorphism (nuclei of variable shape and size see Figure 5.34), together with numerous mitotic figures. There was no alk. phos. staining in any of the tumour cells only in the endothelium of the capillaries in the connective cords. Many cells had PAS positive staining cytoplasmic granules.

The kidneys of all donor animals had frank RPN with a re-epithelialised papilla stump, severe interstitial nephritis throughout the medulla and cortex. The pelvic and ureteric urothelium had hyperplastic lesions varying from mild to severe nodular hyperplasia (in some areas possibly invasive).
Figure 5.33 Transitional cell tumour from bladder of BBN/BEA/BBN treated animal which was one of donor tumours used for xenograft experiments. Giemsa x 216.

Figure 5.34 Higher magnification of tumour shown above illustrating well differentiated pleomorphic cells. Giemsa x 434.
5.6.2.2 Xenografted tissue.

Macroscopically the tumours were of variable shape, encapsulated in a fibrous connective tissue presumably sub-dermal tissue of nude mouse origin (Figure 5.35). One of the tumours had macroscopically had some cystic regions which were hollow containing a colourless fluid. Histologically the tumours comprised of a transitional cell tumour with a solid, nodular, disorganised structure. The cells showed pleomorphism and were well differentiated (Figure 5.36). Interstitial connective tissue cords formed a radiating pattern within the solid tumour, a very similar appearance to the bladder tumour from the donor animal. Similarly to the donor tissues there was no alkaline phosphatase enzyme staining in the xenografted tumour tissue. In contrast there was no positive PAS staining cytoplasmic granules in the xenografted tumour cells. The tumour which was a hollow cyst structure had walls which were an epithelium 2-5 cells thick arranged as in the classical structure of urothelium lining the pelvis, ureter or bladder in control animals. Some regions of the tumour resemble carcinoma in situ with a very disorganised basophilic nuclei within an epithelium of normal thickness. Other areas were loosely arranged with extremely variable staining cytoplasm and nuclei.

5.6.3. In vitro Results and Discussion.

5.6.3.1 Results.

The earliest stage of growth was an outgrowth of cells from the donor tissue, see Figure 5.37. These outgrowths consisted of a flattened plaque of epithelial-like cells. The cells were of varying shape and size with numerous cytoplasmic droplets (whose nature was not determined), the cell nuclei were also of varying size and shape with
**Figure 5.35**  Solid, nodular transitional cell tumour encapsulated in fibrous connective tissue (*) harvested from a Nude mouse. Giemsa x 216.

**Figure 5.36**  Higher magnification of harvested xenografted tumour shown above, note well differentiated cells compare with donor tumour shown in Figure 5.34. Giemsa x 434.
Figure 5.37 Outgrowth from original piece of bladder tumour (arrow) of urothelial cells grown after 4 days in culture. Phase microscopy x 108.

prominent rounded nucleoli see Figure 5.38. A few of these plaques grew to an appreciable size (visible without a microscope), all consistently were composed of epithelial-like cells. Attempts to subculture these colonies failed either because of an excessive trypsinisation period (during sub-culturing process) or that bacterial contamination occurred. Bacterial contamination was a frequent reason for discarding cultures.

5.6.3.2 Discussion.

The reasons for failing to culture a cell line successfully due to the problems created by bacterial contamination may be an inadequate aseptic technique for transferring the media to multiwell plates, or due to endogenous urinary tract bacteria from the donor tissues. Control wells with only media present (no cells) only rarely had contaminating bacteria present.
5.7 DISCUSSION OF RESULTS FROM EXPERIMENTALLY INDUCED CARCINOGENESIS STUDIES.

These investigations were undertaken to test the hypothesis that analgesic associated RPN and UUC are linked by a common pathological sequence of events. Long-term studies with BEA (Bach, 1981) showed that an acute RPN resolved and raised the question that UUC represented a classical initiation-promotion type as a consequence of RPN. The possible "cause-and-effect" relationship was investigated in these studies. Previous research using a 2-stage initiation-promotion model has established that urothelial hyperplasia may develop into tumours (see section 2.3.5.2). The urothelial specific carcinogen N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN) was used in the following series of studies to initiate the urothelium at sub-carcinogenic doses followed by an acutely (BEA) induced RPN in Wistar rats. BBN was
chosen as the carcinogen to facilitate controlled oral dosing (see section 2.3.5.2.B) to reduce the exposure risk to personnel and because of its greater specificity than FANFT with no benign 'extra-urothelial tumours' induced (see section 2.3.5.2.A). The second series of experiments in this study (Methods section 3.6.5; Results-section 5.1-5.5 above) where BBN was used to initiate the urothelium followed by a BEA induced RPN.

5.7.1 Short-term Changes in BBN/BEA Studies:0-6 Weeks.

BBN was chosen as the initiating agent (as explained in section 2.3.5.2.A) and the first priority was to ensure that the response of the rat kidney to BEA was not altered by such treatment. The result from the early time points in this study did not show any differences in the development of the acute RPN as was seen in control animals treated with BEA alone (section 4.1.1 and 4.1.2). The conclusion was that there were no obvious pathological differences and BBN initiation did not alter in any way the response of the rat kidney to BEA.


Compared to the BEA only and BBN only treated control groups, animals from the BBN/BEA treated group had more severe urothelial abnormalities (sections 4.1.2.3-4.1.2.6). Tumours in the renal pelvis and upper ureter were only found in the BBN/BEA treated animals (sections 4.1.2.5 and 4.1.2.6), and bladder tumours appeared earlier (at 21 weeks compared to 30 weeks, sections 4.1.2.4 and 4.1.2.5) and were usually larger with more dysplasia and cellular atypia compared to those observed in the BBN only treated animals. The loss of
alkaline phosphatase staining in urothelium has been reported to be a marker histochemical change in chemically induced urothelial malignancies (Kunze, 1979). The finding in this study that 'nodular' invasive hyperplasia and nodular tumour tissue lacks any positive staining for this enzyme suggests the existence of premalignant and malignant cell populations, based on the experience of Kunze & Schauer, (1977) and Kunze, (1979). Thus the urothelium of animals treated with BBN that had been initiated before an acute RPN was induced. These data suggest that a BBN initiated urothelium can be promoted to pre-neoplastic and neoplastic cells in the upper ureter and renal pelvis by an acute RPN.

Studies by Mattingley and co-workers (Mattingley et al, 1990) using continuous ³H-thymidine administration to animals with a BEA-induced RPN (see section 4.6.1 for more detailed discussion) have shown that the urothelial cells lining the renal pelvis (particularly the fornices region) and ureter were particularly sensitive to proliferative response. This region of urothelia also showed a pronounced hyperplastic response in the marmoset (section 4.3.1) and in the pig (section 4.4.2.3.B and 4.4.3.4) after BEA. These hyperplastic changes may be due to a direct toxic effect of the BEA on the urothelial cells in this region. The hyperplasia may also be due to a number of consequences of the RPN lesion; the excretion of magnesium ammonium phosphate crystals may cause mechanical injury and irritation, the excretion of cell debris including brush border enzymes may also act as an irritant on the urothelial cells, or may result in a loss of protective glycocalx from the urothelium. All these possible factors will be discussed in more detail below and in Chapter 6.
Compared to the BBN only treated group, animals from the BBN/BEA group had more severe urothelial abnormalities earlier, and any tumours were usually larger and with more dysplasia and cellular atypia.

Changes which were common between the BEA only group and BBN/BEA group were; hyperplasia of the urothelium (especially pelvis and ureter) with loss of alk. phos. staining in the urothelium and increased PAS positive glycocalyx staining which progressed with time. The glycocalyx normally provides a protective barrier against bacterial and chemical attack, thus if the urine becomes more chemically hostile, a natural response of the urothelium may be an increase in the thickness or chemical nature of this barrier. The prominent glycocalyx staining with PAS-positive inclusion bodies accumulating too, could represent a long-term aberration of cellular function especially because they were most marked in those regions where the urothelial dysplasia was greatest. It is also possible that the increased glycocalyx staining is a response to changed renal function due to a loss of concentrating ability as a consequence of the RPN. PAS-positive staining inclusion bodies have been reported to be present in malignant bladder carcinomas by Hukill & Vidone (1965). The presence of the granules (inclusion bodies) could be related to the glycocalyx, particularly because changes in these complex carbohydrates have been linked to tumourigenesis and cell surface recognition (Iozzo, 1985; Smets & Van Beek, 1985).

These changes in carbohydrate staining may therefore represent early or subtle changes in the urothelial cells that predispose them to, or are directly responsible for, abnormal growth patterns. Similarly, increased glycosaminoglycans may be an expression of metabolic changes
within the urothelial cells due to direct or indirect biochemical alterations as a result of a circulating (nephro) toxin. Makhailidis et al, (1987) in a review of prostanoids and their role in function, pathogenesis and treatment of disease in the urinary bladder report that "*the link between prostanoids and glycosaminoglycans may be of great relevance, since glycosaminoglycans appear to inhibit the direct contact and adherence of chemicals and bacteria to the bladder mucosal surface*". Certain prostanoids raise intracellular cyclic AMP levels which in turn increases local glycosaminoglycan production. The inclusion bodies may represent a response to increased prostanoid levels within the urothelial cells. However, analgesics and NSAID impair prostaglandin synthesis by inhibiting cycloxygenase enzyme in the arachidonic acid cascade and glycosaminoglycan synthesis is reduced, thus allowing bacterial and chemical attack directly on urothelial cells.

The loss of alkaline phosphatase staining in the hyperplastic urothelium which occurred gradually after about 6 weeks post BEA dosing in BEA only treated group, in nodular 'invasive' hyperplastic areas in BBN/BEA treated group is a histochemical change which has been reported frequently in chemical induced urothelial malignancies. (Kunze 1979; Kunze & Schauer, 1971, Stiller & Rauscher, 1971). The focal loss of alkaline phosphatase from otherwise apparently histologically normal rat bladder urothelial cells follows carcinogenic doses of BBN or FANFT (Kunze & Schauer, 1971). The loss of alkaline phosphatase in the research reported in the published data (Kunze, 1979) was irreversible and occurred after the discontinuation of carcinogen, and could not therefore be a direct toxic effect. These alkaline phosphatase-free cells are considered to be pre-
neoplastic cells that develop into papillomas and carcinomas (Kunze, 1979, 1986). Gamma-glutamyl transpeptidase (GGT) positive cells are already well established as markers for the pre-malignant changes in other organs (De Young et al, 1978; Bannasch, 1986a,b; Hannigan & Pitot, 1985; Moriyama et al, 1983). It has therefore been generally assumed that the presence of foci of GGT in the urothelia is a sensitive and specific marker of malignancy, especially because these changes are present in nodular hyperplasia and carcinoma that develops after BBN (Ozono et al, 1985). GGT foci were not demonstrated in this study which may be due to differing methodologies from previous reported studies, Ozono et al (1985) used wax sections whereas in this thesis glycolmethacrylate sections were used. Alternatively Vanderlaan et al, (1982) has suggested that GGT identifies only advanced carcinomas and large papillomas, and may not be a pathognomonic indicator of premalignant changes under all circumstances.

From the results of the BBN/BEA study (section 5.2) it does appear that an initiated urothelium can be promoted by RPN to develop hyperplasia, dysplasia and finally tumours. This suggests that it is possible for RPN in the clinical situation to progress to UUC assuming the pathogenesis of BEA-induced RPN is the same as that for analgesic induced RPN: which on a morphological basis appears to be true.

The pilot study (see section 3.6.5.2 and 5.4.1) where further doses of BBN were administered to a BBN/BEA treated animal to hopefully mimic continued initiation of urothelium with carcinogens in human clinical situation. It is postulated that many chemicals are carcinogenic when given to embryonic or newborn animals, but not when given to
adults (Warwick, 1971) because the former have populations of rapidly dividing cells within their tissues. The rationale for this study was based to some degree on the results of Mattingley et al, (1990) (who found that the cells of the upper ureter and pelvis were capable of rapid proliferation), and on results from studies in this thesis (section 4.1.1) that have shown a marked hyperplasia of the cells in this region. It was of interest to determine whether administering the carcinogen during the period of intense proliferative activity increased the targeting of the carcinogen to these cells, and/or resulted in an increased tumour yield.

The results from this study (section 5.4.1) show that dosing with the carcinogen during the 3 days after a single dose of BEA causes a propensity to develop pelvic tumours similar to those reported in FANFT studies (Erturk et al, 1969). Hyperplasia of the pelvic urothelium occurs after BEA-induced RPN, it is possible that rapidly regenerating cells are sensitive to chemical attack. However, the main study performed later to confirm these results (sections 3.6.5.4 and 5.4.2) where larger numbers of animals were used, did not show any differences between the BBN/BEA treatment and the BBN/BEA/BBN treatment. No pelvic or ureter tumours were induced and the only observed difference was that bladder tumours from the BBN/BEA/BBN treated group showed keratinisation earlier than the BBN/BEA-induced tumours (section 5.4.2).

5.7.3 Paracetamol Studies.

It appears from this pilot study (section 5.3) that paracetamol on its own could play a significant role in the initiation/promotion of the urothelium to produce severe hyperplasia, dysplasia and tumours
following an acutely induced RPN.

The very rapid development of dysplasias and tumours (within 7 to 14 weeks) in uninitiated animals given an acute RPN and then exposed to paracetamol raises a number of very important health care questions. Vast amounts of paracetamol are consumed (Wing et al., 1989), and it has generally been assumed that it is the most innocuous of the "over the counter" analgesics (except when taken in overdose) and should be taken as a single compound, rather than mixed analgesics. If the data in the rat are real and the same mechanisms apply to man (i.e. paracetamol can cause tumours where there is pre-existing renal papillary necrosis), then the health consequences of continued paracetamol exposure may have a very significant detrimental effect. However, studies performed in the pig to investigate this concept (that paracetamol can promote a urothelial carcinogenesis following an acute RPN in the multipapillate kidney) have been detailed earlier in this thesis (section 3.6.4.6). The results from this study (section 4.4.3.3) suggest that subsequent dosing with paracetamol does not exacerbate the acute RPN (discussed in detail above, section 4.6.5.2). However, a pronounced pelvic urothelial hyperplasia with abnormal PAS-positive intracellular staining was observed (section 4.4.3.4) which may represent a pre-neoplastic change which if allowed to develop over a longer time period could have developed into tumours. To confirm this hypothesis it would be necessary to treat a group of pigs as described (section 3.6.4.6) and monitor the animals for a longer period of time. It may be possible to monitor the tumour development using clinical techniques such as urinary cytology (Eriksson & Johansson, 1976), retrograde ureteropyelography (Oschner et al., 1974) and computerised tomography (Gatewood et al., 1982; Nielsen & Ostri,
5.7.4 BBN Study 5 - An Irreproducible Experiment

The finding (section 5.5) that at every sacrifice time point so far examined only 20-25% of the BEA-dosed animals exhibited gross RPN (loss of papilla tip with stump remaining) and mild RPN changes in 50% of those animals that had intact papillae is puzzling. As stated above previous studies performed in this laboratory over the last decade and all reports from many other studies (see Table 2.6 for references) have shown that BEA > 25-35 mg/kg (Bach et al, 1983) induces a RPN lesion in 100% of the animals dosed. All of the materials used, weighings, injection times etc have been thoroughly checked and no errors or discrepancies (which could explain the anomalous results in this study) have been found. The study was allowed to continue until the 30th week to ensure that the protocol did not induce tumours despite the lack of an obvious RPN. It was felt that the cost of extending the study to 30 weeks in terms of time was justified, relative to the time, effort and expense of the study so far. This was also deemed necessary to obtain results from a time point (30 weeks) to compare to early studies, when tumours had been induced (section 5.2). Similar negative results were, however, found at 30 weeks; there was only a 20% incidence of gross RPN and no tumours were present. The experiment was terminated with no worthwhile results forthcoming.

A possible explanation for the anomalous results is as follows:- The Wistar rat strain at the University of Surrey had been recently "cleaned up" by having new breeding stock barrier-bred from Caesarean-born animals at a commercial animal suppliers. This was performed to
remove a minor, but endemic respiratory infection, while the animal unit was rebuilt, fumigated and re-decorated as part of an improvement and up-grading of the facilities. The BBN study N° 5: Chronic dosing with paracetamol after BBN/BEA treatment (section 3.6.5.4) was performed using these "clean" rats 2-3 months after breeding stock had been re-housed. The most probable explanation for the anomalous response is a reduced sensitivity to bromoethanamine, but the cause cannot be explained.

The dose response curve to BEA of these "new" animals has been checked several times (including at the 30 week time point of this study) and these studies have shown that (within the limits of biological variability) has returned to that has always been previously experienced.

This normal dose-response to BEA was not, however, in itself adequate to re-investigate the problem. A number of urinary markers are being currently developed to allow us to assess non-invasively that papillary necrosis has occurred. The monitoring of urine for assessing magnesium levels, triglycerides, uronic acid and sustained lactate dehydrogenase release (Delacruz et al, 1989; Ding et al, 1989; Moret et al, 1989) provides an unambiguous indication of papillary injury. The increase in urinary triglycerides is related to the accumulation of renal lipid material and the increase in uronic acid relates to the loss of the medullary mucopoysacharide.

5.7.5 Relevance of the BBN/BEA Model of UUC.

Considering the results obtained from this study (section 5.1-5.5) in terms of the criteria necessary to designate a chemical or agent carcinogenic (section 2.3.2) then the RPN lesion within the BBN/BEA
regimen can be considered a carcinogenic process. The BBN/BEA regimen which induces pelvic and ureter tumours can be considered a model of UUC, since tumours are induced in greater numbers than in control animals of this strain (with reference to historical control data Table 2.14). Tumours are also induced earlier than control animals (refering to BBN only treated animals). The fact that the BBN only animals develop tumours means that strictly according to the criteria of Berenblum (1974) for initiation-promotion then this study does not conform exactly to the classical initiation-promotion studies. However, administering the BEA step before the BBN step (as described in section 3.6.5.4) did not induce any urothelial tumours even in the bladder, whereas BBN only treated and BBN/BEA treated animals both developed bladder tumours as described above in results section 5.2-5.4.

Compared to earlier studies used to induce UUC (see section 2.2.5) with analgesics (Johansson et al, 1976; Isaka et al, 1979; Nakanishi et al, 1982) this model system has a number of advantages over the dietary administration of carcinogen and promotor compound.

1. The dose of initiating carcinogen is known and can be controlled and manipulated (Becci et al, 1979; McCormick et al, 1981a), if required, whereas in the dietary fed methods there is little control over the exact dose of the carcinogen each animal is consuming.

2. It is also possible to control the promotion stage (by reducing the dose of BEA to induce a less severe RPN lesion), also when administering additional compounds such as analgesics the oral gavage technique allows a quantitative dose to be given which is similar to a typical daily dose consumed by an analgesic abuser.
These factors make this model system much more adaptable than previous investigations. Compared to the spontaneous tumour models in the DA/Han rat and BN/BiRij rat strains (see section 2.3.5.1) this chemically induced procedure produces tumours earlier although it must be said that the 25-30% incidence of identifiable macroscopic tumours is probably considered low and not very cost-effective.

The weaknesses of this model are:

1. It still requires a considerable period of time to induce tumours for harvesting to use in in vitro studies etc which is costly, both from an economic point of view and one of man hours involved in the maintenance of the animals.

2. The quantitative dosing technique when used in long-term dosing procedures, ie subsequent dosing with analgesics, was laborious, time consuming especially when all the animal maintenance procedures have to performed by the researcher.

3. The final incidence rate of UUC is low (30% maximum), this might not be considered cost-effective. There is a need for further research as to how one may increase the numbers of tumours induced. The pilot study with single analgesics would seem to suggest that this at least reduces the latent period of tumours and also increases the severity of the urothelial pre-neoplastic changes. Other studies have used experimentally induced urinary tract infection together with FANFT initiation to obtain an increased incidence of pelvic tumours (Johansson et al, 1987). Urinary tract infection is associated with RPN and BBN (discussed in detail below, section 6.2.1) it may be possible to increase the incidence of tumours in the pelvis and upper ureter by superimposing a urinary tract infection on the BBN/BEA treatment.
4. There is a high incidence of tumours in the control carcinogen only treated group. One answer here is to reduce the dose of carcinogen, but increase the frequency of dosing. The problem here is that there is no longer a comparison with earlier studies and a new learning curve must be begun.

5.7.6 Nude Mouse Xenograft Studies.

The overall "take" rate of the donor tumours of 10.5% (2/19) is low (section 5.6.1) compared to the generally acceptable "take" rate of 50% for a variety of human tumours xenografted into nude mice (Polvsen et al, 1982). Considering the donor bladder tumours alone then the "take" rate of 29% (2/7) is an improvement, but still low when compared to the "take rate" of 60% in other studies using xenografting chemically-induced (FANFT) bladder tumours into NMRI-nu/nu mice (Weldon & Solway, 1975). There a number of possible reasons why the pelvis and ureter donor tissues failed to take:—there was insufficient tumour tissue present in the original transplanted tissue. This may stem from the technical difficulties of surgically dissecting the very small tumour areas from the pelvis and ureter. If the transplanted tissue was non-malignant or in a regressive state then this may account for the fact that these tissues did not develop in the host. The donor tumour tissues appeared to be well differentiated with well developed connective tissues within the tumour tissue. "Take" rates have been reported to be the highest in tumour tissues which have the least histological differentiation ie invasive metastases (Sharkey & Fogh, 1984). The ability of a tissue to be accepted as a xenograft depends on the how well the tumour cell can adhere/attach/in invade the host tissues and a number of other factors.
including angiogenesis; encouraging capillary growth into the tumour tissue to sustain its growth and development (Gabbert, 1985).

Typically, the "tumour" tissue from BBN/BEA treated rats is papillary in nature and not invasive, this may be a factor in whether the tissue "takes" in the host tissue.

The slow initial growth rate of the successful xenografted tumours is not unusual. Other studies have shown that the primary transplant time is significantly longer than that of subsequent passages and that the chance of non-takes is increased during the primary transplant period (Polvsen et al, 1982). If tumour cells can be transplanted to a third passage then there is a 90% chance of the tumour being established as a cell line. Growth rates have been shown to vary considerably in studies of different human tumours transplanted in nude mice. Polvsen et al, (1982) report that the average passage time to reach a measurable mean diameter of 10 mm was for kidney tumours 5.8 weeks, urinary tract tumours 2.3 weeks and colon tumours 7.8 weeks, although a considerable range can be experienced e.g. kidney tumours: 1.2 - 20.8 weeks. Rapid growth rate has, however, been correlated with an increased success in the "take" of the transplanted tumour (Mattern et al, 1981; Sharkey & Fogh, 1984). The growth rate of the successful tumours was slow (11/12 weeks to reach a mean calculated volume of 200 mm$^3$) compared to studies with transplanted human urothelial cancers (4/5 weeks to reach a mean calculated volume of 500 mm$^3$) as reported by several workers (Huland et al, 1985; Kyriazis et al, 1984,1985), or with pituitary adenocarcinoma tumours which only took 20 days to grow to a mean volume of 200 mm$^3$ (Kyriazis & Kyriazis, 1980). Differences in the "host" nude mice strain have been suggested as one possible
reason for the differences in "take" rates and growth rates of tumours in nude mice.

Many investigators have observed that the health of the nude mouse is of major importance for the successful maintenance of tumour lines. Stress has been shown to affect the health of laboratory animals and the experimental results that are obtained from such stressed animals (Fortmeyer, 1982). Every precaution was taken to minimise the potential environmental and experimental stress that may have been experienced by the nude mice. However, the mice may have induced a type of stress by their own natural territorial behaviour.

As mentioned earlier (section 3.6.6.5) these nude mice were prone to fighting between themselves, particularly immediately after the cages had been cleaned. It is presumed that each male mouse considered the clean fresh unmarked bedding as "his territory" and proceeded to defend it against all intruders (the other 9 mice in the cage). The animals are therefore stressed from fighting and/or injuries. In an attempt to reduce such stress injured mice were separated on their own facilitate healing, but isolation can stress rodents (Fortmeyer, 1982). It is perhaps for this reason that it is recommended that one uses female mice for experimental investigations (Sharkey & Fogh, 1984).

The morphological results and the histochemical results (the lack of alkaline phosphatase staining in both the donor and xenografted tumours, see section 5.6.2), indicate that the tumours harvested from the nude mice were identical to the donor tumours. Limitations in time have meant that micro-cytochemical investigations on the very small tissue samples stored at -70°C have not yet been performed. These investigations together with karyological studies on the cells
grown in vitro may have provided some more evidence for better defining the similarities or differences between the donor tumour and the xenografted tumour.

The mice used to subpassage the successfully grown bladder tumours were old, the same stock group as the original host mice. Older nude mice have a better developed killer cell immunity system (Sharkey & Fogh, 1984) with which to illicit an immunological response and thus prevent the tumour tissue establishing.

The choice of the subcutaneous transplantation site (section 3.6.6.4.B) may not have been the best site for growing these chemically induced tumours. It may have been better to transplant the tumour cells intraperitoneally where the rich, easily accessible, peritoneal blood supply provides a good site for tumour cells to "embed" and grow. This site has the major disadvantage in that it is not possible to monitor the growth of the tumour non-invasively which means it is a single end-point study method. Given that one of our main objectives was to obtain a quickly growing tumour with which to establish an in vitro cell line it may have been prudent (with hindsight) to take a small number of animals and subject them to this type of experimental procedure. Another alternative would have been to transplant the tumour cells directly into the bladder of nude mice, thus providing an environment almost exactly the same as the growth site of the donor tumour with regard to basement membrane, extracellular matrix etc. This technique has been used with moderate success by Huland et al, (1986) who obtained a 33% take rate of a cell suspension of human transitional cell carcinoma injected intravesicularly into the NMRI-nu/nu mouse bladder which had
previously been instilled with N-methyl-N-nitrosurea to induce inflammation of the bladder urothelium. The accepted transplanted bladder tumours showed multifocal tumour growth with 4/5 tumours also showing muscle invasion. Similarly Naito et al, (1987) has shown that transplanting renal cell carcinomas into the renal subcapsule of nude mice produces tumours which grow faster, were larger, had more systemic metastases, had a better vascular system and were more invasive than those transplanted subcutaneously. Naito et al, (1987) suggest that the tissue organ of tumour origin is the best site for implantation and growth of tumours in nude mice. The initial slow growth period in our successful tumours (Figure 5.32) may correspond to a period where the tumour cells are adapting to living in a "foreign" environment with different osmolality, extracellular matrix composition, growth factors, etc. and basement membrane to that of its original tissue site. Another critical factor involved in the success of the growth rate of the transplanted tumour is how quickly the tumour becomes vascularized and thus is provided with the means to sustain rapid and uncontrolled growth. The step of tumour vascularization is essential for the success for spontaneous tumours (Folkman, 1985) so it is likely to be just as important in nude mice xenografts. The delay in rapid tumour growth may correspond to the period where the transplanted tumour is having to "embed" into a different extracellular matrix and establish a blood supply by releasing tumour angiogenesis factor (Folkman, 1985). It can be envisaged how much easier this would be for a tumour cell which had been transplanted into a tissue type which for all instances is identical to it's site of origin, resulting in a better chance of acceptance, rate of growth, sustained growth and invasion. Future
studies of this nature should consider these facts. Although these alternative organ transplantation site methods may initially involve greater technical difficulties they will probably result in an improved experimental system in the long-term.

5.7.7. Discussion of In Vitro Studies.

It was important to attempt to develop in vitro cell cultures of the chemically induced tumours for several reasons:

1. Having such a malignant urothelial cell line would have helped reduce the need for large numbers of animals required presently to investigate the mechanism and factors controlling cell growth in the upper urothelial tumours.

2. The use of in vitro cell lines allows a greater flexibility in experimental design and procedures which is more economical than using animals in long term in vivo experiments. It is possible to change factors which may affect the tumour development; vitamin addition or mineral insufficiency, addition of chemotherapeutic agents to the growth media, alteration of pH, change in oxygen tension. All of these changes could be performed in the in vitro situation prior to repeating any "interesting" results in the in vivo situation.

3. It be desirable to undertake chromosomal analysis, including assessment of oncogene activation products, to determine whether the induction of tumours was due to the activation of these genes.

4. The ability to culture large numbers of tumour cells economically could allow metabolic and biochemical analyses of tumour cells to be performed more cost effectively than in in vivo studies.

5. Such a cell line could be compared with material derived from human tumours to characterise any similarities which may be
diagnostically useful in human analgesic abusers.

5.7.8 Role of Mast Cells and Macrophages in the Development of UUC.

Throughout this study it was noticed that mast cells were frequently associated with the nodular pre-neoplastic hyperplasias (section 5.1 and 5.2, Figures 5.2 and 5.7) in the lamina propria. Also they were frequently observed in the interstitial cords which are a feature of this type of tumour (section 5.2.2). In the late time points macrophages were observed within the superficial layer of urothelium (section 5.23, 5.3, 5.32).

Mast cells play an essential role in tumour invasion as they are able to increase the permeability of local blood vessels by the release of histamine (Gabbert, 1985). Thus the presence of mast cells in the lamina propria close to the nodular hyperplasias may aid the chemotaxic growth of these cells towards blood vessels (Nicolson, 1988). Histamine also promotes non-specific inflammatory reactions including oedema which may help the downgrowth and invasion of nodular hyperplasia by disrupting the lamina propria and distorting the extracellular matrix filaments (Parawaresch et al, 1985). The tumour cells may release an "attractant" to encourage the aggregation of macrophages into the area and then enroll these cells into the tumour progression process (Normann, 1985).

Similarly macrophages may play a role in the tumour progression by stimulating angiogenesis and producing growth factors (De Baetselier et al, 1985). Macrophages when "activated" kill cells by generating reactive species such as hydrogen peroxide, superoxide anion ($O_2^-$) via a number of oxidative pathways. It may be possible for macrophages to metabolically activate substances such as paracetamol, since they have
a considerable potential for oxidation.

Recent studies by Marletta (1988) have elucidated a possible role for macrophages in the early stages of carcinogenesis through the endogenous production of nitrosamines from nitrate. Activated macrophages can perform n-nitrosation reaction and immunostimulation leads to an increased production of urinary nitrate (Marletta, 1988). Since RPN and UUC have both been associated with urinary tract infections (Eknoyan et al, 1982; Johansson & Wahlqvist, 1977) a situation can be envisaged where an increase in macrophages occurs to combat the urinary tract infection, and because of their subsequent "activation" these macrophages are able to produce N-nitrosamines locally to affect the urothelial cells. If these N-nitrosamines have a 4-hydroxybutyl structure then they may specifically initiate urothelial tumours (Okada, 1984). The possibility that bacterial infection per se could have a role to play in the pathogenesis of UUC is discussed in detail below - section 6.2.1.
CHAPTER 6.
GENERAL DISCUSSION INTER-RELATING MECHANISMS OF PATHOGENESIS OF RPN AND UUC, CONCLUSIONS AND POSSIBLE FUTURE RESEARCH DIRECTIONS.

6.1 MECHANISTIC BASIS OF RPN.

6.1.1 Mechanisms Postulated to Have a Role in the Pathogenesis of RPN and Brief Summary of Chemically Induced RPN Studies.

Several hypotheses (see Table 6.1) have been put forward to explain analgesic nephropathy (Rosner, 1976; Shelley, 1978; Duggin, 1980; Prescott, 1982), but it is only recently that the concept of a progression of the condition from an early, silent lesion to end stage renal disease has been given some experimental credence (Bach & Bridges, 1985; Bach & Gregg, 1988; Gregg et al, 1989b; Bach et al, 1989).

Table 6.1: some of the hypotheses which have been implicated in the pathogenesis of RPN and UUC.

1. The counter-current concentration mechanism.
2. Microvascular degeneration and medullary ischaemia.
3. Perturbation of arachidonic acid metabolism.
4. Alterations in intermediary metabolism.
5. Immunological changes.
6. Metabolic activation of biologically reactive intermediates.
7. Xenobiotic metabolism. (Peroxidase-mediated)
8. Cytotoxicity.

It has often been difficult to induce a model papillary necrosis in rodents using mixed analgesics (Bach & Bridges, 1985; Bach & Hardy, 1985, and see section 2.1.3.2.D). 2-Bromoethanamine hydrobromide has, however, been regarded as an ideal chemical with which to induce a
model RPN (see section 2.1.3.2.F). With the exception of the anomalous response of the nude mouse reported above (section 4.2.3). It targets selectively for the renal medulla in both sexes and all strains and species investigated so far. This includes Wistar, Fischer 344, Sprague-Dawley, Holtzman, Donrju, Munich Wistar, Gunn and Battleboro rats (see Table 2.6) in CD-1, Schnider (Bach, P.H. & Gregg, N.J., unpublished observation), Obese, Balb/c and C57Bl/6 mice (Scarlett et al, 1990), Syrian Hamster (Carlton & Englehardt, 1989) and the pig (Gregg et al, 1989a). Both the functional and the pathological changes caused by BEA are virtually identical to those associated with analgesic nephropathy in man and the lesion induced in animals using analgesics or NSAID (Bach & Bridges, 1985). Another advantage of BEA is that it causes a dose related RPN (at doses > 50 mg/kg ip) in 24-48 hr in all rats as opposed to the uncertainty of 3-12 months dosing rodents with mixed analgesics (Bach & Hardy, 1985). It also causes no extra-renal lesions, unlike the NSAID which target for the gastro-intestinal tract (Kaump, 1966).

Light microscopy is the major investigative technique used to identify nephropathic lesions in toxicological studies and in the clinical situation but it has almost exclusively performed on 5 um wax sections. The use of semithin (1-2 um) glycolmethacrylate resin sections within this research project has allowed high resolution microscopy, coupled with selective enzyme histochemistry, to provide unprecedented morphological detail and the identification of the earliest cellular changes that initiate the pathogenesis of such nephropathies (Bach et al, 1987; Gregg et al, 1990a,b).

The results from investigations using BEA-induced RPN suggest...
(sections 4.1-4.4) that this "model" system mimics closely the morphological changes occurring during the pathogenesis of RPN in the clinical situation due to analgesic abuse (Burry et al, 1977; Gloor, 1978; Bach & Bridges, 1985). The earliest degenerative change is located in the renal medullary interstitial cells which undergo necrosis first in the rat (section 4.1.1), and similar changes also occur in the mouse (section 4.2). While there are no time course data on the marmoset and pig these cells are also show the most prominent morphological changes.

The necrosis of the medullary interstitial cells is followed by, or closely associated with, alterations in the alterations in the mucopolysaccharide matrix and followed by degeneration of the endothelium, distal tubules, collecting ducts and loops of Henle, with changes in the enzyme histochemical staining profiles of the proximal tubules and microvasculature. The subtle degenerative changes in the proximal tubule do not appear to be central to the development of the papillary lesion. Exfoliated brush border enzymes and cells are important components of the proteinaceous casts in the distal nephron. Hyperplasia, the formation of distal tubular casts and then the proximal and distal tubular dilatation all appear to be secondary consequences of papillary necrosis, and the delayed increase in alkaline phosphatase staining in the urothelium supports this. The increased ATPase staining of sub-urothelial capillary endothelium suggests that there is a progressive microangiopathy similar to that described in human analgesic abusers with RPN (Mihatsch et al, 1979).

The time course of the complex cascade of morphological and histochemical changes occurring in the pathogenesis of BEA-induced RPN induced in the Wistar rat is schematically represented in Figure 6.1.
Figure 6.1. Schematic diagram summarising the cascade of morphological and histochemical changes occurring after a single ip dose of BEA in the Wistar rat.
The data presented in chapter 4 are the first to document such a cascade for BEA-induced RPN.

Similarly analgesic nephropathy is not a single event, but a dynamic process of renal degeneration and repair in man, as the consequence of multiple exposure renal injury, and the response of the kidney under a continued barrage of insult. It is best considered in terms of the primary injury; RPN, and its secondary consequences. It is likely that the damage to the fine elements of the medulla such as the interstitial cells, loops of Henle, collecting duct, etc; (all of which have been described above in the acute or sub-acute experimental models, see section 2.1.2.3) each contribute to their own series of degeneration-repair consequences as the heterogeneous population of the renal cells normally work in concert to maintain function even after injury (Bach, 1989).

The mechanistic basis of the primary RPN caused by any of the possible experimental manipulations is far from clearly understood. In common with most other toxic lesions a variety of mechanisms have been proposed at one time or another and some of these can be largely disregarded out of hand. For example, there is no link between immunological injury and RPN in human analgesic abusers (Gault et al, 1971) or in animals with a model lesion (Murray & Von Stowasser, 1976), nor with changes in intermediary metabolism (see Shelley, 1978). However, the data from the nude mouse study (section 4.2.3), suggest that the immune system could have some role in the pathogenesis of toxic nephropathies.

6.1.2. Role of Haemodynamic and Vasculature Changes in RPN.

Over the last 20 years there has been a persistent suggestion that the
The medulla is close to anoxia (it is argued that only 10% of the renal blood supply reaches this part of the kidney) and it is therefore prone to ischaemic injury. The potential for analgesic and NSAIDs to reduce haemoperfusion (see Shelley, 1978, Bach and Bridges, 1985) together with microvascular degeneration at the late stages of all RPN has been envisaged as support of this hypothesis. This argument is not supported by a critical assessment of the facts as the medulla is well oxygenated on the basis that the kidneys receive 20-25% of the resting cardiac output. Most of the experimental evidence for the acute models does not support vascular occlusion or degeneration (Ham & Tange, 1969; Sherwood et al, 1971; Bach et al, 1983; Gregg et al, 1990a) [see section 2.1.3.2.D.i], but cannot identify stasis, flow changes or increases in oxygen requirements. Data from Solez et al., (1974) showed increased radiolabelled albumin clearance following BEA, which appears to exclude homeostasis. The data from these studies (section 4.1.1.1) supports the argument that microvasculature changes are not an initiating factor in the pathogenesis of acutely induced RPN, but more likely play a role in the exacerbation of the lesion. There was no extravasation of Monastoral blue B and platelet adherence only occurred at late time points and in areas of the papilla where necrosis of the interstitial matrix and interstitial cells was pronounced (section 4.1.1). Comparing these results with the data of Solez et al, (1974) who reported an early increase in medullary blood flow 6 hr after BEA it seems unlikely that ischaemia and microvasculature changes have an initiating role to play in the early pathogenesis of acute RPN though it seems probably that these changes will help exacerbate the lesion.

Data from the pig study (section 4.4.3.1.B) show that the effective
renal plasma flow to the kidney increases as a consequence of the administration of BEA. However, the determination of the effective renal plasma flow was performed 26 days after the administration of BEA and there is no data to show how these haemodynamic parameters are affected immediately after dosing with BEA. Effective renal plasma flow determination does, however, only provide an average assessment for the whole kidney and not a focal assessment for the medulla.

It would be attractive to do effective renal plasma flow assessment in rats over early time points after BEA and to repeat the assessment of effective renal plasma flow in the pig at earlier time points. The pig has been suggested to be a more relevant species in which to investigate RPN (see section 4.6.5.2 above), however, to perform a time course study on the pig similar to performed in rats (section 3.6.1 and 4.1.1) would be difficult and expensive but the investigation of the morphological and functional changes at an earlier time point could be useful.

6.1.3 Role of Metabolic Activation in Pathogenesis of RPN.

Metabolic activation by the mixed functional oxidase systems has been recognised as a key factor in a number of toxic lesions, including the kidney (Rush et al, 1984), but the absence of cytochrome P-450 from the medulla has suggested that such a process is unlikely. Many of the peroxidative enzymes such as the hydroperoxidase activity associated with cyclo-oxygenase (in prostaglandin synthase) are capable of a similar series of metabolic activations to P-450 (see Bach and Bridges, 1984, 1985). Several peroxidases convert the two major metabolites of phenacetin, p-phenetidine (Anderssen et al, 1983) and acetaminophen (Joshi et al, 1978; Zenser et al, 1979a,b; Nelson et
al, 1981; Mohandas et al, 1981a,b) to reactive intermediates. Despite the fact that this hypothesis is attractive neither acetaminophen nor p-phenetidine cause RPN in the experimental situation.

All of the enzymes involved in prostaglandin synthesis are localised to the medullary interstitial cells, endothelial cells of all arteries and arterioles, the collecting ducts (Smith, 1981; Smith & Wilkin, 1977; Smith & Bell 1978). This almost ubiquitous distribution of prostaglandin synthetase and other peroxidase activities in the medulla make it most difficult to explain why papillotoxins affect predominantly one cell type as the earliest manifestation of target selectivity. The medulla has very low levels of glutathione and therefore once generated any reactive intermediate would not be readily inactivated (Bach & Bridges, 1985). The medullary interstitial cells, are rich in prostaglandin hyperoxidase and other peroxidases which have the potential to produce biologically reactive intermediates (Bach & Bridges, 1984, 1985; Bach & Gregg, 1988). These cells also, however, contain high levels of poly-unsaturated fatty acids (Bojesen, 1974) that would predispose to lipid peroxidation if such reactive intermediates were generated locally (Bach & Bridges, 1984). The interstitial cell does seem to be the target both in BEA-induced RPN and aspirin-induced RPN in Hooded rats (Molland, 1976, 1978), and the reason for this target cell cytotoxicity probably resides in the biochemical characteristics of these cells. These facts, considered together with the fact that interstitial cells do not undergo proliferative changes when damaged, nor is there a stem cell population from which to replace 'lost or damaged', cells (Bach & Bridges, 1985, Mattingley et al, 1990), could lead to the continuous
and insidious loss of these cells with the concomitant cascade of pathological changes (Figure 6.1). The predisposition of interstitial cells to lipid peroxidation is supported by work done by Benns et al (1985) who reported that interstitial cells in culture are sensitive to the toxic effects of BEA and paracetamol. Studies using 3T3, MDCK and HaK cells in culture provide additional evidence that lipid peroxidation could be the initiating mechanism in RPN (Bach et al, 1986). 3T3 and MDCK cells have a high prostaglandin synthetase enzyme activity whereas HaK cells do not, in addition 3T3 cells also contain high numbers of lipid droplets but MDCK and HaK cells are essentially free of lipid droplets. The 3T3 cells which contain both high concentrations of prostaglandin synthetase enzymes and lipid droplets were the most sensitive to the toxic effects of BEA. BEA at a concentration of 0.2mM was cytotoxic to 3T3 cells in 2-4 hr, whereas the MDCK and HaK cells were resistant to BEA at concentrations up to 2.0mM over 24 hr (Bach et al, 1986).

The interstitial cells synthetise the mucopolysaccharide ground substance that serves to support the delicate anatomical elements in the medulla, and also acts as a matrix that controls the availability and movement of liquid, ions and other molecules between different renal compartments. The early loss of this matrix will drastically affect renal function and morphology and play an important role in the cascade of degenerative changes. The loss of matrix could well be a key factor in the loss of concentrating ability. This could increase the urinary excretion of xenobiotic metabolites which affect more interstitial cells (thus perpetuating the lesion) and also the urinary tract urothelium. However it is still not certain from this light microscopy study whether the matrix changes are a consequence, or a
cause of the interstitial cell degeneration, or if a chemical effect on the collecting duct cell plays a role in these matrix changes. Species differences in the glycosaminoglycans/proteoglycans constituents (see Table 6.2) of the interstitial matrix may offer some explanation for the differing susceptibility of different species/strains to BEA-induced necrosis in this region (Pitcock et al, 1988).

<table>
<thead>
<tr>
<th>Species</th>
<th>Type and distribution (%) of glycosaminoglycans in kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HA</td>
</tr>
<tr>
<td>RAT</td>
<td>13-17%</td>
</tr>
<tr>
<td>PIG</td>
<td>30%</td>
</tr>
<tr>
<td>MAN</td>
<td>10-38%</td>
</tr>
</tbody>
</table>

HA, hyaluronic acid; ChS, chondroitin sulphate; HS, heparan sulphate; DS, dermatan sulphate; Hep, heparin.

Table 6.2 Species variation in the type and distribution of glycosaminoglycans in kidney. Modified from Bach & Bridges, (1985).

Future studies utilising immunohistochemistry including lectin markers for the matrix and ultrastructural studies with transmission electron microscopy should hopefully provide some answers to this problem. The focus of such ultrastrutural and immunohistochemical studies should be on the very early time points (with small time intervals), in the cascade of necrosis (0-4 hr). The aim being to observe a more subtle cascade of necrotic changes and thus be able to identify which is the primary change ie interstitial cell necrosis or interstitial matrix alteration.
Interstitial cells also play a prominent role in prostaglandin synthesis, which are substances acting as modulation of local, rather than systemic, homeostasis. Thus prostaglandins can be envisioned as serving a cytoprotective role which is an important factor when the kidney is 'stressed' (either by disease, electrolyte depletion or fluid overload) in protecting the respective renal compartment from injury (Schlondorff, 1986).

Perturbation of the arachidonic acid cascade could by decreasing prostaglandin synthesis (Bach & Bridges, 1984) result in an ischaemic response thus exacerbating the RPN lesion. The prominent necrosis of the thick ascending limb of Henle in the pig paracetamol study may provide some evidence for a possible mechanism linking the initial necrosis of interstitial cells, functional changes and the exacerbation of the RPN lesion. The thick ascending limb of the nephron is concerned with the absorption of salt (primarily sodium chloride) from the lumen as an intergral part of the countercurrent concentrating mechanism. To effect this absorption of sodium chloride a co-transport carrier protein present in the luminal membrane transports one $\text{Na}^+$, two $\text{Cl}^-$, and one $\text{K}^+$ ions into the cells of the thick ascending limb (Greven, 1987). There is some evidence that this carrier protein may be intimately associated with the Tamm-Horsfall glycoprotein (Greven, 1987). The link with the necrosis of the interstitial cells is through prostaglandins particularly $\text{PGE}_2$, which is synthesised mainly by the medullary interstitial cells (Schlondorff, 1986). $\text{PGE}_2$ has a number of potent effects on control of renal haemodynamics, salt and water excretion (Clive & Stoff, 1984) presumably acting via the $\text{Na}^+\text{-}2\text{Cl}^-\text{-}\text{K}^+$ co-transport system, since prostaglandins act at the thick ascending limb (Greven & Farjam,
Clive & Stoff, (1984) have suggested the existence of a prostaglandin-mediated counter regulatory mechanism that could be suppressed by drugs with a resultant impairment of renal haemodynamics producing ischaemia, loss of ions (Na\(^+\), Cl\(^-\), K\(^+\)), loss of Tamm-Horsfall glycoprotein and pronounced diuresis resulting in a cascade of necrotic changes similar to those described in BEA-induced and analgesic induced RPN (Bach & Bridges, 1985; Bach & Hardy, 1985). Greven (1987), in a review of loop diuretics (drugs which affect the thick ascending limb) suggests that adenylate cyclase (through cAMP) may have a role to play in regulating the Na\(^+\)-2Cl\(^-\)-K\(^+\) co-transporter system. Thus necrosis of the thick ascending limb could occur as a consequence of the interstitial cell necrosis because of the loss of regulatory prostaglandins.

The increased mucopolysaccharide staining observed (described in section 4.1.1) could represent the consequences of an increase in prostaglandin synthesis due to the renal stress and toxic injury following BEA-induced RPN. An association between prostaglandin synthesis, an increase in intracellular cAMP and an elevated localised glycosaminoglycan production has been reported (De Asua et al, 1975; Green et al, 1982).

The long-term progression of BEA-induced RPN affects the anatomical elements in the cortex producing an interstitial nephritis including cortical scarring, (a feature which is common in kidneys of human analgesic abusers), general atrophy and sclerosis of glomeruli, proximal and distal tubules, thus severely compromising the functioning capability of the kidney. This sclerosis and thickened basement membrane is first apparent in juxtamedullary nephrons where
their loops of Henle descend deep to the papilla tip. They are thus the first nephrons affected by the cast formation and dilatation following the necrosis (section 4.1.2). Cast formation may occur as a consequence of a transient albuminuria due to a transient change in glomerular permselectivity after BEA (Moret et al, 1989). These intraluminal casts probably cause a blockage and produce a back pressure. With a build up of luminal pressure and unphysiological conditions (including potential toxins) within luminal fluid, thus the sclerosis and basement membrane thickening which is observed as a long-term consequence of a BEA-induced RPN could be a cellular response to these changes.

Hyperplasia of the pelvic urothelium may be a secondary consequence of RPN, a response to a direct cytotoxic injury by BEA, or as a result of mechanical injury from the marked transient excretion of magnesium ammonium phosphate crystals (Bach & Gregg, 1990) and is most evident in areas adjacent and opposite denuded covering papilla epithelium (Gregg et al, 1990a; Mattingley et al, 1985; and section 4.1.1). This rapid proliferative response could be a protective adaptation to mechanical injury (discussed above section 4.6.1 and below section 6.2.5), increased output of cytotoxic urinary metabolites or a response to "growth factors" produced by residual covering epithelium in an attempt to re-epithelialise denuded areas. The increased alkaline phosphatase staining which occurs initially (section 4.1.1) is probably a cytoprotective response since this enzyme is found in increased quantity at the apex of viable cell layers in control urothelia below the "umbrella" cell layer.

This hyperplasia of pelvic and ureteric urothelium is continued for many weeks after the onset of RPN, eventually foci of papillary and
nodular hyperplasia develop which are postulated pre-neoplastic stages in bladder carcinogenesis (Cohen, 1983). This suggests that RPN may in itself be a process that could promote an already initiated urothelium.

6.2 MECHANISM OF BBN/BEA INDUCED UUC.

The data reported above (section 5.1-5.6) is one of the first studies to show that a localised injury to the papilla can "promote" the adjacent urothelia to a neoplastic state after initiation. This suggests a classical two stage process of initiation with BBN followed by an acute BEA-induced RPN resulting in the development of upper urothelial tumours. Promotion is, however, considered to be a multiexposure situation and therefore a single dose of BEA does not fit into this strict definition (see section 2.3.4.1). It is, however, likely that the changes associated with the BEA-induced degenerative cascade produces a sequence of multiple insults, which in concert promote the urothelium. This suggest that analgesic-associated RPN per se may be a key factor in producing a proliferative response in a pelvic or ureteric epithelial cells, especially those that had already been initiated. These proliferating cells could then progress to localised upper urothelial carcinoma. It is uncertain how valid a 2-stage model is for human analgesic abusers, since carcinogenesis is a complex and dynamic process which is dependant on a great variety of interactions between the cells and carcinogenic agents. Since RPN in man is a pathological condition in which the pathogenetic mechanism which initiates the condition probably has a multifactoral basis (see section 6.1.1 above). Then a pathological condition which develops as a consequence of RPN will probably have a more complex mechanism of
pathogenesis. However, there are a number of factors (e.g., hyperplasia and bacterial infection section 2.2.6) which are common to RPN and UUC in the clinical situation and have also been reported (see below) for experimental studies where animals were fed analgesics.

6.2.1 Role of Urinary Tract Infection in the Development of UUC.

Urinary tract infection (UTI) had been associated with RPN and UUC in man (Eknoyan et al., 1982; Johansson & Wahlqvist, 1977). Experimentally UTI induced by the intravesical inoculation of *Proteus mirabilis* in the Gunn rat has been reported to induce RPN and hyperplasia of the renal pelvis urothelium (Thomas & Tange, 1985). Johansson et al., (1987) also found an increased incidence of upper urothelial tumours in female Sprague-Dawley rats initiated with FANFT and then intravesically inoculated with *E. coli* (06K13H1).

The mechanistic basis for these changes are unclear. They could include bacterial toxins or metabolic products, mechanical effects, enzymic effects of the urothelia, etc. More importantly, bacteria have been reported to be able to produce N-nitrosamines by nitrosylating secondary amines in the bladder or urinary tract (Claude et al., 1988). Secondary amines can result from N-glucoronidated metabolites (such as BBN-Gluc, see section 2.3.5.2.B.i) being excreted, undergoing subsequent hydrolysis to N-hydroxylamines (Kadlubar et al., 1977). These N-hydroxylamines may be metabolised by the bacteria to the N-nitrosamines producing localised initiation in the urothelial cells.

The plasma membranes of urothelial cells from BBN-induced pre-neoplastic hyperplasia have been found to contain more unsaturated fatty acids than normal urothelial cells (Mimata et al., 1987). Thus
precancerous urothelia may be more permeable and allow easier invasion by bacteria. Thus, bacterial infection may set up its own cascade of effects in the urinary tract.

The mucoid glycocalyx of the urothelial cells is a protective layer against bacterial attack. E. coli bind to urinary tract cells because their fimbrae have a number of different adhesin molecules which bind to carbohydrate groups in the mucoid glycocalyx thus enhancing the chance of colonising the urothelial cells (Parkkinen et al, 1988). S-type fimbrae are in particular associated with E. coli that produce pyelonephritis and UTI. A major inhibitor of S-fimbrae adhesins is Tamm-Horsfall glycoprotein (THG) (Parkkinen et al, 1988). The mechanism of this protection relates to the normal turnover of THG as soluble carbohydrates in the urine which block the receptors of the fimbrae adhesins thus inhibiting the binding of the bacteria to the urothelial glycocalyx (Parkkinen et al, 1988). It has been reported that the addition of THG at urinary concentrations (1-10 ug/ml) will interfere with 50 ug/ml of E. coli S-fimbrae colonisation (Parkkinen et al, 1988).

THG is completely lost from the distal nephron in BEA-induced RPN (Bach & Bridges, 1984), thus there is an opportunity for bacteria to attach, colonise and induce UTI as the inhibitory mechanism has been disrupted. This UTI can possibly exacerbate RPN and UUC as described in detail above illustrating a possible link between RPN and UUC and the complexity of the whole pathogenic mechanism.

It is also probable that UTI increases the influx of macrophages into the urothelium to combat the infection. It has been suggested that macrophages have the potential to metabolise endogenous nitroamines
which could initiate urothelial cells, and thus add to the complex cascade resulting from UTI, this concept has been discussed in detail above (see section 5.7.8).

6.2.2 Role for Metabolic Activation via Prostaglandin Hydroperoxidase Synthase Enzymes.

Zenser et al (1985) proposed a mechanism by which the prostaglandin hydroperoxidase (PGH) component of the prostaglandin synthetase enzyme system is important in the mechanism of UUC. The papilla is a region of high PGH activity and several renal toxins (e.g., acetaminophen, p-phenetidine, NPAA) could serve as substrates to peroxidative metabolism to produce reactive intermediates (Anderssen et al, 1983; Zenser et al, 1983a,b; Mohandas et al, 1981a,b, Feldman & Bach, 1985 unpublished data) via a free radical mechanism (Boyd & Elling, 1984). In vitro studies on human urothelial cells have shown that PGH activity is present (Danon et al, 1986) thus the PGH activation of substances may occur within the cells. This may explain how activation of urothelial cells could occur in the experimental situation when there is frank interstitial cell necrosis due to BEA (presumably with a loss of PGH activity). A study by Mimata et al (1987) found that precancerous urothelial cells (initiated with BBN) had a higher arachidonic acid concentration that normal urothelial cells. This increased presence of arachidonic acid may stimulate PGH synthetase enzymes system to induce prostaglandin synthesis. The PGH can also metabolise other substrates to further initiate or promote the precancerous cells to progress to cancer. Additional synthesis of prostaglandins may have a positive feedback effect since PGF$_2$ has been shown to initiate DNA synthesis, proliferation and an early elevation
6.2.3 Role of Oncogenes in UUC.

Weinberg (1988), states that the application of chemical carcinogens induces cancer creating mutant alleles (oncogenes) of pre-existing cellular genes (proto-oncogenes). The most simple scenario is that in which a urothelial cell undergoes point mutation which after clonal proliferation results in a tumour, this occurs in bladder cancer with the Ha-ras oncogene (Reddy et al, 1982). In reality the process is much more complex, generally requiring two or more oncogenes to be activated to achieve full transformation at different steps in the carcinogenic process (Figure 6.2).

![The Development of Malignancy](image)

**Figure 6.2** The multistage nature of cancer. Substances involved in the activation of oncogenes in the initiation, promotion, and progression stages are called mutagens, promoters and progressors respectively. From Spandidos, (1985).
This requirement may provide some explanation as to why carcinogenesis is a multistep process occurring over a long time period in vivo (Weinberg, 1988).

The Ha-ras oncogene is the result of a point mutation where guanine is replaced by thymine. This leads to a single amino acid change where valine is encoded (instead of glycine) and this changes the whole function of the Ha-ras product (Spandidos, 1985). Proto-oncogene products are postulated to play a vital role in critical cellular functions (such as regulation of gene expression, control of cellular differentiation by encoding for protein kinases which affect the growth factor receptors), and because of this have remained unchanges for millions of years. The Ha-ras proto-oncogene that is found in mammalian cells is essentially the same gene as that found in yeast and drosophila (Weinberg, 1988). The p21 protein encoded by the proto-oncogene is a guanosine triphosphatase (GTP-binding) membrane associated protein (Willman & Fenoglio-Preiser, 1987). The activated ras p21 protein product (see Figure 6.3) has an impaired GTPase activity (Spandidos, 1985), the proto-oncogene induces differentiation whereas the Ha-ras oncogene induces dedifferentiation (Peehl & Staney, 1986). The N-nitrosamine induction of tumours support the concept that rapid DNA alkylation and DNA repair are essential for possible early activation of the Ha-ras oncogene, since N-nitrosamines are rapidly metabolised (Montesano et al, 1985). BBN is rapidly metabolised to BCPN and BBN-Gluc within 2 min (Bonfanti et al, 1988) (see section 2.3.5.2.B.i) and BBN also induces Ha-ras oncogenes in the NIH3T3 transfection assay (Fujita et al, 1988). Pre-neoplastic nodular and papillary hyperplasia induced by BBN have been reported to express Ha-ras p21 product (Ward et al, 1988).
Figure 6.3 Hypothetical signal transmission from growth factor to adenylate cyclase through p21 and growth factor receptor. From Spandidos, (1985).

One possible mechanism for the UUC in BBN/BEA model is that the BBN activates the Ha-ras oncogene and the proliferation following BEA-induced RPN results in a clonal expansion of those initiated cells to produce tumours.

6.2.4 Role for Stagnation and Pooling of Urine in Renal Pelvis, Ureter and Bladder

A common feature of BEA-induced RPN is a diuresis (section 2.1.3.2.D.i) and a flaccid bladder and evidence of pooling in the ureters. Even when the concentrating ability has been partly regained such changes persist.

A mechanism for the metabolic activation of bladder carcinogens via the prostaglandin synthetase system (Kadlubar et al, 1977) has been proposed and Antrup et al, (1981) also reported that the urinary human
bladder urothelial cells in culture can metabolize several different classes of chemical carcinogens into their ultimate proximate form. Any urinary tract carcinogens are transported to the urothelium via the urine (Cohen et al, 1983). Urine borne chemicals excreted by kidney could reach toxic levels because of the concentrating mechanism involved in the production of urine. The role of urine in altering urothelial carcinoma has been determined by diverting urine away from the bladder (to the lower intestinal tract, via a uretersigmoidostomy) drastically reduced the frequency of bladder tumours which followed FANFT feeding (Rowland et al, 1980). Studies using a heterotopic bladder (where a donor rat provides the bladder which is transplanted into the gluteal muscle of a recipient) have shown that an induced urothelia does not progress to a malignant lesion if it is filled with saline, but does develop tumours when filled with normal urine (Oyasu et al, 1981). It is then feasible to contemplate a situation where potential carcinogenic metabolites are excreted. Such factors occurring after RPN would increase the probability of metabolic activation by urothelial cells. Potential carcinogenic metabolites of analgesics (eg N-hydroxyphenacetin from phenacetin, Veronese et al, 1985) could be present in sufficient quantities to both initiate and promote the urothelium. However, Figure 6.4 shows how 60-80% of phenacetin is metabolised to paracetamol which in turn is excreted in the urine as glucuronides (55%) and sulphate conjugates (30%) (Clissold, 1986). O-Glucuronides may react directly with the bladder epithelium whereas N-Glucuronides can be hydrolyzed to free N-hydroxyaromatic amines, which can be activated to DNA damaging products (McQueen et al, 1987; Kdllubar et al, 1977). This may have occurred
Figure 6.4 Schematic representation of the metabolic pathways postulated to account for the elimination of therapeutic doses of acetanilide, paracetamol and phenacetin. From Clissold, (1986).

in the BBN/BEA experiments where single analgesics were administered after RPN had been induced and more severe tumours developed early than with BBN/BEA treatment alone (section 5.4.1). The "ghost" papilla can detach and form caliculi within the pelvic space or block the ureter thus causing a stagnation of urine in upper urinary tract. The stagnation of urine in the renal pelvis has been shown to be an important factor in the induction of hyperplasias in the pelvis and ureter using experimental unilateral ureter ligation (Ito et al, 1971). Urine stagnation could occur in RPN due to loss of normal pelvic and ureter peristasis (that normally occurs), sloughing papillae or other cellular debris blocking the ureter etc. Each of
these would produce conditions to enhance UUC induction and several may occur simultaneously. Such pooling of urine may have occurred in the animals with pre-neoplastic papillary hyperplasias such as those observed 13 weeks after BBN/BEA treatment (section 5.2.1), since the papillary hyperplasias may also reduce ureteric urine flow. Greatly enlarged bladders were observed in many of the BBN/BEA animals and some had dilated upper ureters giving some credence to the hypothesis and paralleling the ureter ligation experiments described by Ito et al, (1971). Prostaglandins (which are known to be inhibited by aspirin and NSAID) also have effects on the smooth muscle of gut and bladder (Borda et al, 1983). It is possible they also play a part in the modulating pelvic and ureteral peristalsis. A decrease in synthesis of prostaglandins could stop peristalsis with a resultant stagnation of urine in the upper urinary tract thus increasing exposure time to any urinary carcinogens.

6.2.5 Summary of Possible Mechanistic Basis Linking RPN and UUC.

The most attractive hypothesis to explain the selective targeting of malignant change to upper ureter and pelvis resides in the metabolic activation of endogenous or exogenous compounds which have a short half life in the medulla, but still may activate oncogenes to "initiate" the urothelial cells. Other factors such as loss of mucoid covering, delayed passage of urine would exacerbate any pathophysiological changes initiated by the locally generated reactive metabolites. Many patients with UUC have, however, been exposed to potential urothelial initiating agents by a high incidence of cigarette smoking which has been associated with an increased incidence of urothelial malignancies and carcinomas (McCredie et al,
Diet, alcohol, caffeine consumptions have also been associated with an increased risk of developing urothelial carcinomas (Koch et al, 1986; Kunze et al, 1986; Ross et al, 1989). The data from these investigations suggests a number of possibilities:

1) RPN (or chemicals that cause it) may initiate upper urothelial cells and paracetamol (and presumably other chemicals) may act as promoters.

2) Biochemical changes in the kidney or urothelia after an acutely induced RPN predispose to paracetamol (and/or other factors) acting as total carcinogens.

3) BBN initiation prior to RPN induction may target the toxic effects of paracetamol and other analgesics/NSAID to specific areas of the urothelium which are more susceptible to proliferative changes. This warrants further investigation considering the high levels of consumption in single and mixed analgesic compounds by human analgesic abusers (Pommer et al, 1986; Sandler et al, 1989) who develop RPN and subsequently upper urothelial carcinoma.

The disease condition of RPN and the associated condition of UUC pose a large burden both economic and personnel care on the medical services of the world. Thus the determination of the molecular pathogenesis of RPN and UUC is an important goal.

6.2.6 Possible Relevance of the BBN/BEA Model of UUC to Investigating the Pathogenesis of the Relationship Between UUC and Balkan Endemic Nephropathy.

Balkan endemic nephropathy (BEN) is thought to be caused by fungal toxins, more specifically Ochratoxin A (see section 2.2.2). Patients
with BEN are exposed to mixed mycotoxins. Ochratoxin A which has been reported to cause renal cancer in mice (Kaniwasa & Suzuki, 1978) but not urothelial cancer, but ochratoxin A has been found to be very nephrotoxic in rats (Berndt, 1987). UUC is associated with BEN, but as with UUC and RPN there is as yet no clear evidence for a causal relationship. Patients with BEN also have a very high intake of nitrate- and smoke-cured foods and a high fat diet (personal experience, Rumania 1989!), together with a high alcohol intake. Is the UUC associated with BEN due to urothelial initiation from a variety of environmental sources being "promoted" by an acute nephropathy which targets for the proximal tubule and glomerulus (Delacruz, 1988) The concept of an experimentally induced upper urothelial carcinoma (using the classical two stage initiation-promotion process) now has a valid scientific basis from the studies reported above. This concept lends itself to the investigation of the role that Ochratoxin A plays in a similar malignant condition, using, perhaps, BBN as the initiating agent and assessing the response to an acute or subchronic ochratoxin A-induced lesion. The question does ochratoxin A have the potential to initiate the upper urothelial tract and/or to promote an already intiated urothelium needs to be addressed.

6.3 CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS.

Despite the vast amount of clinical and experimental research over the past 40 years, the mechanism of pathogenesis of analgesic nephropathy is still not fully understood. This lack of knowledge has resulted in an inability to diagnose the condition non-invasively at an early stage in the development in order to effect some therapeutic control.
Currently it appears that legislative measures are the only proven successful means of reducing the numbers of analgesic preparations that are abused and altering their formulations. In those countries where legislation has restricted access of mixed analgesic compounds (Australia, Finland, Sweden, Denmark) there appears to have been a marked decline in the number of patients requiring long-term dialysis as a consequence of analgesic nephropathy (Schwarz, 1987, Kincaid-Smith, 1988). This may not, however, reflect the relatively long latent period before the condition becomes clinically apparent, but merely the failure of the early renal lesion to develop to end stage renal disease. It could also, by the absence of renal failure, be allowing sufficient time for upper urothelial carcinoma to develop.

The International Workshop on the Health Significance and Early Detection of Nephrotoxicity made a consensus recommendation that "responsible authorities and private bodies take the appropriate steps to educate the public with regard to the hazards of the use of analgesics and to reduce access to certain analgesics and prohibit inappropriate combinations" (CEC-IPCS, 1989).

While such a recommendation is sound its implementation is greatly hampered by the failure to identify which analgesic(s) or NSAID(s) has/have the greatest papillotoxic potential, what are the risk factors and what combination of analgesics and NSAID (and/or other therapeutic or non-therapeutic agents) may be inappropriate. Taken in combination with the recently postulated role of caffeine (one of the most widely consumed biologically active compounds) in the pathogenesis of analgesic associated nephropathy, there are still many questions which need to be answered.
In the absence of sensitive and selective non-invasive tests to identify early changes that precede clinically identifiable RPN, little progress can be made in future epidemiological studies. This highlights the future value of animal models until such diagnostic criteria are available. Despite some limitations for the acute and sub-chronic model lesions highlighted in this thesis, they are the most cost-effective way for investigation this complex clinical condition. Once the mechanism of the primary papillary lesion and its secondary consequences are fully understood for these models, it should be relative straightforward to extrapolate many of these findings to the human condition. These animal models should also provide the optimal system in which to compare the papillotoxicity of therapeutic agents and chemicals and identify high risk factors. The use of an animal species such as the pig (which is more closely related to man in terms of renal function and anatomy) may provide the model system of choice for extrapolating experimental research findings in order to compliment clinical and epidemiological studies. In view of the fact that the majority of all past and current toxicological investigations on drugs by the pharmaceutical industries and government agencies were/are performed on rodent species (mainly the rat), the findings of this preliminary investigation in the pig raises the question of how relevant is the data obtained from these rodent studies when applied to nephropathies which affect the papilla. The development of purpose-bred miniature pigs which are of more manageable size for laboratory studies and are commercially available, means that one of the major constraints (one of size and pen space required) limiting the use of pigs in toxicological investigations has been reduced. The recently developed micro-pig,
which grows to a maximum size of 25 kg and has a life span of 10-15 years, has been suggested as being ideal for long-term studies because of its size and ease in handling (Panepinto, 1986). However, a micro-pig costs approximately ten times more than a Large White pig and the current lack of a data base on their response to nephrotoxins probably means that the conventional and miniature strains of pig will continue to be the preferred choice of porcine strains to use in nephrotoxicity studies. Such effort will help make worthwhile advances in the understanding of the pathogenesis of RPN in analgesic abusers.

Priorities for future investigations with a high clinical relevance include development of a diagnostic technique to identify the earliest changes involved in the onset of RPN, establishing the role of caffeine in pathogenesis of the primary lesion or its secondary consequences (since caffeine is common to the majority of proprietary most mixed analgesics, added to most without a proven therapeutic basis) and investigating how new technology, such as magnetic resonance spectroscopy and imaging, and molecular biology can help elucidate the pathogenesis of the initiation of RPN.

6.4 FUTURE RESEARCH BASED ON PROGRESS MADE IN THIS INVESTIGATION.

The focus of drug development and chemical safety on the problem of analgesic/NSAID-induced RPN has resulted in a complacency with regard to the increasing evidence that other chemicals can also cause RPN. While some of these compounds could be amenable to peroxidative activation, others (such as L-triiodothyronine, Kennedy and Jones, 1989) do not fit into this hypothesis easily. The mechanistic basis of such a lesion awaits further investigations, but highlights the
need to be aware that a far broader spectrum of chemicals can cause RPN than are currently accepted or than have previously been documented.

These data suggest that the upper urothelial hyperplasia is an acute response to either the renal papillary necrosis, or its secondary consequences the release of cellular debris into the pelvis or a combination of related factors. This localised proliferative change in the upper urothelia could, therefore, represent an injury that promoted uncontrolled cell proliferation in any cell population that had already been subjected to a genotoxic initiation. These findings may help explain the aetiology of analgesic associated of upper urothelial carcinoma.

Results from studies in BEA-induced RPN in the mouse, marmoset and pig suggest that the lesion is initiated in a similar manner to the rat i.e. the medullary interstitial cells are affected first. This finding raises further questions:

i) by what mechanism are the degenerative changes in the medullary interstitial cell caused?

ii) are the changes in other cell types eg loops of Henle, collecting duct and urothelium a consequence of chemical injury, or a result of the medullary interstitial cell degeneration?

iii) if peroxidative metabolism of papillotoxic chemicals is a key factor in the generation of reactive metabolites (Bach & Bridges, 1985), what is the role of lipid droplets found in medullary interstitial cells?

iv) do the carbohydrate containing (PAS-positive) granules/droplets observed in the collecting ducts prior to necrosis have a role in the
degenerative changes?, what is the relevance of the presence of increasing numbers of such PAS-positive granules in hyperplastic? and neoplastic urothelial cells?

v) are the interstitial matrix alterations an initiating factor or a consequence of interstitial cell necrosis?, do they relate to the PAS-positive granules?

vi) is the differing species response to BEA a result of differences in renal function/metabolism?, or differences in the type of interstitial cells found in the papilla?, and the composition of the lipids within the lipid droplets in those cells? Thus is the apparent resistance of the pig to papillotoxins due to the reported lower concentrations of polyunsaturated fatty acids in these cells which makes them less susceptible to lipid peroxidation?, or is the difference due to other renal/extra-renal factors?

vii) are the differences in interstitial matrix constituents (shown in Table 6.2) of the papilla critical to the ability of each species to cope with an acute RPN lesion?

viii) is it possible to determine a "marker change" in the urine of animals with RPN which could be used clinically to detect patients at risk?

ix) with regard to chronic analgesic dosing after an initiated urothelium, RPN promotion protocol, is it possible to isolate and identify any metabolites which could be the proximate carcinogen(s)?

x) would it be possible to culture tumour cell populations derived from BBN/BEA-induced tumours? and use in vitro techniques to determine some of these questions and reduce the need for continued in vivo
xi) would using a strain of rat such as the DA/Han rat which is genetically disposed to urothelial malignancies produce a higher incidence of tumours?, perhaps just using the BEA-induced lesion!

xii) is there a common biochemical pathway that link the seemingly disparate changes of necrosis (RPN) occurring simultaneously with repair (hyperplasia)?, could mediators such as cAMP, cGMP or Ca$^{2+}$ hold the key? These questions need molecular biology techniques to provide the answers.

xiii) the finding that the immune system (or lack of a complete immune system to be correct) can influence the targetting of toxins so radically that it would be interesting to assess the effects of other papillotoxins and nephrotoxins in general on the Nude mouse kidney. In particular the how does the Nude mouse proximal tubule responds to classical nephrotoxins that target for this region of the nephron eg HgCl$_2$, p-aminophenol, hexachlorobutadiene? Also do all papillotoxins cause such a proximal tubular injury?

The elucidation of each of these questions is the next step in this continuing programme. The use of immunohistochemistry (lectins and/or gold-labelled probes), should provide some answers to these and other important questions.

Immunohistochemistry holds the key to several questions arising from the BBN/BEA model of RPN/UUC. This includes:-

xiv) is it possible to identify preneoplastic changes earlier in the pathogenic sequence?

xv) is it possible to relate these changes to structural or functional (eg oncogene activation) alterations in the cell?, utilising monoclonal antibodies to surface antigens, cyto-keratins, or
lectins to glycoproteins.
xvi) does the identification of why early preneoplastic changes occur, provide a basis for establishing preventative and therapeutic measures?
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Mihatsch, M.J., Torhorst, J., Steinmann, E., Hofer, H., Stickelberger, M.,


APPENDIX A: list of papers submitted and in press.


Experimentally Induced Renal Papillary Necrosis and Upper Urothelial Carcinoma

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I. Introduction

The etiology of renal papillary necrosis (RPN) in humans has been associated with the long-term abuse of analgesics and therapeutic doses of nonsteroidal antiinflammatory drugs (NSAID). However, the lesion has not been clearly defined in terms of the exact causative agent(s), how much (of each) was taken to cause a lesion, and over what period. The primary pathogenesis and the role of other complicating factors are also not clearly understood, nor have the secondary pathophysiological consequences of RPN been adequately interrelated, despite the fact that chronic renal failure and upper urothelial carcinoma are frequently associated with analgesic abuse (Bach and Bridges, 1985).

The understanding of the pathophysiology of a chronically developing renal lesion in humans is a major problem in those conditions where the etiology has been clearly defined, because of the strong likelihood of concurrent and complicating secondary (and unrelated disease) factors. There are important anatomical and functional differences between the kidneys of most animals and humans (Mudge, 1982; Stolte and Alt, 1980). The use of experimental models has generally shown a number of very important clinical and morphological differences; therefore, the use of these models has often limited the understanding of similar conditions in humans.

Although RPN (and upper urothelial carcinoma) are examples of renal disease developing chronically in humans, it has been possible to study a number of chemicals that induce these lesions rapidly in experimental animals. These models (Bach and Hardy, 1985; Bach and Bridges, 1985) all have the important pathophysiological hallmarks of the lesion that has been...
described in humans (Burry, 1968; Burry et al., 1977; Rosner, 1976; Bach and Bridges, 1985). The use of these experimental models has therefore fortuitously provided a way to study the development of papillary necrosis and the progression to a series of renal changes similar to those seen in human analgesic abusers. These models are also allowing the interrelation­ship between the primary lesion and its secondary consequences to be defined in terms of biochemical mechanisms. An understanding of the molecular genesis of this syndrome may be highly relevant to improved clinical management of RPN and upper urothelial carcinoma in humans.

II. Renal Papillary Necrosis and Upper Urothelial Carcinoma in Humans

RPN was first described over 100 years ago (Turner, 1885). It is a lesion that may have a number of different causes (Table I), but most often when encountered in the clinical environment before the 1950s, was due to diabetes mellitus or sickle cell disease (Mandel, 1952). The most frequent cause of RPN since then (and in current clinical experience) is chronic, inappropriate, high-dose analgesic intake, especially the addiction to mixed

<p>| TABLE I |</p>
<table>
<thead>
<tr>
<th>Causes of Renal Papillary Necrosis in Humans</th>
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<tbody>
<tr>
<td>Frequently reported causes</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td>Analgesic abuse</td>
</tr>
<tr>
<td>High-dose nonsteroidal antiinflammatory drug</td>
</tr>
<tr>
<td>therapy</td>
</tr>
<tr>
<td>Upper urinary tract obstructive uropathy</td>
</tr>
<tr>
<td>Recurrent urinary tract infection</td>
</tr>
<tr>
<td>Sickle cell hemoglobinopathy</td>
</tr>
<tr>
<td>Acute pyelonephritis</td>
</tr>
<tr>
<td>Less frequently reported causes</td>
</tr>
<tr>
<td>Dehydrated newborn infants (frequently jaundiced)</td>
</tr>
<tr>
<td>Renal vein thromboses</td>
</tr>
<tr>
<td>Chronic alcoholism</td>
</tr>
<tr>
<td>Severe jaundice</td>
</tr>
<tr>
<td>Calyceal arthritis</td>
</tr>
<tr>
<td>Renal transplant</td>
</tr>
<tr>
<td>Systemic candidosis</td>
</tr>
<tr>
<td>Trauma</td>
</tr>
<tr>
<td>Prolonged hypotension</td>
</tr>
</tbody>
</table>
analgesics over a number of years. Therapeutic doses of NSAID may also induce RPN (Nanra and Kincaid-Smith, 1972; Burry et al., 1977; Prescott, 1979, 1982; Bach and Bridges, 1985).

Initially, all of the mixed analgesics that were associated with the pyelonephritis seen in urology clinics contained phenacetin, and the condition was dubbed "phenacetin kidney" (Spuhler and Zollinger, 1953). Subsequently, however, it became apparent that other analgesics had the potential to cause RPN (Gilman, 1964). The early confusion over the cause of RPN, and the fact that most patients abused, or were prescribed, mixed analgesics and/or a number of different NSAID, also served to obscure case history data that might have provided vital information for the more accurate identification of which analgesics and/or NSAID had the greatest potential to cause the lesion (Cove-Smith and Knapp, 1978; Nanra and Kincaid-Smith, 1975; Nanra et al., 1980). The early failure to realize that phenacetin was not the sole cause of RPN shaped the dogma that resulted in the withdrawal of this drug from the market (Shelley, 1967, 1978). This, it was assumed, would remove the major etiological factor in the genesis of the lesion. When acetaminophen (paracetamol) replaced phenacetin in mixed analgesic preparations the incidence of RPN was expected to drop (Gault et al., 1968; Duggin, 1977; Kincaid-Smith, 1979). The occurrence of the lesion did not, however, decrease in those circumstances where the abuse of mixed analgesics continued (Prescott, 1979, 1982), although some decreases have been attributed to the withdrawal of phenacetin and extensive educational programs to discourage the abuse of mixed analgesics (Wilson and Gault, 1982). A variety of indirect evidence (Table II) has now branded acetaminophen as a very important cause of the lesion, but it is still not clear if there is any scientific foundation for this conclusion. By the time it was realized that most (if not all) mixed analgesics (and many on their own) had the potential to cause RPN (Table III), a great deal of the "phenacetin-containing mixed analgesic" dogma had been established in the medical literature. There is, however, strong evidence from several different sources to suggest that therapeutic doses of NSAID may also cause RPN (Prescott, 1979, 1982; Robertson et al., 1980; Shah et al., 1981; Erwin and Boulton-Jones, 1982; Mitchell et al., 1982; Bach and Bridges, 1985). Based on toxicity data, it is obvious that there are other chemical substances (Table III) that have the potential to cause RPN in animals. Many of these chemicals have industrial uses, and some are persistent environmental contaminants. Clinical situations rarely (if ever) focus on these possible environmental causes, or on the potential for substances other than analgesics and NSAID to contribute to RPN in humans.

There is good clinical evidence to show that patients who continue to abuse analgesics (after the condition is diagnosed), have a very poor
Phenacetin dogma—the belief that phenacetin was the only etiological factor in the genesis of papillary necrosis
Phenacetin is metabolized to acetaminophen
Acetaminophen replaced phenacetin in mixed analgesics, and the incidence of pyelonephritis did not decrease
Urinary acetaminophen was used as a measure of phenacetin abuse in patients known to be taking mixed analgesics
Acetaminophen and aspirin are concentrated in the medulla, but phenacetin is not
The genesis of acute hepatic and proximal tubular necrosis following large doses of acetaminophen has been widely studied, and the conclusions extrapolated to a chronic lesion in the medulla

prognosis and rapidly develop end-stage renal disease (Nanra and Kincaid-Smith, 1972; Kingsley et al., 1972; Murray and Goldberg, 1975; Burry et al., 1977; Cove-Smith and Knapp, 1978), while patients who discontinue the abuse of the offending drugs tend to stabilize, or show improved renal function (Bell et al., 1969; Dubach et al., 1978, 1983). It is these patients, however, who may be at risk of developing upper urothelial carcinoma. There is a very high incidence of epidemiologically associated upper urothelial carcinoma in those countries such as Scandinavia, Switzerland, and Australia where RPN has a high clinical prevalence (Bengtsson et al., 1968, 1978; Dubach et al., 1971; Johansson et al., 1974, 1976; Mihatsch et al., 1979, 1980a−c, 1982a−c; Mahony et al., 1977; McCredie et al., 1982a,b, 1983). There is, however, no proved cause–effect relationship between RPN and upper urothelial carcinoma (Bach and Bridges, 1985).

The diagnosis of RPN (Gault et al., 1968; Duggin, 1977, 1980; Kincaid-Smith, 1979; Bach and Bridges, 1985) and of upper urothelial carcinoma (Bengtsson et al., 1968, 1978; Dubach et al., 1971; Johansson et al., 1974, 1976; Mihatsch et al., 1979, 1980a−c, 1982a−c; Mahony et al., 1977; McCredie et al., 1982a,b, 1983; Bach and Bridges, 1985) is most difficult in the clinical situation, and both progress silently. One early clinical sign of analgesic nephropathy is the loss of urine-concentrating capacity (Bengtsson, 1962; Dubach et al., 1975; Nanra et al., 1978; Nanra, 1980). Polyuria may, however, be a consequence of several nephropathies, and
TABLE III
ANALGESICS, NSAID, AND OTHER DRUGS AND CHEMICALS WITH PAPILLOTOXIC EFFECTS*

<table>
<thead>
<tr>
<th>Drug</th>
<th>Drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaminophen</td>
<td>Ibuprofen</td>
</tr>
<tr>
<td>Aclofenac</td>
<td>Indomethacin</td>
</tr>
<tr>
<td>Aminopyrine</td>
<td>4-Isopropylbiphenyl</td>
</tr>
<tr>
<td>5-Aminosalicylic acid</td>
<td>Ketoprofen</td>
</tr>
<tr>
<td>Antipyrine</td>
<td>Ketoprofen</td>
</tr>
<tr>
<td>Aspirin</td>
<td>Meclofenamic acid</td>
</tr>
<tr>
<td>Aspirin, phenacetin, and codeine</td>
<td>Mefanamic acid</td>
</tr>
<tr>
<td>Aspirin, phenacetin, and caffeine</td>
<td>Mono-N-methyleneimine</td>
</tr>
<tr>
<td>2-Bromoethanamine hydrobromide</td>
<td>Naproxen</td>
</tr>
<tr>
<td>3-Bromopropane hydrobromide</td>
<td>Niflumic acid</td>
</tr>
<tr>
<td>Buckoxic acid</td>
<td>Oxynphenbutazone</td>
</tr>
<tr>
<td>2-Chloroethanamine hydrochloride</td>
<td>Phenacetin</td>
</tr>
<tr>
<td>2-Chloro-N,N-dimethylethanamine</td>
<td>Phenothiazine</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>Phenylalkanoic acid</td>
</tr>
<tr>
<td>Dapsone</td>
<td>N-Phenylantranilic acid</td>
</tr>
<tr>
<td>Diphenyl</td>
<td>Phenylbutazone</td>
</tr>
<tr>
<td>Diphenylamine</td>
<td>Propyleneimine</td>
</tr>
<tr>
<td>Diphenylmethyl alcohol</td>
<td>Sudoxicam</td>
</tr>
<tr>
<td>Ethyleneimine</td>
<td>Sulfinpyrazone</td>
</tr>
<tr>
<td>Fenoprofen</td>
<td>Tetrahydroxyquinoline</td>
</tr>
<tr>
<td>Flufenamic acid</td>
<td>Tolmetin</td>
</tr>
<tr>
<td>Gla phenine</td>
<td></td>
</tr>
</tbody>
</table>

*Full references in Bach and Bridges (1985).

loss of the concentrating mechanism may have a number of renal and extrarenal causes. RPN is also associated with electrolyte disturbances. Cove-Smith and Knapp (1978) reported a high incidence of sodium wastage, and Jaeger et al., (1982) showed that patients were hypocalcemic as a result of a urinary Ca\(^{2+}\) loss. Patients with analgesic nephropathy have a pronounced defect in the urinary acidification mechanism following NH\(_4\)Cl administration (Bengsson, 1962; Steele et al., 1969; Krishnaswamy et al., 1976; Nanra et al., 1978; Nanra, 1980), suggesting that damage to the medulla might be synonymous with loss of effective urinary acidification and altered electrolyte balance. Other classical clinical biochemical parameters used to diagnose renal disease only identify incipient renal failure, by which time papillary necrosis has long since occurred and the secondary degenerative changes that follow this lesion have progressed toward end-stage renal disease. There are few telltale clinical symptoms (Table IV), none of which are pathognomonic of the condition. Degenerative renal changes may be identified by radiology (Lindvall, 1978), but these are essentially indicative of an advanced lesion, and they may miss early, but
TABLE IV
CLINICAL FEATURES ASSOCIATED WITH RENAL PAPILLARY NECROSIS

<table>
<thead>
<tr>
<th>Early symptoms</th>
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</thead>
<tbody>
<tr>
<td>Female predominance 3:1 to 8:1</td>
</tr>
<tr>
<td>Psychiatric dependence, emotional instability,</td>
</tr>
<tr>
<td>and anxiety</td>
</tr>
<tr>
<td>Upper gastrointestinal disease</td>
</tr>
<tr>
<td>Anemia</td>
</tr>
<tr>
<td>Intermediate symptoms</td>
</tr>
<tr>
<td>Bacteruria, sterile pyuria, nocturia, dysuria,</td>
</tr>
<tr>
<td>microscopic hematuria, ureteral colic, and</td>
</tr>
<tr>
<td>lower back pains</td>
</tr>
<tr>
<td>Defective concentration and acidification of</td>
</tr>
<tr>
<td>urine, proteinuria, nocturia, and azotemia</td>
</tr>
<tr>
<td>Late symptoms</td>
</tr>
<tr>
<td>Hypertension, ischemic heart disease, and</td>
</tr>
<tr>
<td>peripheral vascular disease</td>
</tr>
<tr>
<td>Calculi and bladder stones</td>
</tr>
<tr>
<td>Decreased glomerular filtration rate, increased</td>
</tr>
<tr>
<td>blood urea nitrogen, renal tubular acidosis, and</td>
</tr>
<tr>
<td>end-stage renal disease</td>
</tr>
<tr>
<td>Upper urothelial carcinoma</td>
</tr>
</tbody>
</table>

frank RPN. The most dependable method of assessing analgesic-related disease is by detailed patient histories, but the stigma of analgesic abuse normally leads to patients giving unreliable or misleading data on their drug usage (Murray, 1974, 1978). Similarly, it is difficult to diagnose upper urothelial carcinoma unless cytology and other diagnostic procedures such as computerized tomography (Gatewood et al., 1982) are applied. The knowledge that a patient has been an analgesic abuser can provide a basis for routine cytological monitoring, but this is rarely carried out even in situations where the incidence of the disease is high (Jackson et al., 1978). The prognosis for the patients with upper urothelial carcinoma is poor, due to the advanced stage of renal parenchymal disease and widespread metastases (Hultengren et al., 1965; Mihatsch and Knusli, 1982) when the condition is first diagnosed.

III. Experimentally Induced Renal Papillary Necrosis

Early attempts to study RPN experimentally using analgesics and NSAID have been plagued with irreproducible experiments and conflicting data, so much so that Rosner (1976) was of the opinion that analgesic-associated
RPN was a lesion peculiar to humans and that animals were remarkably resistant to this type of pathology. Analgesics, NSAID, and a variety of other drugs and chemicals can, in fact, be used to induce RPN experimentally. The objectives of this article are to review briefly the different animal models of RPN that may be useful for the experimental pathologists, and highlight their advantages and limitations. The focus of this article will be on the use of chemicals that induce RPN acutely or subacutely, because these compounds (particularly if they affect the kidney only) provide a most useful way of studying the pathogenesis of RPN. Many of the histological changes that we have studied have been based on the use of high-resolution light microscopy, where semithin sections of glycolmethacrylate-embedded tissue has been assessed by a number of conventional histochemical methods. In addition, several enzyme histochemical methods have also been used to study changes that originate in the proximal tubule, the urothelial cells, and the endothelial cells. Our own interest has been in the application of a multidisciplinary approach to elucidating the biochemical mechanisms of RPN and its related changes such as chronic renal failure and upper urothelial carcinoma.

A. **Spontaneous and Experimentally Manipulated Models of Renal Papillary Necrosis**

RPN occurs in animals as a result of a variety of other conditions. These include age (Gorer, 1940) and amyloid-related (Dunn, 1944; Cornelius, 1970) changes in mice, and changes that are a consequence of medullary bilirubin deposition (and perhaps other biochemical effects) in the Gunn rat (Gomba *et al.*, 1973; Call and Tisher, 1975; Henry and Tange, 1982; Axelsen and Burry, 1972; Axelsen, 1973). In addition, systemic candidosis also causes necrosis of the medulla (Adriano and Schwarz, 1955; Hurley and Winner, 1963; Knepshield *et al.*, 1968; Tomaszewski and Abromowsky, 1981). It has previously been reported that vascular occlusion (Muirhead *et al.*, 1950; Sheehan and Davis, 1959a,b; Baum *et al.*, 1969; Beswick and Schatzki, 1960), ureteral obstruction (Sheehan and Davis, 1959b; Dziukas *et al.*, 1982), and the injection of heterologous serum into rats (Patrick *et al.*, 1964; Kroe and Klavins, 1965; Wizgird *et al.*, 1965; Ljungqvist and Richardson, 1966; Ljungqvist *et al.*, 1967; Gullbring *et al.*, 1966) also cause RPN. Critical analysis of these data (Bach and Bridges, 1985), however, suggests that the medullary infarct associated with all of these methods differs from the chemically induced RPN, and is more comparable to the "warm ischemic" renal lesion (Mason and Thiel, 1982; Wolgast *et al.*, 1982). It would still be valuable for these lesions to be more fully studied by
histochemical methods, at both the light and ultrastructural levels, to establish the nature of the changes, and where they may be similar to the chemically induced lesion. The long-term feeding of rats with a diet deficient in essential fatty acids (Burr and Burr, 1929, 1930; Borland and Jackson, 1931; Molland, 1982) also causes RPN, but the widespread degenerative changes in many of the major organs makes this a most complex experimental model.

B. ANALGESIC AND NONSTEROIDAL ANTIINFLAMMATORY-INDUCED RENAL PAPILLARY NECROSIS

Attempts to induce the RPN using analgesics or NSAID have proved to be difficult (Rosner, 1976). A number of researchers have produced the lesion with mixed analgesics (see Rosner, 1976; Macklin and Szot, 1980), single constituents such as amidopyrine (Brown and Hardy, 1968), acetaminophen (Macklin and Szot, 1980; Furman et al., 1976, 1981), and aspirin (Molland, 1976), and a variety of NSAID, including phenylbutazone and indomethacin (Arnold et al., 1974; Burnett, 1982; Bokelman et al., 1971). Many “second-generation” NSAID also have the potential to cause RPN (Table III).

RPN has been most difficult to study because the renal medulla is inaccessible to investigation, is not well defined biochemically, and consists of a heterogeneous array of cell types. Several problems associated with studying this lesion have been reviewed in detail (Bach and Bridges, 1985; Bach and Hardy, 1985). Three experimental considerations are most essential to the design and interpretation of all investigations into the mechanism of renal papillary necrosis and upper urothelial carcinoma and therefore warrant repeating.

1. There have been no definitive noninvasive criteria by which to diagnose experimentally induced RPN. Polyuria represents one of the early renal functional changes which precede RPN in experimental animals given repeated doses of analgesics (Angervall and Bengtsson, 1968; Brown and Hardy, 1968; Nanra, 1980), NSAID (Booth et al., 1961), and NSAID analogs (Hardy, 1970a,b, 1974), and those chemical probes that cause RPN acutely (see below). Loss of urinary concentrating ability is not, however, a specific functional change peculiar to RPN, but it also accompanies many other renal and extrarenal changes (Berndt, 1975; Piperno, 1981). Similarly, enzymuria (Ellis and Price, 1975; Halman et al., 1986) has been studied in the acutely induced RPN, but this fails to define the location and extent of a renal lesion. It is only once the renal cortex shows degenerative changes that changes are observed in the usual clinical parameters of renal function, such as blood urea nitrogen and serum creatinine. There are therefore no routine clinical biochemical parameters that are pathogeno-
monic of the lesion even under the most stringently controlled experimental conditions in laboratory animals, and the identification of this silent lesion is dependent on recourse to histopathology.

2. Several analgesics and NSAID (and other chemicals) cause an apex-limited RPN that can be easily missed if painstaking sectioning is not undertaken through this region to ensure that a focal lesion is not missed (Fig. 1). All histology should therefore include the papilla tip or the ducts of Bellini to ensure that this important technical prerequisite has been met.

3. The appropriate choice of species can profoundly affect the course of a chemically induced lesion. It has been suggested that the rat is particularly susceptible to papillotoxic chemicals, because of the highly concentrated urine that they produce (Consensus Conference, 1984). This is, however, unproven (Bach and Hardy, 1985), and there is a paucity of published comparative data to establish if any species or strain is most appropriate; however, there are well-defined but subtle differences between rat strains (Bach and Hardy, 1985; Bach and Bridges, 1985). More importantly, the rat is very sensitive to the ulcerogenic effects of analgesics and especially the NSAID. Thus, it is not uncommon for rats to die from gastric perforation before frank renal lesions are apparent (Kaump, 1966). In addition, several of the chemicals with papillotoxic potential also cause discrete cortical lesions when given to rats at the dose regimens commonly used. There are, however, also instances where rats have proved to be particularly resistant to the papillotoxic effect of analgesics and NSAID (Rosner, 1976) for reasons that are still not understood (Bach and Bridges, 1985). When RPN has been successfully induced, the intensity of the lesion at each different time point varies from gross (with marked advanced cortical degeneration), to mild and focal, and often there are also animals in which no lesion has been found at the end of a long-term study. Based on this variability, it has been difficult to assign either time courses or dose–response relationships to pathological change when the lesion is induced chronically.

In summary, most analgesics and NSAID have been implicated as causing RPN in the animals, but many of the chemicals have not proved to be useful for inducing the lesion experimentally.

The use of those therapeutic compounds that have been implicated in the induction of RPN in humans (Table III) has not, in general, proved to be useful in inducing papillary damage in animal models. Most of the analgesics and NSAID have at one time or another been reported to cause RPN in several different species, but these have not provided robust systems for studying the time course of RPN and interrelating the different morphological changes that take place. Many of these compounds cause marked extrarenal toxicity and have an ulcerogenic potential far greater than the
Fig. 1. Transverse semithin kidney section including papilla tip and mouth of ureter. Bar, 1 mm. [From Bach and Bridges (1985).]
nephrotoxic effects (Kaump, 1966). Some of these drugs and their metabo­
lites also have marked toxic effects on the proximal tubule (Green et al.,
1969; Calder et al., 1971; Crowe et al., 1979; Newton et al., 1982, 1983a,b).
While this may be relevant to the clinical situation, overt cortical damage
has not been a prominent feature of RPN in human analgesic abusers. Thus,
there is a complicating factor that obfuscates the study of a primary
medullary lesion if these compounds are used experimentally. More
importantly, there are a number of inadequately identified variables that
have meant that successive sets of experiments may not be reproducible.
For example, whereas Molland (1976) showed that aspirin caused RPN in
hooded rats, there are reports for other species and strains that contradict
this toxic effect (Rosner, 1976). Some of the problems associated with using
analgesics and NSAID to induce RPN in experimental animals have been
reviewed (Bach and Hardy, 1985). In general, the use of analgesics and
NSAID has served to confuse rather than to clarify the pathogenesis of
chemically induced RPN. There are, however, a number of chemical
“probes” that target very selectively for the medulla, and provide model
systems that are preferable for studying the development of RPN and its
secondary sequelae.

C. Nontherapeutic Chemical Probes for Inducing Renal
Papillary Necrosis

The ethos of many studies in experimental pathology has been to use
model toxic agents to induce rapidly a lesion of interest. The advantages of
inducing lesions over a short time course greatly outweigh the study of
chronic lesions (where other factors may obscure the cascade of pathologi­
cal changes), but there is always the question of validity in extrapolating
data from an acute animal model to a chronic lesion that develops in
humans. Despite these limitations, most of our understanding on the
biochemical mechanisms of carcinogenesis and other toxic lesions in the
major organ systems has been built up using this approach.

IV. Use of Model Papillotoxic Probes to Study
the Pathogenesis and Secondary Development
of Renal Papillary Necrosis

The difficulties that have pervaded the use of therapeutically used
compounds for inducing RPN have largely been overcome by the admin­
istration of papillotoxins that are chemically unrelated to the analgesics and
There are also several NSAID analogs that have very little ulcerogenic effect, and have therefore contributed to our understanding of the pathogenesis of RPN.

A. Ethyleneimine-Induced Renal Papillary Necrosis

The papillotoxicity of ethyleneimine, first described by Levaditi (1901), has been used to study various aspects of RPN (Mandel and Popper, 1951; Davies, 1967, 1968, 1970; Davies et al., 1968; Ham and Tange, 1969; Sherwood et al., 1971; Ellis et al., 1973; Ellis and Price, 1975; Axelsen, 1978a). Ethyleneimine caused a dose-related necrosis (Axelsen, 1978a) that first affected the interstitial cells of the papilla tip, and then other "fine" anatomical elements of the medulla (Ham and Tange, 1969). At subsequent time points (or with higher doses), secondary cortical degenerative changes developed (Davies, 1967, 1968). Using colloidal carbon as a contrasting agent, the microvasculature was shown to be patent up to and beyond the time that necrosis developed (Ham and Tange, 1969). The functional changes associated with the ethyleneimine-induced lesion included marked polyuria, low specific gravity urine, and enzymuria (Mandel and Popper, 1951; Ellis et al., 1973; Ellis and Price, 1975).

There are, however, a number of problems with the use of ethyleneimine as a model papillotoxin. The compound is a powerful alkylating agent and a proved mutagen; it is chemically unstable and may also be explosive (Dermer and Ham, 1969), and it is no longer commercially available. Thus, over the past decade, the use of ethyleneimine as a chemical probe for inducing RPN acutely has declined dramatically.

B. 2-Bromoethanamine Hydrobromide-Induced Renal Papillary Necrosis

2-Bromoethanamine (BEA) hydrobromide has largely replaced ethyleneimine as the model papillotoxin. First shown to cause RPN by Oka (1913), this compound has a number of advantages over ethyleneimine. BEA is commercially available; it is a stable, water-soluble crystalline material, although it is unstable in solution. The BEA-induced lesion is dose related and relatively predictable in its intensity for any dose range in the rat (Bach et al., 1983), and has been characterized in terms of over 35 publications on different renal morphological and functional changes (see Bach and Bridges, 1985, for full reference list). It must, however, be stressed that BEA does cyclize to ethyleneimine in vitro under strong alkali conditions (Dermer and Ham, 1969), and this has been proposed as the mechanism of BEA-induced RPN (Murray et al., 1972). There is, however,
no evidence to show that ethyleneimine is excreted in urine following the administration of BEA to rodents (P. H. Bach, unpublished data), although this does not preclude the localized formation of the unstable alkylating molecule extrarenally or in the papilla.

1. **Morphological Changes**

A single 50 mg/kg dose given ip causes RPN acutely in rats (Wyllie et al., 1972; Shimamura, 1972; Bach and Bridges, 1982; Bach et al., 1983; Gregg et al., 1988a,b) and mice (P. H. Bach and N. J. Gregg, unpublished), and higher doses cause a lesion (Fig. 2A) up to but not beyond the cor-

![Image](image_url)

**Fig. 2.** (A) BEA-induced RPN lesion (100 mg/kg ip after 48 hours) showing limit of necrosis affecting the matrix staining. Giemsa staining, ×4. (B) Medullary interstitial cell necrosis at papilla tip 4 hours after a single ip dose of 100 mg/kg BEA showing pyknotic irregular nuclei (arrowheads). Giemsa, 1-μm resin section, ×100. (C) Dilation of distal and proximal tubules 48 hours after a single 100 mg/kg ip dose of BEA. Alkaline phosphatase, ×20. (D) Regenerative zone between viable and necrotic tissue in papilla 48 hours after a single ip dose of 100 mg/kg BEA. Note mitotic figures in collecting duct (arrow) and loops of Henle (arrowhead). Giemsa, 1-μm resin section, ×40. (E) Adhesion of platelets to endothelia in area of interstitial cell necrosis, 8 hours after a single ip dose of 100 mg/kg BEA. Giemsa, 1-μm resin section, ×100.
ticomedullary junction (Bach et al., 1983). Lower doses of BEA do not cause any easily identifiable lesion, and repeated high doses do not exacerbate the degree of RPN. The morphological changes associated with the time course development of BEA-induced RPN have been described in detail elsewhere (Wyllie et al., 1972; Hill et al., 1972; Bach et al., 1983; Gregg et al., 1988a,b) and will only be outlined in brief. Early hydropic changes developed in the proximal tubule 4–6 hours after BEA administration, but these had reverted to normal by 8–12 hours. Within 4 hours of BEA dosing there was a significant collecting duct dilatation which lasted for 24–48 hours.

Medullary interstitial cells had irregular nuclei at 4 hours and lost their cytoplasmic integrity by 8 hours; necrosis spread from the papilla tip to the corticomedullary junction from 12 hours (Fig. 2B). Collecting duct epithelia (and other areas of the distal nephron) showed degenerative changes at 12 hours and cell exfoliation at 18 hours. Cortical changes were confined to PAS-positive casts in the collecting duct and loop of Henle from 8 hours and dilatation of distal and proximal tubules at 8 and 72 hours, respectively (Fig. 2C). There was active repair at the junction between viable tissue and the necrotic papilla from 24 hours with mitoses in the collecting ducts and loops of Henle (Fig. 2D).

Necrotic changes developed as early as 12 hours and had run their course by 24–48 hours. The earliest degenerative changes following low (50 mg/kg) doses of BEA consistently affected the medullary interstitial cells, and the loops of Henle and the microvasculature were damaged later. The urothelial cells covering the papilla and the collecting ducts were left intact with minimal signs of injury. The kidneys taken from animals given higher doses of BEA showed similar early changes, but these were also associated with subsequent total necrosis which included loss of all of the cellular elements which make up the inner medulla.

Eosinophilic casts were present in the collecting duct from 24 hours, at which time reparative changes were evident at the interface between necrosed and normal areas. There was distal tubular dilatation from 8 hours, but this occurred in the proximal tubules after 72 hours. Endothelial platelet adhesion was first noticeable at 8 hours, was very marked at 18 hours, and continued up to 144 hours; but only the capillaries in necrotic regions were affected, and not those in other parts of the kidney or urothelial tract (Fig. 2E).

2. Histochemical Changes

Normally, the renal medullary matrix stains strongly with colloidal iron, Toluidine Blue, and Giemsa (Bach et al., 1983; Gregg et al., 1988a,b).
Following BEA administration there were marked changes in the medullary matrix staining. The earliest changes were an increased staining intensity and a granular appearance around the interstitial cells at the papilla tip 2–4 hours after BEA dosing. The increased staining became diffuse after 8–12 hours, and was progressively lost from those areas where necrotic changes were taking place between 12 and 24 hours. The necrosed areas had totally lost the histochemical staining of the matrix from 24–48 hours (Fig. 3).

There was also an increase in PAS-positive material at the tip of the papilla 4–6 hours after BEA, which increased to a maximum at 48 hours, but at this stage the PAS staining in the mid-medulla was decreased. Even when there was reepithelialization of the affected area the mucopolysaccharide matrix was not reestablished, probably due to the absence of medullary interstitial cells.

Changes in the matrix staining have also been associated with RPN in humans, where both increases (Burry et al., 1977; Burry, 1978) and decreases (Gloor, 1978) have been reported. It is tempting to suggest that these are similar to the early and late changes in the acutely induced BEA model. Rats given aspirin chronically also developed RPN and a dense fibrillary network of PAS-positive material, which became irregular with more deeply PAS-staining fibers and bodies in the interstitium (Molland, 1978). Recently, these histochemical changes have been confirmed bio-

![Fig. 3. Necrotic papilla 48 hours after 100 mg/kg BEA, showing loss of matrix staining in extreme tip where tissue integrity has been lost. Giemsa, 1-μm resin section, ×3.2.](image-url)
chemically as demonstrated by the loss of radiolabeled and covalently bound sulfate from the medulla following BEA administration; in addition, there was a marked perturbation of urinary proteoglycans and glycosaminoglycans (Bach et al., 1988a).

3. Distal Tubular Changes

Tamm–Horsfall glycoprotein (THG) is produced by the ascending thick limbs of the loop of Henle and lines the epithelium of that segment and the distal tubule, where it is thought to prevent water reabsorption but still to facilitate Na⁺ transport (Lewis et al., 1972). It forms the basic matrix material for tubular casts.

THG staining remained unchanged for 6–8 hours after BEA administration, but during the development of the papillary necrosis this glycoprotein was lost from the distal nephron (Bach et al., 1988b), and small casts were found in the collecting ducts. From 12 hours there were more frequent and marked deposits of heavily stained intraluminal material in the inner medullary collecting ducts, some of which appeared to form aggregates against the epithelial cell walls. Only later, when the medullary mucopolysaccharide staining had been lost, were large casts of THG-positive material deposited in the collecting ducts and ducts of Bellini (Fig. 4), where they were associated with cellular debris (Bach et al., 1988b). The nephrons that appear to feed blocked collecting ducts were generally dilated. Tubular dilatation became more marked at 24 hours, when there were THG-positive casts in the ducts of Bellini. These cast-filled ducts appeared to drain those regions of the cortical nephron where tubular dilatation was most marked. Between 24 and 123 hours the cortical staining pattern was essentially unchanged, but there was more extensive tubular dilatation. The number of THG-positive casts—containing significant quantities of cellular debris—increased, and THG staining in the distal nephrons decreased. Some of the THG-positive material was also extravasated (Bach et al., 1988b). Many of the superficial glomeruli thus affected have THG-positive material in Bowman's space; this finding may be related to glomerular sclerosis (Arruda et al., 1979; Sabatini et al., 1982, 1983) that developed after some weeks. The most marked cystic dilatation of cortical nephrons were associated with the most extensive deposits of THG in the ducts of Bellini, and there were also deposits of THG-positive material around the glomeruli (in Bowman's spaces) of the superficial nephrons, following high-dose BEA.

Perturbation of THG distribution does not appear to play a primary role in the development of RPN, but may be important in the pathogenesis of the related polyuria and the secondary tubular changes that follow the BEA-induced lesion.
4. Enzyme Histochemical Changes in the Proximal Tubule and the Suburothelial Capillaries

The staining of a number of enzyme markers has been monitored during the development of a BEA-induced RPN. There were no changes in the proximal tubular marker enzymes alkaline phosphatase, $\gamma$-glutamyl transpeptidase (GGT), and adenosine triphosphatase (ATPase) before 8 hours, from which time there was a time-related progressive loss of staining up to 144 hours, when GGT was almost undetectable (Fig. 5A). Alkaline phosphatase and GGT (from 12 hours) and ATPase (from 18 hours) staining material occurred in the proteinaceous, PAS-positive casts in the loops of Henle and the collecting ducts (Fig. 5B). Lysosomal acid phospha-
tase staining was increased in the pelvic urothelial cells at 12 hours and in the proximal tubules from 12 hours, up to 48 hours.

There was a marked increase in the staining of the pelvic, ureter, and bladder endothelial alkaline phosphatase, and especially ATPase, at 12 hours. The intensity and area of microvascular ATPase staining increased progressively in these regions from 18 hours, and by 144 hours the capillary lumens were almost occluded in the worst affected areas (Fig. 5C). Capillary sclerosis has been described in the kidneys of human analgesic abusers (Mihatsch et al., 1978, 1984) and is thought to be a specific change which has not been described in any other types of renal disease.

5. Lipid Histochemical Changes

The medullary interstitial cells have a very high lipogenic potential and contain numerous lipid droplets rich in long-chain polyunsaturated fatty
acids (Bojesen, 1974). Oil Red “O” (ORO) stains the lipid droplets in these cells heavily, but not other parts of the kidney (Bach et al., 1988c). ORO-positive lipid material accumulates in kidneys of analgesic abusers (Munck et al., 1970; Burry et al., 1977; Burry, 1978), and similar changes occur in aspirin-induced (Molland, 1976) and essential fatty acid-deficient diet-induced RPN (Molland, 1982). Recent studies have shown that in an acutely induced papillary necrosis, early lipid changes take place in the capillaries, followed by a marked accumulation (Fig. 6) of lipid in the epithelial cells. Normally there is no ORO-positive lipid material in these cells. The epithelial accumulation of lipid material extends into those areas of the outer medulla which were not affected by the papillotoxin and appeared to be normal by routine hematoxylin and eosin staining (Bach et al., 1988c). Other chemically induced lesions, such as those caused by hexachlorobutadiene, aminoglycosides, cis-platin, and polybrene, do not produce these ORO lipid changes (Bach et al., 1988c), which suggests that the capillary and epithelial deposits of lipid material may be pathognomonic of RPN.

Biochemically, the lipid changes in the BEA-induced RPN represent a phospholipidosis, in which phosphatidyl- and lysophosphatidyl choline, -inositol, and -ethanolamine were increased (M. J. Duffy and P. H. Bach, unpublished data). The increase in urinary free polyunsaturated fatty acids

![Image](https://example.com/image.png)

**FIG. 6.** Lipid droplet accumulation in papilla covering epithelium cells following single ip dose of 100 mg/kg BEA. ORO stain, fixed frozen section, ×32.
with \( C_{18}, C_{20} \) and \( C_{22} \) suggests a large precursor pool for the synthesis of eicosanoid-related products (D. J. Scholey and P. H. Bach, unpublished data).

6. Microvascular Changes

The subtle control of kidney microvasculature and the shunting of blood to (or from) different regions of the medulla and cortex represent a most fundamental process in normal renal function. This may be altered in nephrotoxic lesions that have been linked to ischemic injury. The introduction of exogenous particulate material into the renal microvasculature gives some indication of the patency of the vessels or the presence of occlusion. Colloidal carbon has been used to show the loss of medullary microvascular filling at an advanced stage of ethyleneimine- and aspirin-induced RPN. The introduction of this foreign particulate material for assessing vascular filling may, however, present some difficulties. While India ink has been used as the common source of colloidal carbon, it contains a variety of additives, including phenols and adhesives (Vernon-Booth, 1972), to enhance its drawing properties. In addition, the colloidal nature of this material also imparts a substantial oncotic pressure. Both of these problems (which may cause artifacts in assessing microvascular filling), can be overcome by using India ink that has been dialyzed against isotonic saline. Colloidal carbon prepared thus has been used to follow the time course of microvascular changes in animals treated with BEA. There was an early shift of microvascular filling from the cortex to the outer medulla (2–4 hours after dosing), and later (at 8–26 hours) the filling of the inner medulla was more pronounced (Fig. 7A), but at the expense of the microperfusion of the outer part of the medullary plexus. These changes coincided with the development of RPN. By 48 hours, when necrosis was complete, the damaged medulla was virtually avascular. During the course of development of RPN, however, the microvasculature was patent in the medullary tissue beyond the regions in which necrosis had occurred (Fig. 7B). These data were interpreted as showing that an acute medullary necrosis can occur without capillary occlusion (Bach et al., 1983). These observations have also been confirmed by high-resolution microscopy, where platelet adherence and microvascular changes did not occur until late in the development of RPN.

The colloidal carbon method cannot identify “leaky” capillaries, an endothelial defect that could play a very important role in disrupting renal compartmentalization. Monastral Blue B is a water-insoluble, nontoxic colloid; it has an uniform size distribution, high contrast for thin and thick sections, an obvious appearance under electron microscopy, and is com-
Fig. 7. (A) Microvascular distribution of colloidal carbon in the corticomedullary regions of (top) control and (bottom) BEA-treated rat kidneys (50 mg/kg at 2 hours). Unstained 100-μm colloidalin section, ×4. [From Bach et al. (1983).] (B) Aggregates of colloidal carbon at the tip of a necrosed papilla (arrows) 26 hours after 50 mg/kg BEA. Hematoxylin-eosin, ×25. [From Bach et al. (1983).]
mercially available (Joris et al., 1982). Recently, this colloid has been used to assess microvascular integrity in the genesis of RPN, where semithin methacrylate sections showed the vascular labeling of glomeruli and capillaries in the pelvic basal epithelia. No Monastral Blue B was present in the papillary matrix (Fig. 8), suggesting that changes in capillary integrity (and the leakage of material into the interstitium) were not involved in the pathogenesis of RPN (Gregg et al., 1988b).

7. Interaction between 2-Bromoethanamine and Analgesic and Nonsteroidal Antiinflammatory Drugs

It has been generally assumed that the mixed analgesics are more papillotoxic than the individual components singly (Nanra and Kincaid-
Smith, 1972; Duggin, 1977), but there has been very little experimental evidence to substantiate chemical interactions exacerbating RPN. Recently, this question has been addressed using the interactions between a subthreshold dose of BEA < 25–35 mg/kg ip, which does not normally cause a discernible lesion), and various analgesics (Bach et al., 1988b). These investigations were undertaken in an attempt to develop a short-term screening method for evaluating and comparing the papillotoxic potential of analgesics and other compounds. Whereas two doses of aspirin and acetaminophen, in the range of 0.1 to 5.0 mmol/kg, had no morphological effect on their own, there were interactions when BEA was given. Aspirin (0.1 mmol/kg and especially 0.5 mmol/kg) plus BEA caused papillary necrosis more severely and frequently, with marked cortical tubular dilatation and very heavy deposits of THG-positive material in the collecting ducts and the remaining interstitium of the medulla. On occasion, THG-positive material was present in Bowman’s spaces of the superficial, but not juxtamedullary nephrons, in those kidneys where papillary necrosis was most extensive, and tubular dilatation very marked. By contrast, animals pretreated with 2.0 and 5.0 mmol/kg aspirin, before BEA administration, showed no difference from controls.

Pretreatment of rats with 1.0 mmol/kg of acetaminophen greatly exacerbated the response to BEA. There was more extensive necrosis, marked
tubular dilatation, loss of THG from the distal nephron, and heavy casts in the ducts of Bellini and some in Bowman's spaces of the superficial glomeruli. By contrast, there were very few changes in animals pretreated with 0.5 mmol/kg of acetaminophen. Increasing the pretreatment doses of acetaminophen from 1.0 to 5.0 mmol/kg dramatically exacerbated the necrotic lesion, the tubular dilatation, and the changed THG distribution. There were large casts in collecting ducts (associated with other cellular debris) and loss of THG from the distal nephron. The presence of THG-staining material in Bowman's spaces of superficial glomeruli was most frequently seen in animals pretreated with 1.0 and 2.0 mmol/kg of acetaminophen (Bach et al., 1988b).

These data are difficult to interpret because of the unique pharmacological and toxicological properties of aspirin and acetaminophen (Lovejoy, 1978; Plotz et al., 1981), differences which are further exemplified by the complex dose–response curves that were associated with each compound when BEA was also administered. These data do, however, strongly suggest that significant synergistic interactions may take place between chemicals in the genesis of RPN.

8. Functional Changes

There are marked similarities between the renal functional changes and the pathomorphological progression of the lesion following BEA administration and those reported for the analgesic-associated lesion in both experimental animals and humans (Bach and Bridges, 1982, 1985; Bach and Hardy, 1985). Prominent among these is the loss of urinary concentrating ability (Fauwa and Waugh, 1968; Wyllie et al., 1972; Murray et al., 1972; Shimamura, 1976; Sabatini et al., 1981, 1983; Vanholder et al., 1981; Bach et al., 1983), loss of nephron function, and severe cortical degeneration (Sabatini et al., 1981, 1983; Bach and Bridges, 1982). Loss of other urinary electrolytes (Na⁺, Cl⁻, PO₄³⁻, and Ca²⁺) has also been reported following BEA-induced RPN (Arruda et al., 1979; Sabatini et al., 1981). The measurement of urinary acidification capacity and electrolyte handling could therefore offer a simple way of monitoring for RPN in toxicology screening programs. Arruda et al., (1979), however, failed to find any differences between control and BEA-treated rats studied 24 hours after dosing, and Sabatini et al., (1982) found neither an acidosis nor a defective urinary acidification in response to NH₄Cl loading 1 month after dosing with BEA.

Wilks et al., (1986) studied the renal functional changes within 3 hours of BEA administration. Their data showed that the excretion of urea increased (from 30 minutes), osmolality decreased (from 90 minutes), and Na⁺
excretion increased at 3 hours, but potassium excretion was unchanged. Glomerular filtration rate and the clearance of the organic anion, p-aminohippurate, decreased over the period of investigation. These data reflect early functional abnormalities, and suggest that BEA may be less target selective for the medullary interstitial cells than has previously been thought. This is supported by the enzyme histochemical changes in the proximal tubular brush border that may reflect subtle injury to the cortex. Alternatively, these changes may represent aspects of the complexity in the renal response to injury, and they may reflect a rapid homeostatic consequence caused by perturbation of the medullary cells.

C. ETHYLENEIMINE AND 2-BROMOETHANAMINE ANALOGS FOR INDUCING RENAL PAPILLARY NECROSIS

Recently, propyleneimine has been shown to cause RPN at doses as low as 20 µl/kg (Halman et al., 1986). Multiple doses of bromopropanamine hydrobromide, 2-chloro-N,N-dimethylethanamine hydrochloride, or 2-chloroethanamine hydrochloride (Powell et al., 1985a) also cause this lesion, but each is either less effective and/or more toxic than BEA, and propyleneimine has many of the limitations of ethyleneimine.

D. N-PHENYLANTHRANILIC ACID- AND DIPHENYLAMINE-INDUCED RENAL PAPILLARY NECROSIS

The biphenyls are structural analogs of the fenamic acid NSAID, but have the advantage of a lower ulcerogenic potential. Hardy (1970a,b, 1974) has shown that a number of these compounds (Table V) produce RPN, but the most useful experimental models were induced by N-phenylandranilic acid (N-PAA) and diphenylamine (DPA). Both compounds did, however, also produce necrosis of the S3 region of the proximal tubule and marked splenomegaly in the case of DPA (Powell et al., 1983).

1. Morphological Changes in N-Phenylandranilic Acid-Induced Renal Papillary Necrosis

N-Phenylandranilic acid is a potent papillotoxin which targets very selectively for the medulla and causes a marked papillary urothelial hyperplasia Hardy (1970a,b, 1974; Hardy and Bach, 1984), but has little extrarenal toxicity (Powell et al., 1983).

The administration of N-PAA to rats for 14 days caused a dose-related lesion from focal RPN (at low doses) to total RPN, and marked cortical changes follow high doses of the biphenyl (Hardy, 1970a,b). The renal
RPN AND UPPER UROTHELIAL CARCINOMA

TABLE V
PAPILLOTOXIC POTENTIAL OF BIPHENYLS AND NSAID AND THE RELATIVE AMOUNT OF PGE INHIBITION*  

<table>
<thead>
<tr>
<th>Biphenyl NSAID and their analog</th>
<th>RPN induced in rats</th>
<th>Inhibition of PGE£ synthesis</th>
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<tbody>
<tr>
<td>Diphenylamine</td>
<td>++++</td>
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<tr>
<td>Flufenamic acid</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td>N-Phenylanthranilic acid</td>
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<td>Diphenyl</td>
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<td>Diphenylmethyl alcohol</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Diphenyl-2-carboxylic acid</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* From T. L. Hardy (unpublished data).
£ PGE = prostaglandin E2.

interstitial cells, the microvasculature, and the loops of Henle at the apex of the papilla were affected at the low doses (0.5–1 mmol/kg); and a progressive destruction of the collecting ducts and epithelium covering the papilla at higher doses (1–2 mmol/kg), and there were also morphological changes in the outer medulla and cortex at the highest dose levels of 3–5 mmol/kg (Hardy and Bach, 1984).

The histochemical changes associated with the N-PAA- and DPA-induced RPN have not been fully described, but include loss of the mucopolysaccharide (MPS) staining from the medullary matrix in those areas where necrosis has occurred. THG-positive material was lost from the distal nephron and progressively accumulated in casts in the necrotic collecting ducts (Powell et al., 1983).

There are some important morphological differences between the N-PAA-induced lesions, which develop subacutely, compared to an acutely induced RPN (Bach and Hardy, 1985). The lesion caused by N-PAA was apex limited and affected no more than 30% of the medulla (i.e., only the papillary tip) for multiple doses of 3–5 mmol/kg of N-PAA in Sprague–Dawley rats, but the lesion was diffuse in Wistar rats. Ethyleneimine (Axelsen, 1978a) and BEA (Axelsen, 1978b; Bach et al., 1983) cause a lesion which varied from an apex-limited focal necrosis (at low doses) to total ablation of the medulla.

2. Functional Changes in N-Phenylanthranilic Acid-Induced Renal Papillary Necrosis

The N-PAA-induced RPN decreased urine osmolality and increased urine volume, but only at dose levels of 2.0 mmol/kg or more. There were
no changes in the urinary acidification after an oral dose of ammonium chloride in animals treated with doses of N-PAA less than 5 mmol/kg. If the urinary chloride excretion was studied between 0 and 2 hours after ammonium chloride dosing, there was a dose-related decrease in the concentration of chloride ions that was significantly different from controls at the lowest dose of 0.5 mmol/kg N-PAA. The importance of choosing an appropriate window in time through which to monitor the dynamics of the renal response to chemical perturbation is clearly shown by the fact that similar changes were not apparent in urine collected from 2–4 hours (Hardy and Bach, 1984). Increased urinary protein, sodium, potassium, and chloride excretion have also been reported in animals treated with DPA, but N-PAA caused a proteinuria only (Powell et al., 1985b).

V. Biochemical Interpretation of the Pathogenesis and Secondary Consequences of Renal Papillary Necrosis

MECHANISM OF RENAL PAPILLARY NECROSIS

A variety of mechanisms have been proposed to explain RPN. There is little evidence from studies using animal models and clinical material to support the suggestion that RPN is mediated by an immunological injury (Gault et al., 1971; Murray and Von Stowasser, 1976). Similarly, most of the evidence to suggest that analgesics alter intermediary metabolism (see Shelley, 1978) was based on data using renal cortical tissues, which are biochemically very different from the medulla. A role for anoxic injury and microvascular degeneration is not supported by the acute-model lesion, as assessed by vascular filling data and morphology (Ham and Tange, 1969; Bach et al., 1983; Gregg et al., 1988a,b). Neither the colloidal carbon nor the Monastral Blue method differentiates between stasis and high flow rate areas. Solez et al., (1974) have shown, however, that the clearance of radiolabeled albumin was faster after BEA, thus there is no impaired papillary blood flow or hemostasis as evidence to support a contributing factor in the development of RPN. Vascular changes appear to follow the lesion. While it has been argued that the ubiquity of analgesics in inducing RPN could be linked to depressed prostaglandin (PG) synthesis and vasoocclusion (see Shelley, 1978; Bach and Bridges, 1985), the evidence against this is extensive (Bach and Bridges, 1985). An active increase in the concentration of papillotoxic compounds by countercurrent concentration could militate in favor of the lesion, but the loss of concentrating ability occurs well before a lesion is histologically apparent (Wilks et al., 1986).

None of the theories that have been postulated (Rosner, 1976; Shelley, 1978; Prescott, 1982) to explain the molecular pathogenesis of RPN have
so far defined the factors underlying the development of the primary necrotic lesion, nor have they addressed themselves to the progressive secondary cortical changes which result in the degeneration of nephron function. In the absence of a hypothesis to explain the genesis of the lesion, it has been suggested that the metabolic activation of analgesics, NSAID, and carcinogens could play a very important role in cell injury, and be an essential factor in the genesis of both RPN and upper urothelial carcinoma. The absence of medullary and urothelial cytochrome P-450 has precluded this metabolic process from direct involvement in the local formation of putative reactive intermediate(s) that could cause the toxic effect. It is unlikely that a reactive product formed in the cortex (where there is cytochrome P-450 activity) would be transported to the papilla and upper urothelial cells and cause injury, because these reactive products are highly labile and their short-lived nature would preclude this “mechanism” (see Bach and Bridges, 1984, 1985).

Recently, the hydroperoxidase activity present in the PG synthase system (and other peroxidative enzymes) have been shown to convert the two major metabolites of phenacetin, p-phenetidine (Andersson et al., 1983; Ross et al., 1985) and acetaminophen (Joshi et al., 1978; Zenser et al., 1979a,b; Nelson et al., 1981; Mohandas et al., 1981a,b), to reactive intermediates. This is highly relevant to the medulla and upper ureter, where PG synthetase is very active (Mori and Mine, 1981). Only unstable chemical intermediates formed by these enzymes will act locally (at the medulla or ureter) to produce toxic effects, possibly via lipid peroxidation (see Bach and Bridges, 1984, 1985). Despite the attractiveness of this hypothesis, neither acetaminophen nor p-phenetidine causes RPN, and there are no published data to suggest their role in the development of upper urothelial carcinoma in rodents. Long-term phenacetin feeding (over 24 months) does, however, cause a marked pelvic hyperplasia (see Section VI.C).

Histochemically, the enzymes associated with PG synthesis are distributed in different regions of the kidney. For example, the cyclooxygenase is localized to the medullary interstitial cells, endothelial cells of all arteries and arterioles, the collecting ducts, and glomerular epithelial cells of some species (Smith and Wilkin, 1977a,b; Smith and Bell 1978). Prostaglandin antibodies demonstrated PGE2 and PGF2a in cortical and medullary collecting ducts, the medullary interstitial cells, both glomerular mesangial and epithelial cells, and endothelial cells of the arteries and arterioles (Mori and Mine, 1981). More PGA2 has also been shown in the tubular cells of the renal medulla compared to the cortex (Perez and McGuckin, 1972). None of these methods serves to measure other types of peroxidative activity, but total peroxidative enzyme activity can be measured by the diaminobenzidine method. There is more peroxidase activity in the collecting duct compared to the medullary interstitial cells, but the absence of
activity in glomeruli suggests that this method does not demonstrate PG hydroperoxidase (Janszen and Nugteren, 1971, 1973; Litwin, 1977; Al-Ani and Fourman, 1979).

The ubiquitous distribution of the PGs and cyclooxygenase and other peroxidase activities in different parts of the kidney fail to explain why papillotoxic chemicals target selectively for the medullary interstitial cells and do not affect regions such as the collecting ducts or the glomeruli, that have similar enzymes. It has been suggested that the sensitivity of the interstitial cells to these chemicals relates to the presence of both an enzyme-activating system and high levels of polyunsaturated fatty acids. This would predispose to lipid peroxidation and the associated cellular degenerative changes selectively in the interstitial cells (Bach and Bridges, 1984).

The importance of the prostaglandins in the peroxidative activation of potentially papillotoxic chemicals remains to be established. Recent studies have shown that cultured rat medullary interstitial cells are sensitive to BEA (Benns et al., 1985), but not to those nephrotoxins that target for the glomeruli or proximal tubule. These isolated interstitial cells contain lipid droplets and peroxidase activity. Interestingly, 3T3 and MDCK cells have a very high PG synthase activity (Hassid and Levine, 1977), whereas this enzyme has a low level of activity in HaK cells (Hull et al., 1976). The 3T3 cells also contain lipid droplets; the other two cell types are essentially free of lipid material. The 3T3 are very sensitive to BEA and show pronounced cytotoxicity at levels of 0.2 mM over 2–4 hours. By contrast, the MDCK and HaK cells are resistant to BEA and appear to be unaffected by exposure to 2.0 mM for 24 hours. This observation also points to the increased BEA cytotoxicity in those cells with both peroxidative activity and lipid droplets (Bach et al., 1986). It must, however, be stressed that at present there are no data available to establish if the lipid droplets in the 3T3 cells are polyunsaturated, nor is there any information on differences in the intracellular activities of protective factors such as catalase and superoxide dismutase.

We have shown that N-PAA undergoes peroxidative conversion to biologically reactive intermediates (Feldman and Bach, 1988). Horseradish peroxidase and PG synthase from pig seminal vesicle and medullary microsomes, in the presence of hydrogen peroxide or hydroperoxyeicosanoic acid (the precursor of PG formed from exogenous arachidonic acid), activate N-PAA. The biologically reactive intermediate(s) bind to protein and nucleic acid, an effect that is quenched by nucleophilic sulfhydryl-protecting agents such as glutathione (Feldman and Bach, 1988). The quantities of N-PAA that are bound to protein and nucleic acid (Feldman and Bach, 1988) are of a similar order of magnitude to those of several established carcinogens (Kadlubar et al., 1982).
VI. Renal Papillary Necrosis and Upper Urothelial Carcinoma

The relationship between RPN and upper urothelial carcinoma in human analgesic abusers has been the topic of much debate and continued speculation, although the two are very strongly associated (McCredie et al., 1982a,b, 1983; Bach and Bridges, 1985). Thus, the exact pathogenesis of these malignancies in humans is uncertain; there have been no data on which factors exacerbate or ameliorate the development and/or the progression of the tumors, and under what circumstances they metastasize most readily. Normally the lesion is diagnosed at such an advanced stage, and with such widely disseminated metastases, that surgical resection may only be palliative (Hultengren et al., 1965; Mihatsch and Knusli, 1982).

A. Role of Urothelial Hyperplasia in the Development of Upper Urothelial Carcinoma in Human Analgesic Abusers

The progression of hyperplasia, through dysplasia, to malignancy is now a widely accepted series of changes in the development of carcinoma in epithelial cells in a number of different organs (Faber and Sporn, 1976). Such events may occur in the genesis of analgesic-associated carcinoma. There are well-documented foci of hyperplastic ureteric epithelia, in addition to malignancies, in patients with upper urothelial tumors (Lomax-Smith and Seymour, 1980a,b) and in analgesic abusers with RPN, but no diagnosed malignancies (Blohme and Johansson, 1981). These data, taken in light of the already established very high incidence of upper urothelial carcinoma in analgesic abusers (Bengtsson et al., 1968, 1978) and the strong association between the two (McCredie et al., 1982a,b, 1983), highlights the possibility of these proliferative changes in the pelvic and ureter epithelial cells as being premalignant.

B. Animal Models of Upper Urothelial Carcinoma

There are several animal models of bladder and renal parenchymal carcinoma (Hicks, 1980, 1983; Cohen et al., 1983; Hard, 1987), but to date there has been relatively little research on experimentally induced upper urothelial carcinoma. Many of the early investigations into the cause of upper urothelial carcinoma concentrated on the role of phenacetin and mixed analgesics (Johansson, 1981; Johansson and Angervall, 1976a,b; National Cancer
Institute, 1978; Isaka et al., 1979; Nakanishi et al., 1982). While several of these studies reported tumors at a variety of renal and extrarenal sites, the only regular and reproducible effect on the kidney was urothelial hyperplasia (Johansson and Angervall, 1976a,b). In the absence of urothelial malignancies, the general conclusion has been adopted that there was sufficient evidence in animals that phenacetin was carcinogenic, but that this was limited for analgesic mixtures containing phenacetin (IARC, 1980, 1982).

The early studies on bladder malignancies caused by feeding animals with 0.188% N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT) also showed the presence of upper urothelial malignancies in a few rats after 46 weeks (Erturk et al., 1967). Subsequently, over half the 35 rats fed with 0.188% FANFT for 46 weeks were shown to have severe pelvic hyperplasia, and 4 cases of upper urothelial carcinoma were also reported (Erturk et al., 1969). The FANFT-induced upper urothelial carcinoma has been shown to be exacerbated by phenacetin feeding, by mechanical perforation of the renal pelvis (Anderstrom et al., 1983; Anderstrom and Johansson, 1983), and by sustained Escherichia coli-induced urinary tract infection (L. Van Schultz, personal communication). There are also data to show that the administration of phenacetin for a period of 30 weeks, after the urothelia had been initiated with N-butyl-N-(4-hydroxybutyl)-nitrosamine (HO-BBN), caused a significantly higher incidence of bladder tumors (Nakanishi et al., 1978).

These data suggest that upper urothelial carcinoma could be induced experimentally with a total carcinogen such as FANFT and/or the combination of initiation and promotion with any agent that causes a hyperplasia of the papilla or pelvic urothelial cells. None of these investigations has, however, adequately addressed the question of the interrelationship between RPN and upper urothelial carcinoma. There is no evidence to suggest that FANFT causes RPN, and where phenacetin was administered after HO-BBN there were no data to suggest that papillary necrosis had been caused by either chemical. In addition, there are several other manipulations such as mechanical injury to the renal pelvis (Anderstrom et al., 1983; Anderstrom and Johansson, 1983), high-salt diets (Lalich et al., 1974), and saccharin feeding (Murasaki et al., 1982) that also cause pelvic hyperplasia in the absence of RPN.

C. Upper Urothelial Hyperplasia in Chemically Induced Renal Papillary Necrosis

Upper urothelial hyperplasia has, in fact, been a common consequence of many papillotoxic chemicals, and varies from mild, following aspirin...
(Molland, 1976) or BEA (Bach et al., 1983), to a florid hyperplasia following substituted indeneacetic acid NSAID analog (Bokelman et al., 1971). N-PAA often caused hyperplasia in the renal pelvic epithelium adjacent to the necrosed papilla. A group of rats dosed with 5.0 mmol/kg N-PAA for 5 days showed significant epithelial changes, which varied from a mild columnar hyperplasia, with minimal inflammatory cell infiltration, to a florid hyperplasia at the papillary apex (Fig. 9), associated with moderate subapical tissue necrosis (Hardy and Bach, 1984).

The protracted period required to induce RPN experimentally with the therapeutically used analgesics or NSAID, and, especially, the variability in

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**FIG. 9.** Apex-limited urothelial hyperplasia following five daily doses of 5 mmol/kg N-PAA. Hematoxylin–eosin, ×5. [From Hardy and Bach, (1984).]
the extent of the lesion caused by these agents (Rosner, 1976; Bach and Bridges, 1984; Bach and Hardy, 1984), have so far precluded the experimental investigation of the relationship between RPN and upper urothelial carcinoma. The acute nature of the BEA-induced RPN, without extrarenal toxicity and with limited effects on the renal cortex, offers a less complicated model to study pelvic urothelial changes and the factors that may exacerbate the process.

D. Upper Urothelial Hyperplasia Following 2-Bromoethanamine-Induced Renal Papillary Necrosis

After BEA treatment the upper urothelia showed marked proliferative changes at the edge of the epithelia covering the papilla (i.e., at the junction between the normal and necrotic part of the papilla). This was five cells thick (whereas it is normally one cell thick in most parts of the urothelia, except for the ureter, where it is four cells thick) at 24–48 hours (Fig. 10A). Hyperplasia was especially marked at the mouth of the ureter (which was seven cells thick) at 18 hours and in the pelvis opposite the region of necrosis, which was seven to eight cells thick at 18 hours (Fig. 10B). This had only partially resolved by 144 hours.

The ureter and the bladder showed minimal histological changes up to 144 hours. There were few morphological changes in the upper urothelia before 24 hours, after which a progressive and very marked hyperplasia was present in the pelvic urothelia (adjacent to the necrosed papilla tip and up to the fornix), in the urothelia covering the papilla (at the junction with the injured area), in the collecting ducts, and at the origin of the ureter. The increased thickness of the urothelia was most marked at 72 hours and continued as such in all of the upper urothelia (except the pelvis opposite the region of papillary injury) for 144 hours after BEA. There were disproportionately fewer mitoses compared to the degree of urothelial thickening (Gregg et al., 1988c).

E. Upper Urothelial Kinetics Following 2-Bromoethanamine-Induced Renal Papillary Necrosis

We have used autoradiography to study cell kinetics in different regions of the upper ureter following BEA-induced RPN.

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**Fig. 10.**  (A) Marked proliferative changes in papilla covering epithelium at leading-edge junction of normal and necrotic parts of papilla. Toluidine Blue, 1-μm resin section, ×40.  (B) Hyperplasia of pelvic epithelia opposite denuded papilla covering epithelium indicative of necrotic region, 24 hours after 100 mg/kg BEA ip. Toluidine Blue, ×40.
The cell kinetics in different parts of the kidney, pelvis, and ureter were based on the incorporation of $[^3H]$thymidine, infused from a subcutaneously implanted miniosmotic pump at a zero-order rate for 144 hours. This method provides data on the total accumulated turnover of epithelial and urothelial cells for the period under investigation. The labeling index for normal cell turnover was highest (and similar) in the proximal and distal tubules, less in the epithelia covering the papilla and the adjacent pelvis, even lower in the ureter and collecting duct, and lowest in the pelvic fornix. Compared to the baseline cell turnover, there was a 2- to 3-fold increase in all the areas 144 hours after BEA, except for the collecting duct (8-fold) and the pelvic fornix, which showed a 16-fold increase (Gregg et al., 1988c). In absolute terms the most active regions of cell division after BEA were the collecting duct, the pelvic fornix (opposite the papilla tip and the margin of papillary injury), and the origins of the ureter. The urothelia at the margin of papillary injury were apparently the least actively dividing cells. Both the proximal and the distal tubules responded to BEA-induced RPN with a markedly increased rate of cell division compared to controls.

These data show that the upper urothelia is very responsive to an acutely induced RPN, particularly as assessed by autoradiography, which was the most sensitive means of demonstrating proliferative changes. Renal tubular epithelia and urothelia each had their own normal cell turnover, and the response of each to the papillary injury differed. The delayed development of urothelial changes for the first 24 hours after the necrosis of the papilla suggests that the upper urothelial hyperplasia may be a consequence of RPN.

F. Changes in the Upper Urothelial Histochemistry Following 2-Bromoethanamine-Induced Renal Papillary Necrosis

Carbohydrate granules have been noted in the epithelial cells associated with RPN induced by aspirin (Molland, 1976) and BEA (Gregg et al., 1988a). The aspirin-induced lesion was associated with an accumulation of dense fibrils of PAS-positive material in the interstitial matrix. There was also an accumulation of PAS-positive granules in the cells of the collecting duct and the covering epithelium in BEA-treated animals (Fig. 11). These granules appeared before cell necrosis and may therefore have been part of the autophagic process responding to the release or breakdown of the extracellular matrix. The presence of similar granules in pelvic and upper urothelial cells 21 weeks after the induction of an acute papillary necrosis suggested that this change was a long-term aberration of cellular function,
especially considering that they were most marked in those regions where the urothelium was most hyperplastic or dysplastic. Similar changes have been described in the upper urothelial carcinoma epithelial cells, and in cells that had metastasized from these regions (Tucker et al., 1959), and in bladder malignancies in humans (Hukill and Vidone, 1965). The presence of these granules could be related to changes in the glycocalyx, which has been linked to tumorigenesis and neoplasia (Iozzo, 1985) and to foreign cell recognition (Smets and Van Beek, 1984). The granules may also represent premalignant changes, although similar staining has been reported in rodents with lithium-induced nephropathy (McAulliffe and Olesen, 1983).

There are a number of enzyme histochemical changes in the urothelial cells following a BEA insult. For example, there was a marked increase in alkaline phosphatase in the pelvic urothelial cells adjacent to the necrosed papilla at 8 hours, that subsequently included other hyperplastic upper urothelial regions (Fig. 12). This pattern was maintained for up to 24 hours and then declined to give a mosaic of staining that was, nevertheless, still strong in the pelvis adjacent to the necrosed papilla at 144 hours.
Marked alkaline phosphatase staining in hyperplastic pelvic urothelium opposite necrotic papilla at 8 hours after 100 mg/kg BEA ip. 1-μm resin section, ×100.

G. UROTHELIAL INITIATION WITH HYDROXYDIBUTYLNITROSAMINE (HO-BBN) FOLLOWED BY THE INDUCTION OF RENAL PAPILLARY NECROSIS

Pretreating rats with HO-BBN for 5 weeks, to a total dose of 800 mg per rat, followed by BEA, produced early diffuse hyperplasia (similar to that described in humans with RPN), which progressed to focal proliferative changes, up to eight cells thick (Fig. 13A) and discrete lesions with pleomorphic, atypical, and disorganized cells (many of which had irregular and bilobular nuclei, and some of which were multinucleated). These also included papillary P1 tumors (Fig. 13B) and several flat carcinomas in situ that were invasive P1 into the submucosa (Fig. 13C) or showed early invasion into muscle P2 (N. J. Gregg et al., unpublished). These features are similar to those of the tumors reported in human analgesic abusers (Johansson and Wahlqvist, 1977; Mihatsch et al., 1980c).

Animals treated with HO-BBN followed by BEA and those treated with BEA alone, for up to 21 weeks, showed the same mosaic pattern of alkaline
phosphatase staining in the hyperplastic urothelia. Those areas of the urothelium in the HO-BBN-pretreated animals that appeared to be invasive (P1 stage) lacked alkaline phosphatase staining, and looked similar to foci of "normal" urothelium (Fig. 14A). Some of the hyperplastic cells in both the HO-BBN/BEA-treated and the BEA-treated animals had superficial acid phosphatase staining (Fig. 14B), suggesting an autophagic response. Whereas staining for GGT was effective on the brush border, there were no foci of this activity in the hyperplastic or dysplastic cells at any time point following BEA treatment.

By contrast, however, there are focal and irreversible losses of alkaline phosphatase from otherwise histologically and cytologically normal rat

Fig. 13. (A) Atypical, disorganized urothelium with multinucleated cells and potential invasive focal lesions following 5 weeks pretreatment with HO-BBN and 13 weeks after a single dose of 100 mg/kg BEA ip. Giemsa, 1-μm resin section, ×40. (B) Papillary P1 tumor in upper ureter following 5 weeks pretreatment with HO-BBN and 13 weeks after a single ip dose of 100 mg/kg BEA. Note invasive finger into submucosa (arrowhead). Toluidine Blue, 1-μm resin section, ×20. (C) Early invasion in muscle layer of ureter from flat carcinoma in situ, seen as an invasive finger in B. Toluidine Blue, 1-μm resin section, ×100.
FIG. 14. (A) P1 invasive urothelium showing (arrowheads) negative alkaline phosphatase staining compared to hyperplastic urothelium. Gomori alkaline phosphatase method, 1-μm resin section, ×32. (B) Acid phosphatase staining in superficial layer of urothelium following a single BEA 100 mg/kg dose at 30 weeks. Acid phosphatase Napthol AS-B1 method, 1-μm resin section, ×32.
bladder urothelial cells following di-N-butyl nitrosamine, HO-BBN, or FANFT treatment (Kunze, 1979; Kunze et al., 1969, 1973; Kunze and Schauer, 1971; Stiller and Rauscher, 1971; Ito et al., 1973). The alkaline phosphatase-free foci are considered to be preneoplastic cells. GGT-positive cells are a well-established marker for premalignant changes in hepatocarcinoma (Hanigan and Pitot, 1985). Foci of GGT-positive cells are present in otherwise normal urothelia after exposure to HO-BBN (Ozono et al., 1985), a feature that has been present in nodulopapillary hyperplasia and carcinoma that subsequently developed. There is also some evidence to suggest that GGT may not be a pathognomonic indicator of premalignant changes, but rather identifies only advanced carcinoma and large papillomas (Vanderlaan et al., 1982). These data suggest that the histochemical changes that take place in the upper urothelia differ from those that occur in the bladder, and raise the possibility that there may be subtle aspects in the pathomechanism that are unique to lesions in different parts of the urinary tract.

The combination of HO-BBN and BEA did not produce any other obvious gross histopathological changes in the heart, lungs, liver, spleen, or pancreas up to 21 weeks. Similarly, there were no changes in the high-resolution light-microscopic features in the kidney, pelvis, ureter, or bladder associated with the use of HO-BBN only.

Thus the combination of HO-BBN initiation followed by the promotion of hyperplasia in the upper urothelial cells by a BEA-induced lesion appears to offer a very rapid model for the induction of upper urothelial dysplasia. This experimental approach may provide a superior model for studying the genesis and development of RPN-associated malignancy. Furthermore, this system offers the potential to improve the diagnosis and management of similar changes in human analgesic abusers. More importantly, these data suggest that the development of upper urothelial carcinoma may represent a classical two-stage model of cancer. If this is the case, and it is relevant to the condition in humans, there is a very important need to establish which of the carcinogens that humans are exposed to represent the initiating agents. While the elucidation of these agents requires very careful study, and cannot at this stage exclude analgesics or NSAID, it is noteworthy that smoking was also very strongly associated with the development of upper urothelial carcinoma in analgesic abusers in Australia (McCredie et al., 1982a,b, 1983). Benzo[a]pyrene is one of the potent carcinogens that is a component of all smokes, and is thought to be a major risk factor in bladder cancer (Mommsen and Aagaard, 1983). Interestingly, the peroxidative enzymes of the type that may be involved in the metabolic activation of analgesics, NSAID, FANFT, and other carcinogens (Rapp et al., 1980; Mattamal et al., 1981; Zenser et al., 1983a,b) also convert benzo[a]pyrene to the highly reactive 7,8-diol epoxide (Marnett et al., 1978).
VII. Summary of the Pathogenesis of Experimentally Induced Renal Papillary Necrosis and Upper Urothelial Carcinoma

The morphological, histochemical, and functional data presented above support several distinct series of pathological changes following the administration of BEA. The earliest histochemical changes take place in the medullary matrix, which appears to undergo depolymerization. The renal medullary interstitial cells are the first cell type to undergo degenerative change, which is rapidly followed by damage to the “delicate” elements (endothelium and loops of Henle) of the medulla. The collecting ducts and endothelial changes are late and generally follow the necrosis of other anatomical regions of the medulla. The lipid changes in the medulla are not at present well understood, but they are similar to those already reported in human analgesic abusers.

The early subtle degenerative changes in the proximal tubule do not appear to be central to the development of the papillary lesion, but the subsequent exfoliation of the brush border and proximal tubular cells are important components of the protein casts that begin to form in the distal nephron. These subsequently appear to play at least some role in the development of functional changes that cause marked proximal tubular dilatation.

The intense alkaline phosphatase staining that develops in those regions of the upper urothelia suggests that hyperplasia is a secondary response to papillary injury. Similarly, the increased pelvic, ureter, and bladder endothelial staining for ATPase suggests that there is a progressive suburothelial microangiography, similar to that described in human analgesic abusers, associated with RPN, but also show that this is a consequence of the other changes that are taking place in the kidney.

The proliferative changes in the urinary tract epithelia were assessed by the number of mitoses and the urothelial thickness. The total number of cell divisions that occurred in the epithelia following BEA was measured by the continuous infusion of [3H]thymidine and autoradiography of the semithin sections. Importantly, autoradiography also showed that there is marked regeneration in the proximal and the distal tubules, although routine histology failed to show these changes. Hyperplasia of the upper urothelial cells appears to be a secondary consequence of papillary necrosis in this acute-model lesion.

The time course of the major pathophysiological changes associated with the development of RPN and its secondary consequences of cortical degeneration and upper urothelial hyperplasia are presented schematically in Fig. 15. These data show that there are discrete series of pathological
changes that appear to start on the medullary interstitial cells, and the degenerative changes then go on to affect a variety of other cell types in different parts of the kidney. While much of the effort in understanding nephrotoxicity has been directed at the physiology, morphology, and biochemistry of the primary renal lesion, there is obviously an equally important role for establishing the cascade of degenerative changes that follow a primary lesion. The complexity of the kidney is such that it is unlikely that any one cell type can be damaged without there being repercussions in the rest of the organ and probably extrarenally.

VIII. Remaining Questions

It is now obvious that a variety of chemicals, in addition to the analgesics and NSAID, have the propensity to cause renal papillary necrosis. The study of RPN and upper urothelial carcinoma therefore is no longer to be

![Diagram showing changes in microscopic features over time](image-url)
regarded as being purely the consequence of long-term analgesic abuse or therapeutic NSAID exposure. In humans RPN may also be a consequence of exposure to industrial and environmental chemicals. There is also some evidence to show that the papillotoxic chemicals and analgesics and/or NSAID may interact synergistically to produce RPN. Despite the large number of pathophysiological similarities between the acute and subchronic models of RPN, and between these and the chronic analgesic disease in animals and in humans, it would not be wise to extrapolate all of the pathophysiological changes reported in the rat model to other species. Instead, there is an important need to develop more subtle criteria to interrelate the cascade of degenerative changes and to develop better experimental techniques to allow a progression from acute, through subacute, to chronic animal models to be followed. Similarly, it is likely that studies on a number of different species can help address the question of anatomical and functional differences between humans and animals. Upper urothelial carcinoma may be a consequence of a total carcinogen, or it may more likely represent an initiation of the upper urothelial cells (due to their endogenous peroxidative activity or medullary enzyme activation), and subsequently the development of RPN and the associated injury to the upper urothelial cells and/or the associated functional changes that act as a promoting factor.

A number of questions still need to be addressed to understand more fully the pathogenesis of RPN and particularly the sequence of events that lead from a primary lesion in the medullary interstitial cells to the secondary degenerative loss of cortical parenchyma and urothelial abnormalities. One area that needs to be addressed in particular is the dichotomy between morphology and cell biochemistry that will describe the lesion in terms of molecular changes, in both animals and humans.

The major questions that still need to be answered can be formulated here:

1. By what mechanism are the degenerative changes in the renal medullary interstitial cells caused, and are the changes in the other cell types (e.g., loops of Henle, urothelial cells) a consequence of chemical injury or a result of the medullary interstitial cell degeneration?

2. If peroxidative metabolism of papillotoxic chemicals is a key factor in the generation of reactive intermediates, what is the role of the lipid droplets in the medullary interstitial cells? While the central role of such peroxidative activation can explain target toxicity associated with acetaminophen, N-phenylanthranilic acid, and other analgesics and NSAID, at present there is no known role for this mechanism in the genesis of the BEA-induced lesion.
3. What are the interactions that occur between analgesics and other therapeutic substances (and/or environmental chemicals) that may exacerbate or ameliorate the development of renal papillary necrosis, or affect its progression to chronic renal failure or to upper urothelial carcinoma? 

4. What is the clinical significance of chemicals other than analgesics and NSAID in causing renal papillary necrosis and upper urothelial carcinoma in humans? Ethyleneimine is a very good example of a chemical that was used extensively in industry long before there was a focus on occupational health and hygiene. 

ACKNOWLEDGMENTS

The authors' research reported in this review was supported by The Wellcome Trust, the Cancer Research Campaign, the International Agency for Research on Cancer, the Kidney Research Fund of Great Britain, Johns Hopkins Center for Alternatives to Animals in Testing, the Smith-Kline Foundation, the Humane Research Trust, the Dr. Hadwen Trust for Humane Research, and the Commission of the European Communities. We are indebted to M. E. van Elk for typing the manuscript and to our colleagues for providing unpublished data or material in press. Dr. Enoch Kwizera provided valuable critical comments.

REFERENCES


INTRODUCTION

Upper urothelial carcinoma (UUC) in analgesic abusers has been closely associated with renal papillary necrosis (RPN) since it was first reported (1). UUC has only rarely been induced in animals using analgesics (2), thus a "cause-and-effect" relationship has not been established (3). The underlying mechanism of RPN is not fully understood and animal models of analgesic-induced lesion are difficult to study because of the:-

i) long latent induction time of the lesion using analgesics
ii) large biological variation in extent of the lesion, and
iii) extra-renal toxicity e.g. gastric ulceration and perforation associated with the prolonged inappropriately high non-clinical doses of analgesics.

In view of these limitations, a rapidly induced model of RPN would be useful to help elucidate and identify factors contributing to secondary renal changes, the association between RPN and UUC and the pathomechanism of UUC. 2-Bromoethanamine (BEA) hydrobromide has no structural relationship to the analgesics, but it is an ideal model probe that induces an acute, dose-related RPN within 24-48 hr after a single injection. This lesion has been widely studied (see 3) and one of its secondary consequences is a simple hyperplasia localised to the pelvis, ureter and the epithelium covering the papilla, but this change does not progress to carcinoma (4).

Previous research using a 2-stage initiation-promotion model has established that bladder hyperplasia may develop into tumours (5). We have developed such a model by initiating the urothelium with a urothelial specific carcinogen, N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN) and then "promoted" urothelial cell proliferation by inducing RPN with BEA. BBN was chosen as the carcinogen to facilitate controlled oral dosing (6), to reduce the exposure risk to personnel and because of its greater specificity with no benign extra-urothelial tumours induced.

METHODS

Two groups of male Wistar rats (University of Surrey strain) weighing 120g were oral dosed twice weekly for 5 weeks with BBN (MRI, Kansas City,
Missouri, USA) to a total dose of 800 mg. Each dose was administered in a 0.5 ml volume of a 20:80 ethanol:water solution. After one week's respite, one of these groups was injected ip with a single dose of freshly prepared BEA (Aldrich, Poole, Dorset, UK) at 100 mg/kg in physiological saline. A control group received a 0.5 ml volume of the ethanol:water solution twice weekly for 5 weeks, and then after one week's respite received a single ip dose of BEA.

Animals were allowed food and water ad libitum, monitored daily and groups of animals (n = 3) were sacrificed periodically up to 40 weeks after BEA. Kidney, bladder and ureter tissues were dissected and fixed for 24 hr at 4°C in 4% (v/v) formaldehyde:1% (w/v) calcium chloride fixative.

Transverse slices of the kidney containing the papilla tip, bladder and the upper 2 cm portion of the ureter were embedded in glycolmethacrylate resin (JB-4, Polysciences, Northampton, UK). Semi-thin sections (1 um) were stained with Haematoxylin and Eosin (H&E), Giemsa, Toluidine blue, or PAS. RPN and urothelial changes were graded according to Burry and Mostofi respectively.

RESULTS

Control urothelium epithelial layer is typically 2-4 cells thick (Fig. 1a). Six weeks after BBN/BEA treatment there was papillary and nodular hyperplasia in the ureter (Fig. 1b) and aggregates of mast cells within the lamina propria. Within the superficial layer of epithelium dysplastic cells with basophilic nuclei were present.

Thirteen weeks after BBN/BEA both papillary and nodular hyperplasia was present along the entire length of the ureter (Fig. 2a), together with foci of nodular hyperplasia that appeared to invade the lamina propria and with multinucleated cells in the superficial layer (Fig. 2b). Dysplasia and "invasive" P2 foci were in the lamina propria and approaching the muscularis layer (Fig. 2c).

Thirty-four weeks after BBN/BEA treatment dysplastic changes were more frequent, extensive and advanced and a large tumorous mass was found in the pelvis, adjacent to the truncated papilla stump of one kidney (Fig. 2d). This tumour arose from the pelvic wall in which there were numerous mitotic figures.

Forty weeks after BBN/BEA treatment dysplasia in the pelvis and ureter was more advanced and another kidney had a nodular tumourous mass filling the upper ureter and extending into the pelvis. The tumour was dysplastic, with the loss of cellular polarity and it was extremely difficult to differentiate between the laminae propria and muscularis, suggesting an "invasive" stage.

Bladder tumours were present in the BBN/BEA treated animals, after 21 weeks, but not in BBN only (up to 30 weeks) or BEA only treated animals (up to 40 weeks).

DISCUSSION

Compared to the BEA only and BBN only control groups, animals from the BBN/BEA group had more severe urothelial abnormalities. Tumours in the renal pelvis and upper ureter were only found in the BBN/BEA treated animals, and bladder tumours appeared earlier and were usually larger with more dysplasia and cellular atypia than in the BBN only. The finding in this study that "nodular" invasive hyperplasia and nodular tumour tissue lacks any positive staining for this enzyme suggests the existence
Figure 1. a Control urothelium from Wistar rat showing three distinct layers; e = epithelium, lp = lamina propria, lm = lamina muscularis. Giemsa stain. Bar = 40 μm. b Nodular and papillary hyperplasia in ureter from animal 6 weeks after BBN initiation and BEA promotion (BBN/BEA). Many areas are dysplastic with extremely basophilic nuclei sloughing into lumen. Giemsa. Bar = 25 μm.

Figure 2. a. Disorganised hyperplastic ureteric urothelium from animal 13 weeks after BBN/BEA treatment. Giemsa. Bar = 80 μm. Inset b shows nodular 'invasive' area (arrow) and large multinucleated cells (arrowhead). Giemsa. Bar = 20 μm. c. Papillary and nodular hyperplasia in ureter, 13 weeks after BBN/BEA treatment. Toluidine blue. Bar = 20 μm. d. Macroscopic photograph of tumour found in pelvis 40 weeks after BBN/BEA treatment. Bar = 1 mm.
of premalignant and malignant cell populations within the urothelium of animals that had been initiated before an acute RPN was induced. These data suggest that a BBN initiated urothelium can be promoted to preneoplastic and neoplastic cells by an acute RPN. This classical 2-stage process suggests that analgesic-associated RPN may be a key factor in producing a proliferative population in a pelvic or ureteric epithelial cells that had already been initiated. These proliferating cells could then progress to localized upper urothelial dysplastic foci and with time a carcinoma. It is presently uncertain how valid a 2-stage model is for human analgesic abusers. Many of these patients have, however, been exposed to potential urothelial initiating agents by a high incidence of cigarette smoking which has been associated with an increased incidence of urothelial malignancies and carcinomas (10).

REFERENCES


Epidemiology and mechanistic basis of analgesic-associated nephropathy

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SUMMARY

End-stage renal failure (ESRF) due to analgesic nephropathy is still a common clinical condition in several countries, but the prevalence in dialysis patients shows large geographical differences. The frequency of ESRF of unknown aetiology is the inverse of that linked to analgesic abuse, and data suggest that the occurrence of analgesic nephropathy may be underestimated. The study of analgesic nephropathy is difficult because the earliest damage to the kidney is a renal papillary necrosis (RPN), which cannot easily be diagnosed. Continued analgesic abuse generally leads to a progressive secondary cortical degeneration which is easier to diagnose. If analgesic abuse is stopped at an early enough stage in nephropathy, clinical symptoms stabilize or improve, and ESRF may be averted. A high incidence of upper urothelial carcinoma (UUC) is also observed in individuals with a history of analgesic abuse, but it is still not clear if the two have a related pathogenesis.

Study of the mechanism of RPN in animals administered analgesics and nonsteroidal anti-inflammatory drugs (NSAID) has been difficult owing to their extrarenal toxicity. Several model compounds cause identical clinical changes and have as their selective target the renal medullary interstitial cells; subsequently, other changes (including cortical and glomerular degeneration) develop as a secondary cascade. A number of mechanisms have been proposed to explain RPN (e.g., counter-current concentrating mechanism, ischaemic injury, altered prostaglandin metabolism, immunological changes), but peroxidative metabolism of papillotoxic chemicals within the interstitial cells seems to be the most likely cause.

Analgesic abuse is a costly socioeconomic condition for which there is currently no clinical treatment. If it is diagnosed early enough, severe renal degeneration can be prevented. Additional epidemiological information is needed to establish the causative role of analgesics and other chemicals, in order to determine the relative risk of each. Additional animal experiments are needed in order to clarify the molecular pathogenesis of RPN and UUC, to differentiate the stages in progression to ESRF and to develop more sensitive and selective diagnostic criteria.
Renal papillary necrosis (RPN) was first described as a frequent consequence of the abusive intake of mixed analgesics in which phenacetin was a common component. This observation led to the incorrect description of RPN as 'phenacetin kidney'. It has since become apparent that a variety of analgesics, nonsteroidal anti-inflammatory drugs (NSAID) and industrial and environmental chemicals can cause RPN and interstitial nephritis [1-6].

During the last decade, phenacetin has been removed from most proprietary analgesic mixtures on the basis of decisions taken by the pharmaceutical industry and regulatory agencies; however, after such action in Australia, the number of deaths from RPN was not reduced significantly although analgesic abuse continued [7]. In Belgium, the sale of phenacetin-containing analgesics declined dramatically from 1972 to 21% of intake in 1976 and 9% of intake in 1983; nevertheless, the prevalence of analgesic nephropathy in dialysis patients remained at the same level between 1979 and 1984. A prospective study in Belgium has shown decreased renal function in analgesic abusers who never took analgesic mixtures containing phenacetin [8]. Paracetamol (acetaminophen, which largely replaced phenacetin in analgesic mixtures), other therapeutic agents and industrial and environmental chemicals are assumed to be involved in the pathogenesis of RPN [1].

The aetiology and epidemiology of RPN has been complicated by poly-pharmacy, failure to document the intake of nonphenacetin analgesics, ignorance of the role of other analgesics, NSAID and other chemicals in causing papillary necrosis and the difficulty in diagnosing RPN. In addition, most patients deny analgesic abuse. Most of the epidemiological data that have become available over the last 30 years reflect the intake of phenacetin and not of other agents which could be implicated. Estimates of the amount of analgesic required to produce papillary necrosis vary from <1 to 35 kg of phenacetin, but information is not available on the quantities of other analgesics taken or on exposure to other papillotoxic chemicals.

Attempts to explain analgesic abuse [1-3] include addiction to caffeine and the misguided use of these drugs to increase productivity and reduce strain in the working environment. Only a minority of patients take the drugs for genuine indications, and the origins of abuse are usually psychosocial. Patients are neurotic, dependent, immature, introverted, anxious or depressed, and up to 20% may have psychiatric illnesses and other addictive habits such as smoking, alcoholism and the use of psychotropic drugs and sleeping tablets. The majority of the patients are women over 30 years of age from lower socioeconomic and educational groups, with a history of heavy consumption of analgesic mixtures over five to 30 years. Several environmental factors such as dehydration, high temperatures and bacterial infection have been implicated in the development of the renal lesions [9]. The prognosis for patients who continue to abuse analgesics after RPN has been diagnosed is poor, but those who stop taking these substances show stabilization of, or improvement
in, renal function, and progression to ESRF may be prevented [3]. Apart from these renal lesions, there are also increased incidences of anaemia, gastric ulcers and coronary heart disease [10].

PREVALENCE OF ANALGESIC NEPHROPATHY

There are very marked differences in the incidence of analgesic-associated end-stage renal failure (ESRF) between countries (Table I). The disease has apparently disappeared in some of the high-incidence countries due to legislation on analgesics restricting over-the-counter sales; however, despite withdrawal of phenacetin in Switzerland, Belgium and the Federal Republic of Germany, analgesic abuse still remains one of the main causes of ESRF.

The overall incidence of ESRF in any one country may, however, be misleading, since high concentrations of analgesic abusers occur in specific geographical locations, such as the Winston-Salem area of the U.S.A. and in northern Belgium and the Federal Republic of Germany. In Belgium, the epicentre of the high-frequency abuse area is situated in the northern part of the country, where a prevalence of up to 51% is observed (Fig. 1), while analgesic nephropathy remains at a markedly lower level in the southern part of the country [8]. Similarly, in the Federal Republic of Germany (Fig. 2), there are high prevalences in West Berlin (up to 50%), Hamburg and Bremen [12].

Several studies indicate that the prevalence of this disease is underestimated. Whereas Pommer et al. [12] showed a 13% prevalence of analgesic-associated end-stage nephropathy in the Federal Republic of Germany, the European Dialysis and Transplant Association found no more than 6%. The proportion of nephropathies

<table>
<thead>
<tr>
<th>Region</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>South Africa (1978)</td>
<td>22</td>
</tr>
<tr>
<td>Switzerland (1980)</td>
<td>20</td>
</tr>
<tr>
<td>Belgium (1984)</td>
<td>18</td>
</tr>
<tr>
<td>Australia* (1985)</td>
<td>15</td>
</tr>
<tr>
<td>F.R. Germany (1983)</td>
<td>13</td>
</tr>
<tr>
<td>Canada* (1976)</td>
<td>3</td>
</tr>
<tr>
<td>Scandinavia* (1979)</td>
<td>3</td>
</tr>
<tr>
<td>France (1979)</td>
<td>2</td>
</tr>
<tr>
<td>U.K. (1979)</td>
<td>1</td>
</tr>
<tr>
<td>Italy (1979)</td>
<td>1</td>
</tr>
<tr>
<td>Spain (1979)</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Fig. 1. Analgesic nephropathy as a cause of end-stage renal failure in Belgium (1984). From Ref. 11. Population, 10 million; area, 30,515 km²; chronic dialysis units ( ), 54; total numbers of patients under chronic dialysis, 2334; ( ) and number, patients with end-stage renal failure caused by analgesic nephropathy expressed as % of patients treated in the dialysis unit(s).

of unknown aetiology noted in the registry of the Association for the dialysis population of the European countries (1980–84) may also be indicative of the underestimation of analgesic nephropathy. This observation is supported by the inverse relationship seen between the established prevalence of analgesic nephropathy and nephropathy of unknown aetiology, which is low in Switzerland and Belgium, more than 10% in the Federal Republic of Germany, France and Italy, and more than 20% in Portugal [13].

CLINICAL SYMPTOMS AND DIAGNOSIS OF RENAL PAPILLARY NECROSIS IN MAN

The diagnosis of analgesic nephropathy is difficult because of its silent progression over a long period. Usually, the symptoms are nonspecific or minimal until secondary damage affects the cortex and advanced renal failure occurs. One early clinical sign of analgesic nephropathy is the loss of urine-concentrating capacity [10,14,15], but this may be a consequence of several renal and extrarenal causes. Analgesic nephropathy is also associated with electrolyte disturbances, such as sodium wastage and hypocalcaemia, and a pronounced defect in urinary acidification after ammonium chloride loading [14,15]. Increased blood urea nitrogen or serum creatinine levels are indications only of incipient renal failure, by which time
papillary necrosis has long since occurred and the secondary degenerative changes have progressed toward ESRF. Irregular shrinking of the renal tissue and medullary calcifications may be identified by radiology and ultrasound, but these represent advanced degenerative changes. The histological changes in the kidney are confined to the medulla, which is generally assessed only at autopsy. Other changes in advanced renal failure are aspecific, and, while biopsies may show pyelonephritis, they will not establish the underlying cause.

Post-mortem studies [1,4-7] have served to confirm an RPN-related disease and to establish that 'early' degenerative changes begin at the tip of the papilla and affect interstitial cells, the loops of Henle, capillaries and the staining interstitial ground substance and cause an accumulation of lipid material, especially in collecting duct cells. Intermediate RPN affects more anatomical elements, including atrophy, sclerosis and inflammatory response in the outer medulla, and calcium deposits in the necrotic tip, which may have sloughed off or remained whole or fragmented in the calyces. The papillary stump re-epithelializes. Total RPN affects the
other medulla to the corticomedullary junction, and cortical changes are characterized as chronic interstitial nephritis, tubular dilatation, atrophy, basement membrane thickening, sclerosis, fibrosis, inflammatory cell infiltration and vascular degeneration. Suburothelial capillary sclerosis appears to be pathognomonic [6]. Pelvic, ureteric and bladder urothelium changes include thickening of capillary walls, sclerosis of lamina propria together with altered fat and collagen deposition, and, especially, disordered epithelial states, from hyperplasia through to malignancy and tumours [1,4–7].

EXPERIMENTAL MODELS OF RENAL PAPILLARY NECROSIS

Most of the studies that describe pathological and histological changes in patients with RPN have been retrospective – performed on autopsied post-mortem tissues. The complex heterogeneous structure of the kidney undergoes rapid autolytic degradation after death, which alters the morphology and compromises the interpretation of the progression of and interrelationship between degenerative changes. In addition, extrarenal complications and disease factors may adversely affect the interpretation of histological data. The best means of approaching this problem is to study the mechanism in animal models of RPN.

Analgesics such as aspirin, phenacetin and paracetamol may not induce RPN in rats, or may do so only after inappropriately high doses given over prolonged periods [1,2]. Even then, the biological variation within groups has been unacceptably high. Further, many therapeutically used NSAID cause death due to extrarenal toxicity, such as gastric ulceration and perforation [16]. Thus, rodents are not good experimental models for studying the mechanism of analgesic-induced RPN.

These problems have led to the use of alternative systems, such as chemicals with known papillotoxic potential. There are marked similarities between the renal functional changes and pathomorphological progression of lesions in several acute model systems and those reported for analgesic-associated lesions in both experimental animals and man [1,17]. The most commonly used chemical agents fall into two categories:

(i) Analgesics and NSAID analogues: N-Phenylanthranilic acid, an analogue of the NSAID fenamate, which has been studied extensively [18,19], causes a dose-related apex-limited lesion after oral gavage of 1–2 mmol/kg body weight for 14 consecutive days, or after a single intravenous dose.

(ii) Nonanalgesic-induced renal papillary necrosis: 2-Bromoethanamine hydrobromide induces a dose-related RPN within 24–48 h after a single dose [20], as reported in more than 35 studies (see Ref. 1 for full list). The tremendous reduction in time needed to produce this lesion, compared to those induced using analgesics, NSAID and their analogues (which may take weeks or months), has several logistically important advantages. The histological changes induced in experimental RPN follow the same pattern of early, intermediate and total RPN as has been...
described in man, and are dose- and time-dependent. The earliest morphological changes induced by papillotoxins occur in the renal medullary interstitial cells; the medullary glycosaminoglycan matrix also undergoes changes, with an increase and then a decrease in staining intensity. It is only subsequently that there are platelet adhesions, blocking of blood vessels, degenerative changes in the collecting ducts and proximal tubules and the accumulation of lipid material in capillaries and epithelial cells. At the same time as repair and re-epithelization are taking place, there is an increase in the presence of tubular casts, proximal and distal tubular dilatation, and hyperplasia of collecting ducts and of covering and pelvic urothelia; suburothelial capillaries undergo sclerotic changes. When the repair phase is advanced or complete there are also degenerative changes in the cortex, including fibrosis, glomerular sclerosis and cystic dilatation. The use of high-resolution light microscopy and ultrastructural studies (in conjunction with histochemistry and immunohistochemistry) can help establish the changes in adjacent cells and interrelate the cause-and-effect relationship in the sequence of degenerative events.

MECHANISM OF ANALGESIC-INDUCED RENAL PAPILLARY NECROSIS

Progress in the understanding of the pathogenesis of RPN has been assisted by use of model lesions [1], and some of the factors that may be implicated in the molecular changes have been defined. There is no evidence to suggest that the lesion has an early immunological basis or that it is a consequence of renal hypoxia or vasoconstriction, and there is no experimental basis to suggest that the altered intermediary metabolism is a critical factor [20]. The concept that altered prostaglandin metabolism gives rise to vascular (or other) changes is an attractive one, but the exceptionally low levels of these hormones, combined with their instability, has made it very difficult to test this hypothesis. The counter-current concentration mechanism is an important normal renal function and may be a primary factor in concentrating chemicals to toxic levels within the medulla. One of the earliest changes in the development of RPN, however, is loss of concentrating processes, which detracts from this hypothesis. Furthermore, the concentration of a compound in the medulla cannot explain the molecular mechanism by which it causes RPN [1].

At present, the most attractive explanation for the development of RPN is related to metabolic activation within the kidney. There are two major oxidative systems for xenobiotic metabolism in the kidney: the cytochrome P-450 system, localized to the cortex, and the prostaglandin hydroperoxidase system, located almost exclusively to the medulla. The latter has been shown to convert a number of chemicals, including paracetamol, N-phenylanthranilic acid and several carcinogens, to reactive intermediates that bind to both nucleic acids and protein [1]. The reasons for the selective targeting of particular chemicals to renal medullary interstitial cells are uncertain but may relate either to the absence of free-radical scavengers or nucleophiles or to the presence within these cells of large numbers of lipid droplets.
containing polyunsaturated fatty acids. These would form an ideal substrate for extensive lipid peroxidation within the renal medullary interstitial cells once a reactive species had been generated within them.

UPPER UROTHELIAL CARCINOMA

Upper urothelial carcinoma (UUC) has been associated with RPN and analgesic abuse, but a cause-and-effect relationship between the two is not proven [1]. The incidence of UUC among analgesic abusers is very high, especially among women, with a female: male ratio of 2.5:1 [21], in keeping with the ratio of analgesic abusers. Analgesic abusers also develop UUC at a younger age than nonanalgesic abusers [22-24], and their distribution in analgesic abusers has a distinct pattern, with tumours of the renal pelvis, ureter and bladder being induced 80 times, 90 times and seven times more frequently than in nonanalgesic abusers. Tumours are typically multiple, diffuse, poorly differentiated and rapidly spreading [24].

Patients who discontinue abuse of analgesics are at a greater risk of developing UUC, often after a latent period of 10-20 years since initiating analgesic abuse. Greatly improved dialysis techniques may increase survival of analgesic abusers who would otherwise develop end-stage renal disease and die [22]. It has therefore been suggested that the incidence of UUC will increase.

The clinical diagnosis of UUC is difficult because there are only a few nonspecific clinical symptoms [1,12,21-28]. The prognosis is poor, and patients with UUC have a mean survival time of only 22 months [22] because of compromised renal function and multifocal sites of rapidly developing, widespread invasion and metastases [26].

Experimental evidence links exposure to analgesics with the development of urothelial tumours [1,21,29]. Although bladder tumours have been studied extensively in animal models, the practical difficulties of looking for malignancies in the ureter and pelvis has limited studies in this area. There is little evidence from long-term carcinogenicity studies to establish a clear relationship between exposure to analgesics and UUC; there is, however, evidence that these carcinomas can be induced using a classical two-stage initiation/promotion regimen [30]. This suggests that localized injury associated with RPN adjacent to urothelium that has already been initiated will result in a proliferation of changes which develop into malignancies.

At present, the full significance of these findings in terms of the human analgesic problem is not clear, although a high incidence of smoking among analgesic abusers could be one of the factors that initiate the urothelium and contribute to the development of this condition after RPN caused by analgesics. Other factors, such as loss of mucoid covering and delayed passage of urine, would exacerbate any pathophysiological change initiated by locally generated reactive metabolites. The selective targeting to the upper ureter and pelvis could also be due to the metabolic activation of endogenous or exogenous compounds to reactive intermediates in the medulla.
CONCLUSIONS AND FUTURE OBJECTIVES

RPN and the associated condition, UUC, represent a large economic and health burden on the medical services. The recent demonstration that chemicals and drugs other than analgesics may also cause RPN warrants special consideration in any assessment of the health significance of this lesion.

More than 30 years after the first publications linking analgesic abuse with pyelonephritis, ESRF and UUC, the causal relationship remains controversial. It is still not clear which analgesics or chemicals are the causative agent(s), the quantity of each that has to be taken to cause the primary lesion, whether different types of papillotoxic chemicals can interact (and, if so, whether additively or synergistically), whether chemicals interact with other diseases or what the predisposing factors are for the development of nephropathy and its clinical course. Only when these questions have been answered will we be able to assess the amount of analgesic that can cause RPN and the relative and absolute risks of developing analgesic nephropathy, ESRF and UUC. Additional epidemiological research and long-term prospective studies are needed. Elucidation of the molecular pathogenesis of RPN and UUC would be an important step in developing safer chemicals or in modulating the course of the condition. Furthermore, there is a need to develop more sensitive and selective diagnostic criteria for RPN and to define adequately the stages in the progression of ESRF in order to identify individuals at risk, monitor renal degeneration, establish the efficacy of therapeutic and dietary manipulations to prevent degenerative changes, and characterize the factors that might exacerbate the development of the lesion and the risk that degenerative changes will progress toward malignancy.

ACKNOWLEDGMENTS

The authors received research support from the Wellcome Trust, the National Kidney Research Fund of Great Britain, the Cancer Research Campaign, the International Agency for Research on Cancer, the Johns Hopkins Center for Alternative to Animals in Testing, and the European Commission (NJG and PHB); and the National Unemployment Programme of Belgium, contract number 20019, and a grant from the Ministry of Health, Belgium (MME and MDeB).

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HIGH RESOLUTION MICROSCOPIC CHANGES IN AN ACUTELY INDUCED RENAL PAPILLARY NECROSIS: MORPHOLOGY AND ENZYME HISTOCHEMISTRY

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INTRODUCTION

Renal papillary necrosis (RPN) has been associated with an excessive consumption of analgesics and nonsteroidal anti-inflammatory drugs (1). The course of development of this lesion in man has been documented from post mortem tissue, where autolysis, other diseases and marked variability in the extent of lesion have made it difficult to determine the progression of pathological changes. There is some evidence to suggest that the renal medullary interstitial cells are the primary site of toxic injury and that microvascular, collecting duct and tubular degeneration are secondary changes. 2-Bromoethanamine (BEA) hydrobromide is a chemical which induces RPN rapidly and has allowed the progression of RPN to be studied. There are marked similarities between this acute BEA-induced lesion and changes that have been reported in animals dosed chronically with analgesics and non-steroidal anti-inflammatory drugs, and also the clinical condition in human analgesic abusers (1,2).

We have re-evaluated the detailed time-course of an acute papillary necrosis using semi-thin glycolmethacrylate embedded sections to provide detailed pathological changes. Furthermore, the ability to combine "routine" staining with selective enzyme histochemistry on serial semi-thin (1um) sections enhances the ability to inter-relate changes in different cell types during the course of the development of degenerative processes that lead to RPN.

MATERIALS AND METHODS

Male Wistar rats, University of Surrey strain, were injected ip with a single dose of BEA 100 mg/kg body weight (BDH, Poole, Dorset, UK) freshly prepared in physiological saline (3) and control animals were injected with vehicle only.

Animals were sacrificed by cervical dislocation in groups of 3 at 2, 4, 6, 8, 12, 18, 24, 48, 72 and 144 hr and pairs of control animals were similarly sacrificed at 12, 48 and 144 hr. All tissue processing was performed at 4°C. The right kidney, ureter and the inflated bladder were immersion fixed in formal calcium fixative (4% v/v formaldehyde:1% w/v calcium chloride). After 24 hr fixation 1-2 mm thick slices of kidney including the papilla tip, cortex, and a longitudinal slice of bladder
were cut. Serial semi-thin (lum) sections were cut to include the papilla tip, mouth of the ureter and the cortex and of the bladder using glass "Ralph" knives. Sections were stained for with haematoxylin and eosin (H & E), Giemsa's stain, Toluidine Blue and PAS. Enzyme histochemistry was performed to show the distribution of alkaline phosphatase (Alk Phos), acid phosphatase (Acid Phos), gamma-glutamyl transpeptidase (GGT) and adenosine triphosphatase (ATPase) using methods modified by Burnett (4).

RESULTS

Morphology and enzyme histochemistry results are summarised in Tables I and II.

Table I. Time course of morphological changes

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Morphological Changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Normal with papilla interstitial matrix increasing in volume towards tip. Urothelium 2-4 cells thick, paler superficial layer which is PAS positive.</td>
</tr>
<tr>
<td>2 hr</td>
<td>No changes discernible.</td>
</tr>
<tr>
<td>4 hr</td>
<td>Medullary interstitial cell nuclei becoming acutely irregular and pyknotic (Fig. la).</td>
</tr>
<tr>
<td>6 hr</td>
<td>Mild hyperplasia of papilla covering epithelium to 2-3 cells thick.</td>
</tr>
<tr>
<td>8 hr</td>
<td>Marked pyknosis of interstitial cell nuclei, sloughing of hyperplastic covering epithelium. Platelets adhering to endothelium dilated distal tubular profiles.</td>
</tr>
<tr>
<td>12 hr</td>
<td>Leading edge of sloughing covering epithelium showing mild 2-3 cell thick hyperplasia. Degenerative changes in tubular elements in papilla. Hyperplasia in upper ureteric urothelium.</td>
</tr>
<tr>
<td>18 hr</td>
<td>50% of papilla affected by RPN. Cytoplasmic granules in necrotic collecting duct cells, mitotic figures present. Hyperplasia of pelvic urothelium opposite leading edge of covering epithelium (Fig. lb).</td>
</tr>
<tr>
<td>24 hr</td>
<td>Medulla affected by necrosis, loss of tissue integrity with cellular debris and proteinaceous casts in papilla. Casts in dilated distal tubules in cortex. Fornix area of urothelium, hyperplastic, mitotic figures present.</td>
</tr>
<tr>
<td>48 hr</td>
<td>Covering epithelium leading edge 5 cells thick, cells very irregular nuclei, cytoplasmic granules present. General hyperplasia of pelvic and ureteric urothelium (Fig. 1c).</td>
</tr>
<tr>
<td>72 hr</td>
<td>75% denudation of papilla covering epithelium. Tubular necrosis extending to outer medulla. Marked dilatation of proximal and distal tubules.</td>
</tr>
<tr>
<td>144 hr</td>
<td>100% loss of covering epithelium, papilla is a mass of necrotic tubules, (Fig. 1d) lymphocytic infiltration. Sloughing of pelvic and ureteric urothelium, bladder urothelial superficial layer becoming increasingly basophilic. Numerous granules in proximal tubule segments.</td>
</tr>
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</table>
Table II. Time course of histochemical changes

<table>
<thead>
<tr>
<th>Time point</th>
<th>Histochemical changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Occasion PAS positive granules in urothelium Alk Phos and ATPase enzyme staining in proximal tubule brush borders (also GGT), intermediate and basal layer of urothelium with superficial layer devoid of stain (Fig. 2a).</td>
</tr>
<tr>
<td>2 hr</td>
<td>No changes discernible.</td>
</tr>
<tr>
<td>4 hr</td>
<td>Increase in interstitial matrix staining with Giemsa. Slight increase in ATPase staining around pyknotic nuclei.</td>
</tr>
<tr>
<td>6 hr</td>
<td>Slight decrease in Alk Phos and GGT proximal tubule brush border staining.</td>
</tr>
<tr>
<td>8 hr</td>
<td>Increase in Alk Phos staining in pelvic urothelium opposite denuded covering epithelium (Fig. 2b). Acid Phos increase staining in the S1 and S2 proximal tubule segments.</td>
</tr>
<tr>
<td>12 hr</td>
<td>Increase interstitial matrix staining at papilla tip. Hyperplastic urothelium has increased apical Alk Phos staining and increased ATPase staining of endothelium sub-urothelial capillaries.</td>
</tr>
<tr>
<td>18 hr</td>
<td>Mosaic pattern of Alk Phos and ATPase staining in hyperplastic urothelium (Fig. 2c). ATPase endothelial staining increase, moderate Acid Phos staining of interstitial cells in medulla.</td>
</tr>
<tr>
<td>24 hr</td>
<td>Decreased interstitial matrix staining, loss of proximal tubular brush border enzymes Alk Phos, ATPase and GGT coincide with an increase of these enzymes in proteinaceous casts in the necrotic papilla. Loss of Acid Phos staining from S2 proximal tubule segment.</td>
</tr>
<tr>
<td>48 hr</td>
<td>PAS positive staining material in proteinaceous casts. Continued loss of Alk Phos, ATPase and GGT from proximal tubule brush borders, also loss of Alk Phos and ATPase to slight degree from pelvic and ureteric urothelium.</td>
</tr>
<tr>
<td>72 hr</td>
<td>Variable enzyme staining in proteinaceous casts, GGT most pronounced. Urothelial staining in ureter reduced to few Alk Phos positive cells, no bladder staining at all. Sub-urothelial capillary ATPase staining increased (Fig. 2d).</td>
</tr>
<tr>
<td>144 hr</td>
<td>Granular Alk Phos and GGT staining in interstitium adjacent to hyperplastic fornix urothelium. Increased Acid Phos lysosomal staining in proximal tubule segments. Sub-urothelial capillary ATPase endothelial staining increase in quantity to occlude lumen.</td>
</tr>
</tbody>
</table>

DISCUSSION

The renal medullary interstitial cells are the first morphological feature to be affected in the cascade of degenerative changes that leads to RPN. These are followed by loss of the mucopolysaccharide matrix and degeneration of the endothelium, distal tubules, collecting ducts and loops of Henle.
Figure 1  a. Medullary interstitial cell nuclei (arrowheads) becoming acutely irregular and pyknotic 4 hr after BEA (100mg/Kg). Giemsa. Bar = 10 um. b. Hyperplasia of pelvic urothelium (arrowhead) opposite leading edge of covering epithelium (arrow) 18 hr after BEA. Toluidine blue. Bar = 100um. c. Hyperplastic pelvic urothelium, 72 hours after BEA (100 mg/kg). Giemsa. Bar = 40 um. d. Papilla tip is a mass of necrotic tissue, with casts in loops of Henle and collecting ducts consisting of exfoliated cell debris. Giemsa. Bar = 25 um.
Figure 2  a. Alkaline phosphatase (Alk. Phos.) staining in control bladder urothelium (arrow). Superficial "umbrella" cell layer devoid of stain. Bar = 10 um.  b. Increased Alk. Phos. staining in pelvic urothelium (arrowheads) opposite denuded covering epithelium (arrow) 8 hr after BEA. Bar = 20 um.  c. Mosaic pattern of Alk. Phos. staining in hyperplastic urothelium, 18 hr after BEA. Bar = 10 um.  d. Sub-urothelial capillary endothelium stained with ATPase, thickness increasing to occlude lumen (arrowheads). Bar = 10 um.
The sensitivity of the medullary interstitial cells to BEA may be due to one or more of the following:

- The interstitial cells are rich in prostaglandin hydroperoxidase and other peroxidases, which have the potential to produce biologically reactive intermediates (5).
- The medulla has very low levels of glutathione and therefore once generated and reactive intermediates would not readily be inactivated (5).
- The medullary interstitial cells contain a large amount of lipid droplets which are very rich in polyunsaturated fatty acids (5).

Thus the consequence of generating a reactive intermediate could be that of a sustained lipid peroxidation that was localized to the target cells, but also affected adjacent anatomical elements of the medulla. The interstitial cells synthesise the mucopolysaccharide ground substance that serves to support the delicate elements (loops of Henle and capillaries) in the medulla and also acts as a matrix that controls the availability and movement of liquid, ions and other molecules between the different compartments. Thus an early loss of the mucopolysaccharide matrix will drastically affect renal function and morphology, and play an important role in the cascade of degenerative changes that follows. Furthermore, whereas epithelial cells have a significant regenerative capacity, the medullary interstitial cells do not undergo proliferative changes when damaged, nor is there a stem cell population from which to replace "lost or damaged" cells (1).

The subtle degenerative changes in the proximal tubule do not appear to be central to the development of the papillary lesion. Exfoliated brush border enzymes and cells are, however, important components of the proteinaceous casts in the distal nephron.

Hyperplasia, the formation of distal tubular casts and then the proximal and distal tubular dilatation all appear to be secondary consequences of papillary necrosis, and the delayed increase in Alk Phos staining in urothelium support this. The increased ATPase staining of sub-urothelial capillary endothelium suggests that there is a progressive microangiopathy similar to that described in human analgesic abusers with RPN (6).

ACKNOWLEDGEMENTS

This research is supported by the Cancer Research Campaign of Great Britain, International Agency for Research on Cancer and Wellcome Trust.

REFERENCES


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MORPHOLOGICAL CHANGES IN THE PIG KIDNEY ASSOCIATED WITH AN ACUTELY INDUCED RENAL PAPILLARY NECROSIS

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INTRODUCTION

Analgesic abuse leads to the development of renal papillary necrosis (RPN) and may cause upper urothelial carcinoma in man. Animal models offer a potentially important means by which to define the underlying pathogenesis of RPN. Moreover, their use could improve early diagnosis, allowing the identification of which analgesic has the greatest papillotoxic potential and define what factors exacerbate this lesion (1). Many analgesics induce gastro-intestinal toxicity in rats which limits the use of this species for studying analgesic-induced RPN (2). The use of the non-analgesic papillotoxic chemical 2-bromoethanamine (BEA) hydrobromide, has helped define the mechanistic basis of RPN, particularly because this compound targets selectively for the medulla and causes a lesion in rodents within 24-48 hr (1). The BEA-induced RPN in the Wistar rat shows most of the pathological changes described in human analgesic abusers (3). The primary choice of rodents for nephrotoxicity studies reflects their low cost, ready availability, ease of handling and the considerable baseline data on renal function and toxicity. However, the extrapolation of nephrotoxicity data from rodents to man is complicated by the marked renal anatomical and functional differences between the two species. In contrast the human and pig kidney are remarkably similar in terms of physiological and anatomical characteristics (Table I).

Table 1. Comparison of some morphological and functional parameters in kidneys of man and pig.

<table>
<thead>
<tr>
<th>Species</th>
<th>GFRa</th>
<th>Glomeruli</th>
<th>Proximal tubule</th>
<th>Maximal %</th>
<th>Long loops</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Numberb</td>
<td>Radiusc</td>
<td>Lengthd</td>
<td>Radiusc</td>
<td>Osmolality</td>
</tr>
<tr>
<td>Man</td>
<td>75</td>
<td>2 x 10^6</td>
<td>100</td>
<td>16</td>
<td>36</td>
</tr>
<tr>
<td>Pig</td>
<td>72</td>
<td>2 x 10^6</td>
<td>83</td>
<td>30</td>
<td>35</td>
</tr>
</tbody>
</table>

Data from (5) & (6).
Both man and the pig are multipapillate, produce urine of similar osmolality, and have GFR values, numbers of glomeruli per kidney and nephron dimensions that are comparable (4-6). The pig is thus an attractive model to study clinically important nephrotoxicity, particularly RPN. These preliminary findings show that BEA causes a RPN in the pig kidney, which is morphologically similar to that in the Wistar rat.

METHODS

Four female Large White pigs, six months old (weight 50 kg), were given freshly prepared 2-bromoethanamine (BEA) hydrobromide (BDH, Poole, Dorset, UK) by a slow iv infusion (over 2-3 minutes) while anaesthetised with a mixture of 2-3% halothane, ±30% nitrous oxide plus ±70% oxygen. BEA was given at dose levels of 50 (n = 2) or 100 mg/kg (n = 2). The animals were allowed access to food and water and monitored daily for any obvious signs of distress.

After 7 days the animals (n = 3) were killed after an overdose of halothane anaesthesia and autopsied. The kidneys, ureters and bladder were removed intact. The kidneys were then bisected longitudinally along the lateral border through the hilus. All tissues were fixed in calcium formaldehyde (4°C) for 72 hr. Control kidneys and bladder were obtained from abattoir material.

The cut interior surfaces of the kidneys were examined macroscopically and tissue blocks of renal pyramids were prepared to include the papilla tip as described by Lomax-Smith and Seymour (7). Together with pieces of ureter and bladder, the blocks were processed and embedded in glycol methacrylate resin (JB-4, Polysciences, Northampton, UK). Semi-thin sections (1 μm) were routinely stained with Haematoxylin and Eosin, Giemsa and Toluidine Blue, using modified standard methods (8).

RESULTS

General observations. Neither animal given 50 mg/kg BEA showed any signs of distress, but those receiving 100 mg/kg had an emetic response 15 min after recovering from the anaesthetic. This lasted for several hr, and 1 animal died (from unknown causes) approximately 24 hr later.

Macroscopic changes. There were prominent casts in the medullary rays, and dark areas at the papilla tips from which the epithelium covering had exfoliated in pigs given the high dose. There were no pronounced changes apparent in the pigs given 50 mg/kg BEA.

Microscopic changes. Interstitial cells, the interstitial cell matrix and columnar collecting duct epithelium in control papillae showed no abnormal histology (Figure 1). Morphological changes that were concomitant with the onset of early RPN were present after dosing with 50 and 100 mg/kg BEA (Figure 2 a-d). These included disruption of interstitial matrix (Figures 2a,b), focal interstitial cell necrosis (Figures 2b,d), exfoliation of the papilla covering epithelium (Figure 2c), and necrosis of collecting duct epithelial cells (Figure 2c,d). These changes were more pronounced in the animals given 100 mg/kg BEA.

DISCUSSION

This study shows that BEA causes a dose related RPN in the pig. The morphological changes observed were essentially the same as in the rat and included foci of interstitial cell necrosis together with disruption of
Figure 1  Semi-thin section of control papilla tip showing intact interstitial matrix and collecting ducts. Giemsa. Bar = 40 um.

Figure 2  a Papilla tip from pig kidney 7 days after single iv dose BEA (100 mg/kg) showing disruption of interstitial matrix (arrowhead) with a decrease in staining. Giemsa. Bar = 100um.  b Focal interstitial cell necrosis (arrowheads) together with disrupted interstitial matrix (*) and necrotic collecting ducts (arrows) in papilla tip, 7 days after single iv dose of BEA (100 mg/kg). Giemsa. Bar = 40um.  c Papilla tip, 7 days after single iv dose BEA 50 mg/kg showing exfoliated covering epithelium and necrotic collecting duct cells with basophilic nuclei. Giemsa. Bar = 100 um.  d Focal interstitial cell necrosis (arrowheads) and necrotic collecting duct epithelial cells (arrow) in papilla, 7 days after single iv dose BEA (100 mg/kg). Giemsa. Bar = 25 um.
the extra-cellular matrix, loss of the covering epithelia and necrosis of the collecting ducts. In the rat these anatomical elements are also the earliest to show degenerative changes, but the lesion was less pronounced in the pig compared to Wistar rats given the same dose.

The decreased effects of BEA on the pig medulla may be due to a number of causes. The multipapillate anatomy of the pig kidney may be less prone to the papillotoxic effects because BEA is not "concentrated" in a single papillae as it is in the rat, or because the urine concentrating capacity of the pig is less than that of the rat (5). Alternatively, both the hepatic and renal metabolism of several chemicals differs in rats and pigs (9,10). These and other factors may account for the reduced severity of the renal lesion in the pig and serves to illustrate marked species differences in the renal handling of nephrotoxic compounds in the multipapillate kidney. Thus in conclusion BEA is less papillotoxic to the pig, but there is a greater systemic toxicity at high doses.

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Reprinted from:

Nephrotoxicity in the experimental and clinical situation

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THE APPLICATION OF HISTOCHEMISTRY AT THE LIGHT MICROSCOPIC LEVEL TO THE STUDY OF NEPHROTOXICITY

P.H. BACH, N.J GREGG AND E.D. WACHSMUTH

I. INTRODUCTION

Light microscopy continues to represent the major method by which nephropathies are identified in chemical safety assessment and in the clinical situation. While ultrastructural studies provide a very important technique for detailed subcellular investigations, they generally do not contribute to diagnosis or treatment. Furthermore, these specialized methods are both time-consuming and costly. Histochemical techniques at the light microscopic level provide a broad approach to the study of renal injury that cannot, at present, be investigated as conveniently by electron microscopy or any biochemical method. Haematoxylin and eosin (H&E) is the routine histochemical stain used to visualize cells, cellular structures and changes associated with tissue injury. Once cell changes have been identified by H&E a host of other histochemical techniques can be applied to help interpret the cause(s) of a lesion. There are, however, examples where subtle or specific cellular changes have not been identified by H&E, and hence more sophisticated techniques are needed. This full range of "routine" to highly "specialized" techniques can all be used to address the question of the molecular changes associated with a toxic insult.

There is also a need to understand more about the factors involved in the degenerative changes that follow a primary renal lesion. These often occur in a discrete anatomical region of the kidney, and subsequently lead to the involvement of other areas. Histochemical methods may help to define and inter-relate the cascade of degenerative changes that follow primary injury and provide information on morphology, cell constituents and biochemistry, from which the "final" renal lesion can be understood.

Normal renal morphology at the light microscopic level has been reported by Moffat and Kriz for the rat, Kaissling and
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Kriz for the rabbit and by Kriz and Koepsell for the mouse. This chapter will discuss those applications of renal histochemistry that have been useful in identifying, localizing and understanding a variety of chemically induced renal lesions. Both conventional histochemical methods and the specific receptor-mediated techniques (e.g. antibody, lectin, radiolabelled chemicals) will be considered. Where appropriate attention will be focused on how these techniques can be used to help understand the primary lesion.

A. Nomenclature and inter-relating histochemical and biochemical changes

This chapter will use the term histochemistry to cover those techniques that involve the microscopic assessment of tissue "biochemistry". Immunohistochemistry is a sub-branch which is highly selective and sensitive. The microdissection of material from the different regions of the nephron will not be considered.

There is a great deal of difficulty in inter-relating the findings of histochemistry with those from pure biochemistry. This is not surprising because of the difficulties in analysing materials in a complex environment such as tissue sections. Also the mass of the material available is small and instrumentation to quantify data is extended to its limits. There are a number of analytical constraints using tissue sections that have been exposed to various treatments (e.g. fixation, freeze-drying, embedding, etc.), to conserve morphological features.

Another problem in interpreting histochemistry is the inconsistency in terminology. The often confused nomenclature for complex carbohydrates has highlighted the need to exercise caution in naming material assessed by histochemistry in biochemical terms. Similarly, "lipid" material may be a measure of hydrophilicity; the high affinities of a binding site present on an antibody are of little value if the antigen is masked; even a monoclonal antibody may bind to non-specific sites and the binding of lectins may be changed by a number of factors. It is well established that proteolytic or cytolytic processes accompany cell damage, and the associated changes may destroy or unmask reactive sites, or alter the cell's microenvironment. Histochemical methods may, however, cause reproducible artefacts, which are of value for diagnosis and may even lead to the better understanding of the pathomechanism of injury. Validation of each method is needed for the most relevant interpretation of data, and several criteria have been put forward. Thus all data derived from histochemical methods should be interpreted with caution. It is also necessary to understand the potential and limitations of each of the histochemical methods being used.

B. Frozen or fixed tissue

Different techniques of preparing tissue may affect histochemical methods because of the unique chemical process upon which each functional group or biological activity is based. Increasingly, two
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or more histochemical methods are used in tandem (either on serial sections or the same section), thus the possibility can arise of an optimal fixation procedure for one method that precludes the use of other techniques.

II. ENZYMES

Enzymes have been determined in nephron segments by microdissection using fluorimetric or radiochemical assay methods, or by incubating whole kidney sections with substrates that are specific for the particular enzyme (together with a coupling reagent if necessary).

Table 1 Typical enzyme activities that can be shown in the kidney by several different methods

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid phosphatase</td>
<td>13-15</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>13,14,16</td>
</tr>
<tr>
<td>D-Amino acid oxidase</td>
<td>17</td>
</tr>
<tr>
<td>Aminopeptidase</td>
<td>16,18,19</td>
</tr>
<tr>
<td>Mg²⁺-ATPase</td>
<td>14,20</td>
</tr>
<tr>
<td>Carbonic anhydrase</td>
<td>21,22</td>
</tr>
<tr>
<td>Cytochrome oxidase</td>
<td>23</td>
</tr>
<tr>
<td>Diaphorase</td>
<td>24,25</td>
</tr>
<tr>
<td>Glucose-6-phosphatase</td>
<td>26</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>23</td>
</tr>
<tr>
<td>β-Glucuronidase</td>
<td>13</td>
</tr>
<tr>
<td>Gamma-glutamyl-transpeptidase</td>
<td>16,27</td>
</tr>
<tr>
<td>Glutamic dehydrogenase</td>
<td>28</td>
</tr>
<tr>
<td>β-Hydroxybutyric dehydrogenase</td>
<td>24</td>
</tr>
<tr>
<td>Inosine 5'-diphosphatase</td>
<td>29</td>
</tr>
<tr>
<td>Invertase</td>
<td>30</td>
</tr>
<tr>
<td>Lactic dehydrogenase</td>
<td>31</td>
</tr>
<tr>
<td>Leucine aminopeptidase</td>
<td>32</td>
</tr>
<tr>
<td>Non-specific esterase</td>
<td>13</td>
</tr>
<tr>
<td>Succinic dehydrogenase</td>
<td>23,25,33,34</td>
</tr>
</tbody>
</table>
NEPHROTOXICITY IN THE EXPERIMENTAL AND CLINICAL SITUATION

The final reaction product (FRP), gives data on the distribution and relative activity of the enzyme if it has adequate contrast and is localized to the site of formation. Traditionally enzyme histochemistry has been undertaken in fresh frozen or fixed sections, where the range of enzymic activities that have been assayed are very large (Table 1).

Pitfalls in using the enzyme histochemical approach are numerous. For example, lactic dehydrogenase (LDH) isoenzymes type A4 (M-LDH) diffuses approximately six times slower than type E4 (H-LDH) in muscle and since the molar substrate turnover of M-LDH is twice that of H-LDH it will produce more FRP, even if its concentration at the tissue site had been originally smaller. Both of these isoenzymes are present in the different parts of the kidney, and failure to differentiate between the two could lead to erroneous conclusions. Intracellular peptidases appear to be less affected by pathological changes than brush-border membrane peptidases, but the difference may not become apparent due to intracellular enzyme diffusion artefacts.

Table 2 The effects of different classes of lytic enzymes on the normal expression of renal membrane bound marker enzymes

<table>
<thead>
<tr>
<th>Renal marker enzymes</th>
<th>5'-Nucleotidase</th>
<th>ATPase</th>
<th>Alkaline phosphatase</th>
<th>Leucyl-β-naphthylamidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipolytic enzymes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospholipase C</td>
<td>+++</td>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lipase VII</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glycolytic enzymes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>β-Glucosidase</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Proteolytic enzymes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trypsin</td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>++</td>
</tr>
<tr>
<td>Papain</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>++</td>
</tr>
<tr>
<td>Protease</td>
<td>++</td>
<td>+</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>α-Chymotrypsin</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>δ-Chymotrypsin</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>Collagenase</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

0 = No changes, + = slight, ++ = moderate and +++ = strong decrease in activity compared to controls.

The interpretation of changes in the amount of FRP also presents a problem, where it is often difficult to be certain whether the increased activities represent de novo synthesis, unmasking, or the loss of factors that normally inhibit the reaction. Hardonk et
al. described the influence of a variety of lytic enzymes on renal membrane-bound markers. Exposure of normal renal cryostat sections to lipolytic, glycolytic and proteolytic enzymes showed that some renal marker enzyme activities were decreased, while others were unaffected (Table 2). By contrast liver sections showed increased enzyme activities. These data illustrate the complexity in interpreting changes induced by similar enzymes.

Another problem that pervades enzyme histochemistry is that cryostat sections less than 4 μm can rarely be cut from large pieces of tissue, and more often sections are 8-10 μm, as a result of which microscopic resolution may be unsatisfactory. The introduction of the hydrophilic methacrylate-based embedding media has facilitated production of 1 μm sections routinely. Provided mild fixation protocols are followed (see below) an unknown fraction of the original enzymic activity may be maintained, which (if it is more than 10%) suffices for histochemistry. Fixation may, however, totally change enzyme distribution compared to frozen sections. The alkaline phosphatase staining pattern in proximal tubules may be reversed by fixing. Similarly, aminopeptidase is localized to the brush-border when assessed in frozen sections by the localization of FRP and antibodies directed against the enzyme, but the cytoplasm of low-temperature fixed tissue. Alternatively, other enzymes such as human carbonic anhydrase are not greatly altered.

A. Frozen sections

The proximal tubules can be identified by the wide variety of brush-border membrane enzymes, but other nephron segments (such as the distal tubule or the connecting and collecting ducts), have specific immunologically reactive markers (see below). A variety of other oxidative enzymes have been characterized in the kidney, notably the medullary collecting duct and interstitial cells. LDH has been widely used as a urinary marker of renal injury, and early studies showed that its distribution in the kidney was ubiquitous, although the collecting duct and proximal tubule stain most strongly. This has been of little use in the histochemical identification of the target of renal injury. The addition of 4 M urea to the incubation system inhibits LDH isoenzyme(s) and has allowed the distribution of renal LDH to be defined in terms of its different activities. The thick ascending limb and the distal tubule are rich in H-LDH, whereas the convoluted tubules of the inner cortex, collecting ducts, glomeruli and vasa recta contain largely M-LDH, a finding that is consistent with immunohistochemical data.

Heterogeneity in histochemistry is also evident in each part of the nephron in different species. Figure 1 illustrates the difference of FRP concentrations of three enzymes in the rat, rabbit, dog, marmoset and baboon kidney. This comparison shows that none of these enzymes are ideal markers for any nephron segment in these species. GGT appears to be the best general marker to identify proximal tubule segments. In addition to species differences, sex-linked, ontogeny and differentiation-dependent differences occur.
Figure 1  Enzyme histochemistry of normal kidney. The concentration of the FRP of a
given enzyme is different in each nephron segment and the distribution is not the
same in different species. The FRP pattern also varies with each enzyme. Frozen,
acetone-fixed serial sections stained for the brush-border enzymes alkaline
phosphatase (APP) and aminopeptidase (AP), and the mitochondrial enzyme succinic
dehydrogenase (SDH). Cortex at the top and outer stripe of outer medulla at the
bottom of each photograph. Arrow head pointing to arteria arcuata. Same magnification
throughout.
Figure 1 continued
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For instance, sex differences in P₁ and P₂ segments of the proximal tubule have been described for various cytoplasmic NAD- and NADP-dependent oxidoreductases and some mitochondrial enzymes in the rat. Differential increases of brush-border membrane enzymes and cytoplasmic enzymes during ontogeny have also been demonstrated.

Renal lesions can be detected by changes in renal (enzyme) catalytic FRP following exposure to a variety of chemicals including mercury, D-serine, and cephaloridine. In general chemical insults may be visualized by a reduced FRP in the area of lesion, but it has been very difficult to interpret, particularly in relation to changes in urinary enzymes. Cottrell and co-workers showed that loss of alkaline phosphatase, lactate dehydrogenase and leucine aminopeptidase FRP all closely paralleled the dose- and time-related damage to the renal cortex caused by mercuric chloride and p-aminophenol. By contrast, increased glucose-6-phosphatase FRP, in the inner cortex (where it is normally not detected), following mercuric chloride-induced injury, was taken as evidence for the utilization of glucose needed for energy production during the repair phase, and supports the concept of cell regeneration. FRP formed by the catalytic activity of succinic dehydrogenase, non-specific esterase, and in particular alkaline phosphatase and aminopeptidase have been shown to be valuable indicators of acute nephrotoxicity after cephaloridine administration to rats and rabbits.

FRP concentrations of a well chosen enzyme may be suitable for defining the site and quantifying the severity of renal lesions. In mice, proximal tubules of the outer stripe of the outer medulla contain twice the concentration of aminopeptidase compared to the cortical tubules. Thus the enzyme cannot be used to demonstrate lesions in the proximal tubule or the proximal convoluted tubule in general, whereas alkaline phosphatase, which does not show this difference in mice and rats, has proved useful. So it can be clearly demonstrated by means of alkaline phosphatase FRP that the P₃ segments of rats, but not of rabbits, are affected by cefsulodin. By contrast, cephaloridine was shown to affect mainly the P₁ and P₂ segments in both species. This can be automated by means of television image analysis which compares areas with high FRP alkaline phosphatase concentrations (e.g. brush-border membrane) within areas of low FRP concentrations (e.g. proximal tubule cross-sections). The loss of alkaline phosphatase FRP is a measure of the number of proximal tubules with lesions. For instance, the area occupied by the proximal tubular marker decreased with increased doses of cephaloridine in rats and rabbits. Pretreatment with probenecid prevented the lesion, in agreement with the protective effects of this anion transport inhibitor, and the area of FRP was closer to controls.

The changes in acid phosphatase and succinic dehydrogenase FRP following a single dose of cis-platin (Figure 2) demonstrate the unsuitability of these two staining techniques for quantification, because the resolution for the different segments is poor, and there is no discrimination on the FRP level between damaged tubules and those with naturally low enzymic activity. In this instance the use of brush-border membrane enzymes is better.
Figure 2  Histochemistry showing the loss of enzymic activity and proximal tubular cell necrosis in the outer stripe of the outer medulla of the male rat kidney over several days after a single i.v. dose of 6 mg/kg cis-platinum. Sections show control (d0), and changes after 2 days (d2) and 6 days (d6). Frozen, acetone-fixed sections stained for the mitochondrial enzyme succinic dehydrogenase (SDH). Baker-fixed frozen sections stained for the lysosomal enzyme acid phosphatase (P). Cortex (C), outer stripe (O) and inner stripe (I) of outer medulla. Same magnification throughout.
Figure 3 Enzyme histochemistry in semi-thin sections of fixed renal tissue.
A. Alkaline phosphatase staining of proximal tubule brush-borders in a 1 μm section of control kidney embedded in glycol methacrylate. Methyl green pyronin counterstain. Insert shows endothelial staining with alkaline phosphatase (arrowheads).
B. Distribution of adenosine triphosphatase (ATPase) in control kidney proximal tubule brush border in cortex only, in 1 μm semi-thin section of glycol methacrylate embedded kidney. Counterstained with methyl green pyronin. C. Gamma-glutamyltranspeptidase (GGT) staining of proximal tubule brush border in control kidney embedded in glycol methacrylate, counterstained with haematoxylin.
Figure 3 D. Increased ATPase staining of endothelium (arrow) in basal sub-urothelial capillaries 144 h after a single 100 mg/kg i.p. dose of 2-bromoethanamine (BEA). Kidney embedded in glycol methacrylate, 1 μm section counterstained with methyl green pyronin. E. Alkaline phosphatase staining of proteinaceous casts in necrotic loops of Henle (arrows) and collecting ducts (arrowheads) in papilla 144 h after single 100 mg/kg i.p. injection of BEA. Kidney embedded in glycol methacrylate counterstained with methyl green pyronin. F. GGT staining in necrotic papilla 144 h after single i.p. injection of BEA (100 mg/kg). Kidney embedded in glycol methacrylate 1 μm section counterstained with haematoxylin.
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Renal enzymic changes may also be associated with abnormal kidney physiology in the absence of an overt lesion. Ammonium chloride (0.25 mol/l) given to rats in drinking water over 6 days induces acidosis and renal enlargement. Histochemically, increased glutamic dehydrogenase FRP was found in the straight and proximal convoluted tubule, whereas in control rats it was seen in the straight portion. This finding is compatible with nephron adaptation or expression of renal functional reserve following exposure to injury or abnormal demands, such as the increased ammonia production in these NH4Cl-loaded animals.

B. Methacrylate-embedded fixed tissue

Glycolmethacrylate has recently been widely adopted for routine high-resolution microscopy, where it is used particularly advantageously for enzyme histochemistry. A large number of fixation protocols have been used, but the common factors for successful use of high-resolution enzyme histochemistry has been low temperature (typically -25 to +4 °C), low concentration of fixative (typically less than 5% glutaraldehyde, paraformaldehyde and/or calcium:formaldehyde) and the minimal period of fixation. Excellent ATPase, acid and alkaline phosphatase, GGT (Figure 3), non-specific esterase, β-glucuronidase, aminopeptidase, and cytochrome C oxidase distribution has been reported and changes have been followed in target selective renal injuries caused by 2-bromoethanamine, indomethacin, adriamycin, hexachlorobutadiene and polybrene.

Similarly, the same high-resolution enzyme histochemistry has been used to demonstrate a number of key marker enzymes in normal and diseased human renal tissue. These include α-naphthylacetate esterase (ANAE), acid and alkaline phosphatase, and ATPase, all of which are confined to the tubule and collecting ducts in the normal kidney following paraformaldehyde fixation. Whereas severely damaged allografts generally retained this staining pattern, there are histochemical differences in renal malignancies. For example, it was possible to differentiate 90% of renal carcinoma based on reduced reactions for acid and alkaline phosphatase, and ANAE. By contrast 90% of Wilms' tumours were weakly positive for acid phosphatase and ANAE. Other enzyme activities (e.g. 5'-nucleotidase) have also been studied in non-renal human tissue using acetone or periodate-lysine paraformaldehyde fixation for 4 h at 4 °C in calcium:formaldehyde.

C. Enzymic changes in parenchymal and urothelial malignancies

Renal cell carcinoma accounts for about 85% of all primary kidney malignancies in man, where the histogenesis is thought to be from the epithelium of the proximal tubule. These carcinomas can be induced in experimental animals by the use of N-hydroxyethylnitrosamine (EHEN) and dimethylnitrosamine. Jaszmin and Riopelle reported that the enzymic profile of the tumours
suggested their proximal tubular origins, although glucose-6-phosphatase, 5'-nucleotidase and alkaline phosphatase stained much less intensely than the normal adjacent epithelial cells. Subsequently, there was little GGT and alkaline phosphatase FRP compared to the adjacent tissue\(^{72,73}\). The presence of PAS-positive brush-border on the carcinoma cells also served to confirm their origins\(^{73}\). The GGT-FRP is also much reduced in human and rat fetal cells\(^{74,75}\). Taken together these data suggest that the EHEN-induced neoplasia represents similar undifferentiated and rapidly dividing cells\(^{72}\).

The histochemical changes associated with chemically induced urothelial malignancies are also widely studied. Kunze\(^{6,79}\) and others\(^{80,81}\) have shown the focal loss of alkaline phosphatase from otherwise apparently histologically normal rat bladder urothelial cells follows carcinogenic doses of di-N-butylnitrosamine, N-butyl-N-(4-hydroxybutyl)-nitrosamine (HO-BBN) or N-[4-(5-nitro-2-furyl)-2-thiazoyl]formamide. The loss of alkaline phosphatase was irreversible and occurred after the discontinuation of the carcinogen; it could not therefore be a direct toxic effect. These alkaline phosphatase-free cells are considered to be preneoplastic, and develop into papillomas and carcinomas (Figure 4).

![Figure 4 Mosaic pattern of alkaline phosphatase staining in bladder papilloma from animal initiated with HO-BBN then promoted with BEA and sacrificed 18 weeks later. Arrows show "preneoplastic" areas with focal loss of staining. Bladder embedded in glycol methacrylate 1 µm sections counterstained with methyl green pyronin.](image)

Ozono\(^{82}\) has also shown that a high frequency of GGT-positive cells is present in otherwise normal urothelia after exposure to HO-BBN. GGT-positive cells are already well established as markers for the premalignant changes in other organs\(^{83-89}\). It
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has therefore been generally assumed that the presence of foci of GGT in the urothelia is a sensitive and specific marker of malignancy, especially because these changes are present in nodular hyperplasia and carcinoma that develops after HO-BBN. These enzymic changes may not be pathognomonic indicators of premalignant changes under all circumstances. For example, Vanderlaan et al. suggested that GGT-FRP identifies only advanced carcinoma and large papillomas. Similarly, whereas Kunze has reported a variable reduction in NADPH-diaphorase in about two-thirds of the urothelial cells with reduced alkaline phosphatase activity, Vanderlaan et al. found increased NADPH-diaphorase in focal nodular hyperplasia, under similar experimental conditions.

III. Carbohydrates

A number of different types of carbohydrate material predominate in discrete anatomical areas of the kidney, and the histochemistry of each shows their unique localization. The basement membrane of the proximal tubule, and especially the glomeruli, are filled with a mucopolysaccharide (MPS) matrix, the medulla interstitium is very rich in MPS (Figure 5), and the distal tubule is coated with glycoproteins and glycolipids. The urothelial cells are also covered by mucin or the glycocalyx.

A. Chemical reactions

The individual classes of carbohydrate may, to some extent, be
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differentiated by simple histochemistry. For example, basement membrane, proximal tubule brush-border and surface mucin are rich in poly-vic-glycols and give a high-contrast stain with periodic acid and Schiff (PAS) or methanamine silver$^{91,92}$. Some of the complex carbohydrates, such as Tamm-Horsfall glycoprotein, only stain weakly by this method and immunohistochemistry is much more effective (see below).

1. Mucopolysaccharides

Mucopolysaccharides (MPS), which in biochemical terms are considered to be glycosaminoglycans, and their supramolecular structures, the proteoglycans, are present in:

(i) the glomeruli, where they form an essential part of the glomerular basement membrane, and (through their polyanionic nature) impart permselectivity to the glomerular apparatus;

(ii) the medulla, where the very extensive quantities of MPS represent a tissue of transition for binding water and cations that are in the process of being reabsorbed.

(iii) The binding capacity for both water and cations is very high and if the matrix is disrupted (see below) the homeostasis of water and electrolyte will be markedly altered.

A number of histochemical methods have been used to show the strongly acidic nature of this matrix$^{92,93}$. The histochemical demonstration of these molecules depends to a significant extent on the method used for fixing tissue$^{94}$, and it may be preferable to the use of cetylpyridinium chloride to insolubilize MPS$^{95}$.

Several early publications reported that the decrease of MPS staining by the addition of magnesium chloride could be used to identify the type of glycosaminoglycan in biochemical terms$^{96}$. This is not the case. On the other hand, selective enzymic digestion$^{97,98}$ of MPS reported for non-renal tissue may help define the presence of a specific glycosaminoglycan in a matrix.

The autoradiographic distribution of labelled glycosaminoglycan precursors may not help substantiate the presence of MPS$^{99}$, because these molecules are also taken up into glycoproteins, and the carbohydrates may be extensively metabolized to other molecules.

(a) Disruption of glomerular carbohydrate. The glomerular basement membrane is thickened in diabetes and nephrotic syndrome when assessed by the PAS stain$^{100}$. The proteinuria associated with a number of chemical insults may be caused by damage to the glomerular basement membrane, altering the foot process and the loss of the polyanionic matrix. This contributes to a loss of permselectivity and the increased leakage of proteins. For example, adriamycin causes a proteinuria that may result from the disruption of the basement membrane-related MPS. Bertani et al.$^{101}$ have shown that within 3 h of adriamycin administration the polyanionic sites of the glomerular epithelial cell decrease progres-
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sively, a change that precedes proteinuria and ultrastructural abnormalities.

(b) Disruption of medullary MPS. The medullary MPS serves as a tissue of transition for water and electrolyte homeostasis, supports the delicate elements of the microvasculature and loops of Henle, and provides some support for the collecting ducts. Loss of medullary MPS histochemical staining has been observed in several instances. For example, McAuliffe\textsuperscript{102} has shown that the intensity of the medullary interstitial MPS staining is greatly reduced in the homozygous Brattleboro rat, but returns to near normal if these animals are treated with antidiuretic hormone. Similarly, the interstitial matrix MPS staining is greatly reduced in rodents with lithium-induced nephropathy\textsuperscript{103}. Such observations are consistent with lithium blocking the actions of antidiuretic hormone, but appear to be a secondary consequence of the lithium toxicity rather than a cause.

The loss of MPS staining from the medulla has also been reported in association with renal papillary necrosis. Medullary MPS staining has been reported to be both increased\textsuperscript{104} and absent\textsuperscript{105} in human analgesic abusers. Molland\textsuperscript{106} described a dense fibrillary network of PAS-positive material in animals with an aspirin-induced renal papillary necrosis that became irregular with deeply staining fibres and bodies in the interstitium (see below). Using an acute model of renal papillary necrosis it has been possible to establish that shortly after chemical insult there is a marked increase in the staining of the medullary matrix, at the same time as the earliest changes are taking place in the interstitial cells\textsuperscript{107,108}. Subsequently, a time-dependent loss of medullary staining occurs in those areas where necrosis develops. About 6 h after a dose of 2-bromoethanamine hydrobromide there is a more intense PAS-positive material at the tip of the papilla, which increased to a maximum at 48 h, at which stage the PAS staining in the mid-medulla was decreased. Even when there is a re-epithelialization of the affected area there is a failure to re-establish the presence of the MPS matrix, probably due to the absence of medullary interstitial cells. The loss of non-specific staining could represent either masking of the functional groups, the loss of those chemical moieties responsible for colour reactions or marked physicochemical changes in the glycosaminoglycan/proteoglycan. Recent biochemical studies\textsuperscript{109} have shown that there is a very marked loss of sulphate groups from the medullary matrix and urinary macromolecular carbohydrate turnover showed changes in the molecular weight polydispersion; taken together these data suggest that the matrix is increasingly disrupted, and eventually lost from the medulla.

(c) Epithelial carbohydrate granules. Tucker et al.\textsuperscript{110} and Alroy et al.\textsuperscript{111} have reported the presence of PAS and Alcian Blue positive granules in the human pelvic epithelial cells, where carcinoma was present in the upper ureter, and in cells that had metastasized from these regions. A series of similar changes have been described by Hukill and Vidone\textsuperscript{112} for bladder malignancies. Intracytoplasmic glycogen or intercellular lakes of mucin were common. Similar changes have also been noted associated with renal papillary necrosis induced by aspirin\textsuperscript{106} and 2-bromoethanamine (Figure 6)\textsuperscript{108}. There is also an accumulation of PAS-positive
granules in the cells of the collecting duct and the covering epithelium. These granules appear before cell necrosis, and may therefore represent the autophagic processes. The presence of similar granules in the pelvic and urothelial cells 21 weeks after the induction of an acute papillary necrosis suggests that this change is a long-term aberration of cellular function, especially because they were most marked in those regions where the urothelial dysplasia was greatest.

Figure 6 PAS-positive granules in the superficial layer of ureteric urothelium from animal treated with BEA (100 mg/kg). Ureter embedded in glycol methacrylate 1 μm section counterstained with haematoxylin.

The presence of these granules could be related to the glycosalix, particularly because changes in these complex carbohydrates have been linked to tumorigenesis, and cell surface recognition. These changes in carbohydrate staining may therefore represent early or subtle changes in the urothelial cells that predispose them to abnormal growth patterns. On the other hand these changes may not be indicative of malignancy or hyperplasia, because they also occur in rodents with lithium-induced nephropathy, where amylase digestion suggested the material was glycogen. The marked changes in the medullary matrix caused by renal papillary necrosis and lithium toxicity may therefore reflect the shunting of simple carbohydrate material (normally used for MPS synthesis) into stored glycogen.

2. Glycoproteins

There are a number of other anatomical regions of the kidney in
which the glycoproteins and/or glycolipids predominate. These will be considered below, under immuno- and lectin histochemistry, although it must be appreciated that some of the less selective stains also demonstrate the presence of these molecules.

B. Affinity techniques with lectins

Lectins bind to well-defined sugar residues\textsuperscript{116} wherever these are available; i.e. they occur on glycoproteins, mucopolysaccharides, glycolipids, etc. The cell surface, intracellular and interstitial carbohydrates in fresh or cryostat sections of one cell type may be constant and therefore visualized with specific fluorescent or enzyme (e.g. peroxidase-linked) lectins. This unique relationship has been reported for both human and animal tissue, where the binding of a number of lectins is associated with one or more renal cell types\textsuperscript{117-123} (Table 3).

Table 3 Selective staining of nephron segments by lectins

<table>
<thead>
<tr>
<th>LECTIN</th>
<th>Soybean agglutinin</th>
<th>Winged pea agglutinin</th>
<th>Peanut agglutinin</th>
<th>Luex europeaus</th>
<th>Dolichos biflorus</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPECIES</td>
<td>Rat</td>
<td>Rabbit</td>
<td>Rat</td>
<td>Rabbit</td>
<td>Man</td>
</tr>
<tr>
<td>Glomeruli</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Proximal S\textsubscript{1}</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>tubule S\textsubscript{2}</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>S\textsubscript{3}</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Henle loop</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Distal tubule</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Collecting duct</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Vascular</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>endothelia</td>
<td></td>
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</table>

The binding of peanut agglutinin to the intercalated cells (or dark cells) of the collecting and connecting ducts in cryostat sections of rabbit kidneys is particularly noteworthy\textsuperscript{119}, because there are very few markers for these cells at a light microscopic level. By contrast, Stoward et al.\textsuperscript{124} reported that peanut lectin bound to the brush-border of the rat proximal tubule and collecting ducts, and it was variable in the distal tubule. Glomeruli only reacted positively after sialidase digestion. In the mouse\textsuperscript{125}, however, peanut lectin stained Bowman's capsule, the tubules and basement membrane and the collecting ducts, but the lectin from Dolichos biflorus stained only Bowman's capsule and the collecting ducts. Some selectivity also exists in any single species, but this may be different in another species (see Table 4 for differences in soy bean and winged pea agglutinin). Thus a nephron segment specific
lectin is not available, nor are there any systematic relationships between species, although the mouse, rabbit and man have similar lectin staining patterns, which differ from the rat. Some lectins such as limulin have not yet been studied in a number of different species, and therefore its binding to sialoglycoproteins of the rat glomeruli, and to a lesser degree both the proximal and the distal tubule, may not preclude its usefulness in studying other species.

Table 4 The differentiation between proximal and distal tubules using lectins

<table>
<thead>
<tr>
<th>LECTIN</th>
<th>Species where only one tubule type is stained</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proximal tubule</td>
</tr>
<tr>
<td>Wheat germ</td>
<td>guinea pig, quail, frog,</td>
</tr>
<tr>
<td>Ricinus communis</td>
<td>rabbit, guinea pig, frog</td>
</tr>
<tr>
<td>Soy bean</td>
<td>0</td>
</tr>
<tr>
<td>Peanut</td>
<td>0</td>
</tr>
<tr>
<td>Ulex europeus</td>
<td>guinea pig, rat, frog</td>
</tr>
<tr>
<td>Dolichos biflorus</td>
<td>0</td>
</tr>
</tbody>
</table>

Fixation may drastically alter lectin binding, winged pea agglutinin binds to rat tubular epithelium after fixation in Carnoy solution, whereas only vascular structures were seen after glutaraldehyde. By contrast Faraggiana and co-workers have reported that fixation and wax embedding had little effect on the binding profile of lectin-peroxidase conjugates that were used for human tissue. The lectin from Lotus tetragonolobus bound exclusively to the proximal tubule, while peanut and soy bean lectin were confined to the collecting duct. Wheat germ lectin bound several parenchymal components including the glomerular capillary wall and its podocyte cell coat. These workers also unmasked glycoproteins using sialidase digestion, after which the glomeruli stained with soy bean and peanut lectin. Lectins can also be used on normal semi-thin methacrylate embedded sections, where they have been used coupled to colloidal gold particles or fluorescent probes.

It is reasonable to assume that a variety of pathological processes, such as enzymic degradation of carbohydrates, could alter or destroy lectin receptor sites, whereas loss of lipid or protein from complex macromolecules unmasks different binding sites. The main problem with the use of lectins is that their staining pattern can only be related to the availability, masking and unmasking of specific sugar moieties, and that further molecular
interpretation is precluded.

IV. ANTIGENS

A. Application of antigen-based histochemistry

There has been an increasing interest in the use of antibodies directed against unique or novel characteristics along the nephron. These antigenic determinants may be present on enzymes, glycoproteins or other molecules, associated with membranes or soluble cytosolic constituents. Many of the monoclonal antibodies so far reported react with one or more regions of the nephron and/or parts of the cell. There are some major disadvantages to using antibodies; their production is time-consuming and their specificity may be doubtful, only a few antibodies will detect the same antigen in an unrelated species, and each antibody is a unique entity, the production of which cannot be exactly reproduced. Even when taken from the same source, polyclonal antibodies may vary in titre between batches, and their production depends on the longevity of the animal producing them.

Visualization of the site of antibody binding to the antigenic determination is done by labelled antibody, e.g. labelled with enzymes, fluorescent dyes or radioactive groups. It is evident that proximal tubules can easily and specifically be stained by anti-brush-border membranes, and distal tubules by means of antibodies directed against tonin and Tamm-Horsfall glycoprotein. However, not all enzymic antigens present in the brush border membrane can be demonstrated histochemically, e.g. whereas aminopeptidase binds its antibody alkaline phosphatase does not, which may indicate hidden antigenic determinants.

Provided the concentration is sufficiently high, the antibody will act as a native cross-linking agent in frozen, unfixed sections. The use of high antibody concentrations may, however, be inappropriate due to the presence of antibodies against undesirable antigens giving rise to misleading staining, if the binding site is not specifically monitored. For example, monospecific anti-renin diluted 1:1000 not only stained the juxtedudillary apparatus in mice, but also stained the epithelial cells of the afferent arterioles and some cells of the proximal tubule and collecting duct. A 10-fold dilution resulted in the loss of proximal tubule and collecting duct staining, whereas an additional 100-fold dilution (i.e. 1:1,000,000) resulted in the disappearance of the arteriole staining. These data were interpreted in terms of the quantity of renin released from the juxtedudillary apparatus and its uptake by pinocytosis, but equally they may represent decreased binding to the biotransformed product angiotensin.

1. Polyclonal antibodies

Antiserum raised against purified epithelial cells may be useful to stain particular nephron segments, but may contain antibodies with various antigen specificities. If only one of these reacted with
antigenic determinants shared by cells of other nephron segments or the interstitium, the staining is not specific. If, on the other hand, the specificity of an antiserum has been established, antigen concentrations can be quantified in sections using antibody concentrations close to saturation levels. This approach is different from the conventional one, which aims at the number of antigenic sites by diluting out the antiserum.

2. Monoclonal antibodies

More recently, specific renal antibodies have been produced by cloning techniques, and appear to be the most promising technique for detecting specifically defined antigenic determinants in renal sections. The applicability of monoclonal antibodies may be restricted if the density of antigenic determinants is too low.

Figure 7 Immunohistochemistry of normal male rat kidney showing the different concentrations of the cytoplasmic isoenzymes aldolase-A and aldolase-B within and between nephron segments. Frozen, acetone-fixed serial sections stained for aldolase (ALD) activity after treatment of sections with antibody against ALD-A and consecutively with antigen ALD-A (A), antibody against ALD-B and antigen ALD-B (B), and both antibodies and antigens (AB). Cortex (C), outer stripe (O) and inner stripe (I) of outer medulla and inner medulla (M).
Figure 8 Enzyme- and immunohistochemical changes in rat kidneys 24 h after a single 1.3 g/kg i.v. dose of cephaloridine. FRP of enzymes is normally only present in proximal tubules, but following cephaloridine insult it is also demonstrated in distal tubules where it is associated with hyaline casts. Frozen, acetone-fixed sections stained for the brush-border enzymes alkaline phosphatase (APP) and aminopeptidase (AP), and using immunohistochemistry for the cytoplasmic isoenzymes aldolase-A (A) and B (B). Outer and inner stripe of outer medulla, with stained proximal tubules and casts (top) and serial sections through distal tubules of inner stripe containing FRP of enzymes of the proximal tubule (bottom). Arrowheads point to tubular casts.
Figure 9 Histochemistry of human hypernephroid carcinoma. The FRP concentration of each enzyme is heterogeneously distributed in the tumour cells. Frozen, acetone-fixed serial sections immunohistochemistry stained for aldolase-A (A) and aldolase-B (B) and using enzyme histochemistry for alkaline phosphatase (APP) and aminopeptidase (AP). Tubular lumen (T).
3. Antibodies to enzymes

The use of antibodies is also of advantage in the investigation of enzymes with the same catalytic activity, but different molecular properties, i.e. isoenzymes. The mixed aggregation immunocytochemical technique\(^{35}\) has been used to localize and quantify specifically renal isoenzymes of lactate dehydrogenase\(^{35}\) and aldolase\(^{35}\) in nephron segments. For instance, during renal maturation aldolase-B monomers increase in the proximal tubules of rats, but not in the distal tubules. By contrast, aldolase-A monomers increase in the distal tubules, but not in the proximal tubules\(^{35}\). It can be concluded that renal casts in adult rats that contain mainly aldolase-B monomers are derived from proximal tubules (Figure 7). Similarly, the presence of proximal tubule markers can be shown in hyaline casts after a nephrotic insult (Figure 8). Moreover, isoenzyme determinations may be useful in the study of tumorigenesis (see below) and development\(^{139}\), and help established tumour cell heterogeneity (Figure 9) with other antisera and lectins\(^{122}\).

4. Fixed or frozen sections

Immunohistochemical techniques have been applied to both frozen and fixed sections. Increasing use is being made of semi- and ultra-thin sections of epon- or methacrylate-embedded materials. Ultra-cryostat sections are also being employed, but are beyond the scope of this chapter. The literature on the optimal methods to be used is not consistent and depends on the technique and type of molecule being studied. Generally protease treatment reverses the loss of antigen binding sites caused by a variety of fixation methods\(^{140}\). Increasingly, however, short fixation of fresh tissue is becoming popular, especially at low temperatures for the duration of fixing, dehydration and embedding\(^{141}\).

B. Renal immunomarkers

Immunohistochemical methods have also been used to demonstrate a variety of functional protein epitopes in discrete localizations of the kidney\(^{132,133,137,140-162}\) (Table 5), but very few of these techniques have been applied to help elucidate the mechanisms of nephrotoxicity. There are a number of macromolecules that are present in the glomeruli\(^{163-165}\) (Table 6), some of which are also present throughout the tubular basement membrane. Under pathological conditions a variety of immunodeposits have been observed in a large number of glomerulopathies, and immunofluorescence monitoring provides the standard means of diagnosis. Glomerular immunochanges\(^{166-169}\) are beyond the scope of this chapter. There are several tubular epitopes that warrant special comment because of their importance in studying target cell toxicity. The use of antibodies for assessing arachidonic acid metabolites and the enzymes responsible for their bioconversion is covered below in Section VI.
Table 5 The distribution of structural and functional proteins, exogenous filtered proteins and enzymes in the kidney as assessed by immunohistochemistry

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Distribution</th>
<th>Species</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>Proximal convoluted tubule</td>
<td>Guinea pig</td>
<td>142</td>
</tr>
<tr>
<td></td>
<td>basement membrane</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>apical vesicles and</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>lysosomes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aminopeptidase IV</td>
<td>Proximal tubule brush-border</td>
<td>Rat</td>
<td>143</td>
</tr>
<tr>
<td>Na⁺,K⁺-ATPase</td>
<td>Proximal tubule weak</td>
<td>Mouse</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>basolateral</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Distal tubule strong</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>basolateral</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Absent intercalated cells</td>
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</tr>
<tr>
<td>Atrial natriuretic factor</td>
<td>Intercalated cells of the</td>
<td>Rat</td>
<td>145</td>
</tr>
<tr>
<td></td>
<td>collecting ducts - homogeneous</td>
<td></td>
<td></td>
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<td></td>
<td>in some and apical in others</td>
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<tr>
<td>Carbonic anhydrase</td>
<td>Weakly in the proximal tubule</td>
<td>Rat</td>
<td>146</td>
</tr>
<tr>
<td></td>
<td>Strongly in the loop of Henle</td>
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<tr>
<td></td>
<td>Strongly in the collecting ducts</td>
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<tr>
<td></td>
<td>Distal convoluted tubule -</td>
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<tr>
<td></td>
<td>a mosaic of very strong and</td>
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<tr>
<td></td>
<td>absent in adjacent cells</td>
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<td></td>
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<tr>
<td>Carbonic anhydrase</td>
<td>Only isoenzyme II in the loop</td>
<td>Rat</td>
<td>146</td>
</tr>
<tr>
<td>isoenzymes</td>
<td>of Henle and distal nephron</td>
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<td></td>
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<tr>
<td>Cathepsin D</td>
<td>Cortical and medullary</td>
<td>Rat</td>
<td>147</td>
</tr>
<tr>
<td></td>
<td>collecting ducts</td>
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<tr>
<td></td>
<td>Mesangial cells</td>
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<tr>
<td></td>
<td>Proximal tubule weak</td>
<td></td>
<td></td>
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<tr>
<td>Clathrin</td>
<td>Apical portion of the</td>
<td>Rat</td>
<td>148</td>
</tr>
<tr>
<td></td>
<td>proximal tubule</td>
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<td></td>
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<tr>
<td>Enoyl-CoA hydratase</td>
<td>Proximal and distal epithelial</td>
<td>Rat</td>
<td>149</td>
</tr>
<tr>
<td></td>
<td>cell mitochondria</td>
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<td></td>
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<tr>
<td></td>
<td>Absent from glomeruli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heat-stable</td>
<td>Proximal and distal epithelial</td>
<td>Rat</td>
<td>149</td>
</tr>
<tr>
<td></td>
<td>cell mitochondria</td>
<td></td>
<td></td>
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<tr>
<td>Heat-labile</td>
<td>Proximal tubule epithelial</td>
<td>Rat</td>
<td>149</td>
</tr>
<tr>
<td></td>
<td>cells</td>
<td></td>
<td></td>
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<tr>
<td>Ferredoxin*</td>
<td>Glomerulus and proximal</td>
<td>Chick</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>convoluted tubule</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Glucosidase F₁</td>
<td>Proximal convoluted tubule</td>
<td>Human</td>
<td>151</td>
</tr>
<tr>
<td></td>
<td>brush-border and loop of Henle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kallikrein</td>
<td>Distal tubule cytoplasm</td>
<td>Rat</td>
<td>152,153</td>
</tr>
<tr>
<td></td>
<td>Sometimes vascular poles</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>glomeruli and collecting ducts</td>
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<td></td>
<td>Apical regions some distal tubules</td>
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<td></td>
<td>Reabsorption droplets proximal tubules</td>
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<td></td>
<td>Some collecting ducts in medulla</td>
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<tr>
<td>Characteristic</td>
<td>Distribution</td>
<td>Species</td>
<td>References</td>
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<td>------------------------</td>
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<tr>
<td>Metallothionein</td>
<td>Epithelia of collecting duct and distal convoluted tubule in controls</td>
<td>Rat</td>
<td>154</td>
</tr>
<tr>
<td></td>
<td>After Cd loading - strong proximal convoluted and collecting duct epithelia staining in both nuclei and cytoplasm</td>
<td></td>
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<td></td>
<td>Weak to moderate staining in glomerular mesangial and visceral epithelial cells and vascular smooth muscle cells</td>
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<td></td>
<td>There was no staining in vascular endothelial cells</td>
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<tr>
<td>Renin</td>
<td>Juxtamedullary apparatus</td>
<td>Mouse</td>
<td>155</td>
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<tr>
<td></td>
<td>Epithelioid cells of the juxtamedullary apparatus</td>
<td>Rabbit</td>
<td>156</td>
</tr>
<tr>
<td></td>
<td>Dog</td>
<td>156</td>
<td></td>
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<tr>
<td></td>
<td>Mouse</td>
<td>157</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Epithelioid cells of the afferent arteriole in the juxtamedullary apparatus</td>
<td>Human</td>
<td>158,159</td>
</tr>
<tr>
<td>Cu-Zn superoxide</td>
<td>Thick ascending limb of the loop of Henle</td>
<td>Dog</td>
<td>160</td>
</tr>
<tr>
<td>dismutase</td>
<td>Proximal convoluted tubule</td>
<td>Rat</td>
<td>161</td>
</tr>
<tr>
<td>Tissue polypeptide</td>
<td>Strongly positive lining renal pyramid thin segment of loop of Henle collecting ducts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>antigen</td>
<td>Weakly positive parietal cells of Bowman's capsule</td>
<td>Human</td>
<td>140</td>
</tr>
<tr>
<td>Trehalase</td>
<td>Proximal tubule brush-border</td>
<td>Rabbit</td>
<td>162</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>162</td>
<td></td>
</tr>
</tbody>
</table>

* Iron-sulphur part of 25-hydroxy-vitamin-D₃-hydroxylase.
** Also stains most extra-renal tissue.
Table 6 The distribution of structural proteins in the kidney glomeruli as assessed by immunohistochemistry

<table>
<thead>
<tr>
<th>Structural protein</th>
<th>Distribution</th>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen type IV</td>
<td>GBM* laminae densa Mesangial matrix</td>
<td>Rat</td>
<td>163</td>
</tr>
<tr>
<td>Entactin</td>
<td>GBM peripheral capillary loops Tubular basement membrane</td>
<td>Rat</td>
<td>164</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Strongly mesangial matrix GBM laminae rara at the endothelial-mesangial interface</td>
<td>Rat</td>
<td>163</td>
</tr>
<tr>
<td>Glycoprotein GP-2</td>
<td>GBM tubular basement membrane</td>
<td>Guinea pig</td>
<td>165</td>
</tr>
<tr>
<td>Laminin</td>
<td>GBM lamina rara Mesangial matrix GBM peripheral capillary loops Tubular basement membrane</td>
<td>Rat</td>
<td>163</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
*GBM = Glomerular basement membrane

1. Cytochrome P-450

The role of oxidative metabolism in the generation of biologically reactive intermediates has received much attention\textsuperscript{170,171}, and anti-cytochrome P-450 has been used to show the localization to the proximal tubule, particularly the P\textsubscript{2} and P\textsubscript{3} segments, and the induction following exposure to dioxin\textsuperscript{172,173}.

2. Ligandin and brush border antibodies

Ligandin or glutathione-S-transferase B is located in the proximal tubule of both animals and man\textsuperscript{174-176} and the thick limb of the loop of Henle in man\textsuperscript{176,177}. Similarly the distribution of anti-aminopeptidase to the brush-border has been described for several species\textsuperscript{18,36,59} and brush-border antigens in rats\textsuperscript{178,179}.  

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3. Tamm-Horsfall glycoprotein and other distal tubular macromolecules

Tamm-Horsfall glycoprotein (THG) is localized to the distal nephron, where it plays an essential, but not yet fully understood, role in urinary concentration\textsuperscript{183,180}. The distribution of this glycoprotein is perturbed by a number of nephrotoxins. Potassium dichromate\textsuperscript{180} caused a biphasic release of THG and deposits along the luminal borders of the epithelial cells within 12 h, loss from the distal nephron and an increase in the number and size of casts from 48 h after dosing. Luminal casts accounted for all of the THG-positive staining material by 144 h, but distal epithelial THG increased from 192 h, and the distribution was normal by 14 days.

\textbf{Figure 10} Aggregates of immunohistochemically positive Tamm-Horsfall glycoprotein hyaline tubular casts present in medullary collecting ducts 7 days after the induction of an acute papillary necrosis using 100 mg/kg i.p. of BPA.
THG is also lost from the distal nephron at an early time point during the course of development of an acutely induced renal papillary necrosis (Figure 10)\textsuperscript{181}. Only later when the medullary MPS staining had been lost do large casts of THG positive material deposit, especially in the collecting ducts and ducts of Bellini in the necrosed areas, where they are associated with cellular debris\textsuperscript{181}. The nephrons that appear to feed blocked collecting ducts are generally dilated. Some of the THG-positive material is extravasated, and many of the superficial glomeruli thus affected have THG-positive material in Bowman's space. The presence of THG in Bowman's space may be related to glomerular sclerosis\textsuperscript{182}, following an acutely induced medullary injury. Alkaline phosphatase-, ATPase- and GGT-FRP are present in these casts, which supports the idea that there are proximal tubular changes during the development, or as a result of renal papillary necrosis\textsuperscript{188}.

There are several examples where the cross-selectivity of antibodies to proteins derived from different species has been useful. For example, much of the research on the changes in animal THG has been based on the use of antibodies raised to human THG. There are also instances where data that are difficult to interpret have been generated from the application of antibodies to tissue other than the one of interest. Pich et al.\textsuperscript{183} reported the very strong binding of an anti-human casein antibody to the mammary glands and sweat gland, and also the distal tubule and the collecting duct. The unexpected renal distribution of this protein suggests that it may be involved in the control of electrolyte, water and other fluid movement. Identification of the antigen had not been undertaken and cross-reactivity with other antigens could not be excluded.

THG and the anti-casein positive material are not the only proteins that function in different regions of the body to modulate ion and water permeability and transport. Molin et al.\textsuperscript{184} showed that antibodies to the α-subunit of the S-100 protein (originally found in the central nervous system) also stained the thin limb of the loop of Henle, and the connecting and collecting ducts in the rat. No other part of the kidney or urothelium reacted positively to this antibody, and the antibody to the β-subunit of S-100 did not react with the kidney or urothelium. The distribution of S-100 in the kidney closely parallels the distribution of the carbonic anhydrase - isoenzyme C\textsuperscript{184}. The S-100 protein has been strongly implicated in calcium binding, and its presence in the distal part of the nephron suggests that it plays some role in modulating Ca\textsuperscript{2+} reabsorption, perhaps similar to the role played by THG in Na\textsuperscript{+} uptake. A vitamin D-induced calcium-binding protein (first described in the chick intestine) has also been reported in the rabbit, rat and chick kidney\textsuperscript{185,185a}. There were some species differences, but in general this calcium-binding protein appeared to be distributed almost identically with that described for the S-100. The potential perturbation of these novel proteins by chemicals, and the resulting disruption of calcium and other electrolyte homeostasis, warrants further investigation. At present there are no data to confirm that the S-100 and vitamin D-dependent calcium-binding protein are the same molecules, and very little is known.
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about the relationship of either of these to THG.

4. Renin and kallikrein distribution

There are a number of other enzymes that play major roles in renal homeostasis, the most important of which are renin and kallikrein. These two enzymes produce essentially opposite functional effects in the kidney. Renin-secreting cells appear to be on the outer aspects of the vessel wall, and immunoreactive angiotensin II is present in high concentrations with renin granules, and is therefore assumed to be excreted with them. Drstavik and Inagami showed that the localization of the individual mediators is separate; whereas kallikrein is localized in the thick ascending limb of the loop of Henle (up to the distal tubule), renin was always associated quite separately in the epithelioid cells of the afferent arteriole. Renin is also localized to this position in the normal human kidney. More importantly, in those kidneys with ischaemic injury or Bartter's syndrome the renin-positive epithelial cells showed increased staining intensity, and in afferent arterioles that were some distance from glomeruli. The pattern of staining was normal in localized parenchymal areas where ischaemic injury had not occurred and in other types of nephropathy, even when plasma renin levels were high. This novel observation needs to be confirmed in the experimental situation, where this abnormal distribution may help identify those injuries where chemicals cause a direct or indirect anoxia of the renal parenchyma. It may also give some indication of the mechanisms involved and the consequences of renal ischaemic injury.

5. $\alpha_2U$-Globulin distribution in the kidney

The development of renal carcinoma in only male rats exposed to branched-chain light hydrocarbons has heightened interest in the hepatic synthesis and renal excretion of $\alpha_2U$-globulin. In the proximal tubule the reabsorption of this low molecular weight protein gives rise to the hyaline droplet that is the characteristic feature of "old rat" and light hydrocarbon nephropathy. Roy and Raber used a rhodamine-linked anti-$\alpha_2U$-globulin to show the distribution in the liver (cytoplasmic in the parenchymal cells) and the kidney, where it was localized to the cells of the proximal tubule, the loops of Henle and the distal tubule. The distribution of rhodamine-labelled $\alpha_2U$-globulin showed the presence of the protein along the length of the nephron. More recently, Simpson et al. used an indirect immunoperoxidase assay and showed that the presence of $\alpha_2U$-globulin was not confined to the hyaline droplets, but was also present in the cytoplasm and lumen of the proximal tubule of male rats treated with the branched-chain hydrocarbon 2,2,4-trimethylpentane. $\alpha_2U$-Globulin is also synthesized by the duct cells of the submaxillary gland, where it is not under sex hormone control, although the protein is immunologically identical to that produced by the liver.

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6. Tumour antigens

Cordon-Cardo et al.\textsuperscript{194} used a variety of human urothelial and renal cancer cell lines to produce monoclonal antibodies which were then shown to be selectively associated with different parts of the nephron. Each of these antibodies was novel and did not cross-react with other previously identified antigens such as THG, fibronectin, laminin, etc. There has been some interest in the potential use of these regio-specific markers to identify areas of renal necrosis using the presence of urinary excreted antigens from damaged cells as a measure of injury.

Monoclonal antibodies raised to cytokeratin polypeptides have been shown to react with various renal sites, including the proximal and distal tubule and the urothelia\textsuperscript{195}. More importantly, the pattern of renal carcinoma and type I and II carcinoma were each unique and different from the rest of the kidney and urothelial tract\textsuperscript{195}. Wang and Krueger\textsuperscript{196} also reported an antibody that is selective for rapidly proliferating cells in different regions of the body. The identification of rapidly dividing cells could be used to show the presence of proliferative bursts of activity that were indicative of repair, hyperplasia or malignancy, especially if combined with other methods.

The ABO isoantigens normally associated with blood groups have also been used to differentiate between normal and non-invasive transitional urothelia carcinoma (which stains for the tissue isoantigen) from invasive carcinoma which frequently did not stain\textsuperscript{197}.

There is also limited clinical data to suggest that anti-ligandin reacts with renal adenocarcinoma, but not with undifferentiated carcinoma, papillary adenoma, well-differentiated papillary adenocarcinoma and Wilm's tumours in man\textsuperscript{197}. Immunoreactive renin-containing cells are present in most renal tumours where they are also intimately associated with blood vessels\textsuperscript{187}. Another series of markers in a very large number of renal tumours is the co-presence of the intermediate-sized filaments of cytokeratin and vimentin. While cytokeratin is present in other parts of the normal kidney, it is never present with vimentin. This suggests that vimentin is expressed as part of the neoplastic transformation\textsuperscript{198}.

G. High-resolution immunohistochemistry

Most of the immunohistochemical methods described make use of wax sections. Generally these are 5 \(\mu\)m or thicker (often 7-10 \(\mu\)m), but the use of special techniques may facilitate semi-thin sections. For example Clyne et al.\textsuperscript{199} reported the immunohistochemical tubular localization of albumin in those patients with proteinuria, where the tissue was freeze-substituted, paraffin-embedded and cut at 0.5 \(\mu\)m.

The use of low-density methacrylate resins has also opened the potential of undertaking immunohistochemistry on semi-thin sections, which provides vastly improved resolution and precise localization of the antigen labelling\textsuperscript{67}. There is loss of antigenicity as a result of the processes involved in embedding material in
glycol methacrylate resin. Hemming et al.\textsuperscript{200} reported that cryosections used an antibody titre that was 1000 more dilute than that necessary for semi-thin sections, but the superior morphological detail seen in the methacrylate material made it most useful. Several of the technical difficulties associated with low-temperature embedding of tissue have been made more simple by the device reported by Wells\textsuperscript{201}.

The most important criterion for successful immunohistochemistry in methacrylate material is the use of low-temperature fixation and processing to preserve both morphology and antigenicity\textsuperscript{67,141,168,200,202-207}. Protease treatment helps to improve the availability of antigens\textsuperscript{67,168}, but these tend to be the extracellular binding sites, and etching the resin may be necessary to detect intracellular antigens. The double antibody technique\textsuperscript{208} has been used successfully to contrast antigens in glycol methacrylate, where the secondary antibody has included immunogold\textsuperscript{209}, fluorescent labels\textsuperscript{168,206}, avidin-biotin-peroxidase\textsuperscript{67} and peroxidase-anti-peroxidase\textsuperscript{200}. In general, the most successful application of immunohistochemical methods to glycol methacrylate has been with those antigens that are most resistant to fixation\textsuperscript{67,168,209-211}.

V. LIPIDS

There are many aspects of lipid histochemistry that require more extensive or renewed evaluation, because of the subtle differences that may be introduced by fixation and staining properties of the different lipid stains. Thus fixation procedures have been evaluated for their ability to unmask lipid from lipoproteins\textsuperscript{212} or to stabilize membranes\textsuperscript{213}, and several of the approaches have been compared\textsuperscript{214}. While it is generally appreciated that dehydration for wax or methacrylate embedding will remove lipid, the remaining vacuoles may be interpreted as structures in their own right\textsuperscript{219}. Some lipids are also water-soluble and may be leached out of tissue that has been subjected to prolonged fixation\textsuperscript{214}. The other important aspect that has to be re-evaluated are the differences between adipose fat globlets, fat droplets, the different types of lipid material such as membranes, free neutral and fatty acids, sterols; the more complex lipids such as lipoproteins, glycolipids and also the question of cytoplasmic "lipid domains", which show varying degrees of lipophilicity\textsuperscript{212-216}. Depending on the stain used any one or more of these different characteristics can be shown\textsuperscript{216-218} and various artefacts have been described\textsuperscript{215}. Berg\textsuperscript{216} used the now-established carcinogen 3,4-benzpyrene (benzo[a]pyrene) and showed that there was a very strong fluorescence associated with the brush-border and basal filaments (probably the mitochondria of the proximal tubule) in formaldehyde-fixed frozen mouse kidney. Sudanophilia was also present in these epithelial cells. By contrast Oil Red "O" stains the lipid droplets in the interstitial cells of the medulla heavily, but not other parts of the kidney\textsuperscript{219}. The medullary interstitial cells have a very high lipogenic potential and the numerous lipid droplets are rich in polyunsaturated fatty acids, especially those with C18 to C24 chain lengths\textsuperscript{220}. These
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lipid droplets are also apparent as osmophilic material in semi-thin sections.*

A. Abnormalities in lipid distribution following chemical insult

While lipid changes are well described following liver injury there is a paucity of data on nephrotoxicity. The outstanding histochemical changes in patients with analgesic abuse-related renal papillary necrosis is the accumulation of very large quantities of Oil Red "O" positive lipid material. Similar changes have also been reported in experimentally induced papillary necrosis following aspirin and a prolonged essential fatty acid-deficient diet.* More recent studies have shown that these changes also occur in an acutely induced papillary necrosis (Figure 11), where the earliest changes took place in the capillaries.

![Figure 11 Accumulation of lipid in collecting duct epithelial cells in papilla from rat 48 h after single i.p. injection of BEA (100 mg/kg). Fixed-frozen section stained with Oil Red "O". Insert shows lipid accumulation in proteinaceous casts in loops of Henle and covering epithelium of papilla (arrows).](image-url)
There was also a marked accumulation of lipid in the epithelial cells (normally there is no lipid material in these cells as assessed by Oil Red "O"). The epithelial accumulation of lipid material extended into those areas of the outer medulla which were not affected by the papillotoxin and appeared to be normal by routine H&E staining. A comparison with a variety of other nephrotoxic lesions, such as those caused by hexachlorobutadiene, aminoglycosides, cis-platin and polybrene, suggest that the capillary and epithelial deposits of lipid material are pathognomonic for renal papillary necrosis. Chemical assay of the medullary tissue has recently suggested that these histochemical changes represented a phospholipidosis. The greatly increased levels of lipid material in the hyperplastic urothelia may be pathognomonic of, or associated with, malignant or premalignant changes. These have been described in other malignant tissues and in exfoliating urothelial cells.

Other published data suggest that the development of renal lipid changes is associated with specific types of nephrotoxicity. Aminoglycosides cause a proximal tubule phospholipidosis, which in common with other renal lesions caused by chromium, carbon tetrachloride, tetracycline and aflatoxin also caused a localized increase in Oil Red "O" staining. Puromycin aminonucleoside targets selectively for the glomeruli and causes an accumulation of Oil Red "O" material. The rubeanic acid method has also been used to show the increased free fatty acid levels in the nephrotic syndrome. Recently, there has been a report that the immunosuppressive cyclosporin causes an accumulation of Oil Red "O" positive lipid material in cultured proximal tubular cells, although there appears to be no published evidence from histochemical studies on animals treated in vivo to show that similar changes are associated with this nephrotoxicity.

At present there is very little information to explain the increased staining of lipid material in these damaged cells. In the case of aminoglycoside nephrotoxicity the phospholipidosis has been explained on the basis of decreased degradation and lysosomal accumulation of phospholipids as a result of membrane turnover, but obviously other "lipid" changes may represent lipid unmasking, degradation of membranes, and/or accumulation of lipid material due to increased synthesis or decreased utilization in those instances where it is changed.

VI. PROSTANOIDs

The importance of the prostaglandins (PGs) in normal renal function, and their controversial role in the development of a variety of nephropathies such as nephrotic syndrome, renal papillary necrosis and hydronephrosis, have been widely studied. Much of the information on the distribution of prostaglandin metabolism has come from biochemical studies on medullary and cortical tissue slices, isolated glomeruli and cultured cells, and by the use of classical microdissection studies. For example, the presence and distribution of NAD- and NADP-15-hydroxyprostaglandin dehydrogenase, and other enzymes related to arachidonic acid metabolism,
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has been established using tissue fragments from cryostat sections. It is important to stress that the absence of a particular PG or one of the enzymes that metabolize arachidonic acid from any one cell type does not exclude the potential for the cell to produce the arachanoid of interest. Three distinct approaches have been used:

A. Antibodies raised to the enzyme

Smith and Wilkin used an anti-cyclo-oxygenase to show that the distribution of cyclo-oxygenase was confined to the medullary interstitial cells and the collecting ducts in the rat, rabbit and guinea pig, and in the cow and sheep. In the cortex, cyclo-oxygenase antigenicity was localized in the endothelial cells of all arteries and arterioles, and the collecting ducts in the rat, rabbit, guinea pig, cow and sheep. This enzyme was also present in glomerular epithelial cells of the rabbit, cow and sheep.

B. Antibodies raised to the prostaglandins

Mori and Mine used antibodies raised to each of the prostaglandins, and showed the PGE and PGF were present in the cortical and medullary collecting ducts, the medullary interstitial cells, both glomeruli, mesangial and epithelial cells and endothelial cells of the arteries and arterioles. By contrast PG-6-keto-F (the stable metabolite of PGI) was localized in both mesangial and epithelial glomerular cells, and the endothelial cells of arteries and arterioles. The tissue localization of PGA has also been shown to be more marked in the tubular cells of the renal medulla compared to the cortex, but there was no discrete localization. These data suggested that PGA was localized to the cell membrane rather than the cytoplasm as appears to be the case for the other PGs.

C. Substrate oxidation to a chromophore

Janszen and Nugteren based their histochemical method on cyclo-oxygenase-mediated arachidonic acid oxidation of diaminobenzidine (using cyanide as a blocking agent for mitochondrial oxidation). They showed intense staining of the secretory epithelia of seminal vesicles, and especially the renal collecting ducts and medullary interstitial cells. It has proved difficult to confirm that this colour change relates to the enzyme PG cyclo-oxygenase, although the distribution parallels were described by Smith. Treating animals with analgesics or non-steroidal anti-inflammatory drugs, or the addition of these inhibitors of PG synthesis to the incubation medium, did not alter the intensity or formation of polymerized benzidine reaction product. Litwin has suggested that the colour reaction represents "total" peroxidative enzyme activity, based on the fact that diaminobenzidine oxidation is blocked by 3-amino-1,2,4-triazole, a well-established inhibitor of catalase and peroxidase. This was supported by the fact that the
renal enzymic activity, demonstrated by this method, was the same with hydrogen peroxide and arachidonic acid (there was no reaction with unsaturated fatty acids), it is heat-stable and the pH optima parallel other peroxidases. Also, whereas glutathione abolished the colour reaction, catalase had no effect. The main question that remains to be resolved is which of the several possible different peroxidases can be visualized by this method, especially because of the recent importance that has been accorded the role of metabolic activation by these enzymes. Litwin suggested that the activity was "a special peroxidase related to PG synthesis". This suggests PG hydroperoxidase, which together with cyclo-oxygenase forms PG synthase, or a lipoxygenase. Unfortunately the higher activity in the collecting duct compared to the medullary interstitial cells, and the fact that no activity was reported for glomeruli, does not support the biochemical data already available for these enzymes in the different regions of the kidney.

D. Perturbations of prostanoid metabolism in response to nephropathy

Despite the implicated pathophysiological role for these lipids (and their related products) in several nephropathies, and the number of techniques available to study prostanoid metabolism, only very limited published material is available. Smith et al. showed that there was a time-related increase in cyclo-oxygenase staining in the cortical collecting tubule and the loops of Henle of rabbits with surgically induced hydronephrosis. Contrary to expectations there were no vascular or glomerular changes, nor were there any changes in the medullary staining for this enzyme. Hydronephrosis is associated with phospholipidosis and the very marked increase in thromboxane $A_2$ synthesis, which suggests that a full understanding of this change may only come from the application of histochemical, microdissection and other techniques.

VII. OXIDASES AND ANTI-OXIDANTS

The presence of oxidative enzymes includes a series of mixed functional oxidase systems such as the cytochrome P-450 enzymes, the distribution of which is shown by immunohistochemistry (see above). The distribution of cytochrome P-450 can also be shown by direct microspectrophotometric measurement, but this approach does not appear to have been applied to the kidney. There are other oxidase systems in the kidney. Large numbers of peroxisomes are localized in the P3 portion of the proximal tubule, but they are absent from the glomeruli and the distal nephron. There are highly selective methods for their visualization, and their origins and development have been well documented. The full renal functions of the peroxisomes remain ill-defined. It is generally assumed that urate oxidase is responsible for the conversion of uric acid to urea. In addition to urate oxidase, these organelles also contain catalase, and D-amino acid oxidase. D-amino acid oxidase activity has been
demonstrated in fixed and fresh renal proximal tubules using D-proline or D-alanine, peroxidase and diaminobenzidine (among other methods)\textsuperscript{17}, but the physiological function of the enzyme is far from certain. Reddy\textsuperscript{259} has speculated on the role of D-amino acid oxidase in the genesis of the highly localized necrosis to the P\textsubscript{3} region of the nephron following the administration of D-serine. There is also evidence that oxalate and polyamines are oxidized in the proximal tubule peroxisomes\textsuperscript{260}. The presence of these two oxidases is particularly important during incipient renal failure when the filtered load of oxalate and polyamines is known to be high.

The level of catalase is greatest in the proximal tubule, less in the distal tubule and very low in the glomeruli (there were no data on the medulla) in Syrian hamsters. Furthermore, the catalase levels in the proximal tubule were reduced at the same time as diethylstilboestrol-induced renal adenocarcinomas. Subsequent progesterone treatment reversed the carcinogenic effect\textsuperscript{261} and also restored the catalase levels to normal.

Using the controlled staining of frozen sections with mercury orange, Ashgar et al.\textsuperscript{262} showed that glutathione (GSH) was localized to the proximal convoluted tubule. Recently, Chieco and Boon\textsuperscript{263} used low temperatures to decrease the diffusion of the mercury glutathione complex, which also reduced the colour intensity, but this decreased sensitivity was partially offset by the fluorescence of the complex. The "Prussian blue" method of Smith et al.\textsuperscript{263a} forms much more rapidly that the mercury orange complex and will therefore show better localization. It has been used for determining GSH in liver, but produces an artefactual GSH distribution in the kidney, where the medulla stains very intensely. This probably represents staining of the medullary mucopolysaccharide matrix by colloidal iron\textsuperscript{264}. There is little histochemical data on the distribution of other molecules that are likely to protect the cell from the effects of reactive intermediates.

The distribution of superoxide dismutase has recently been established using immunohistochemical techniques, which showed marked species differences in the dog\textsuperscript{160} and rat\textsuperscript{161} (see above). There are, however, at least three distinct types of superoxide dismutase, and until the distribution of each is defined it will be difficult to relate the absence of enzymic activity to target cell injury in the kidney.

VIII. CATECHOLAMINES

A significant proportion of the control of renal blood flow is resident in the nervous system, and the presence of catecholamine-containing neurons has been shown in many areas of the kidney\textsuperscript{265}. These data are generally based on the fluorescence complex formed between formaldehyde and catecholamines using the Falck-Hillarp method\textsuperscript{266}, but this fails to differentiate between chemically distinct representatives within this group. Recently, Dinerstein et al.\textsuperscript{267} showed that norepinephrine-fluorophore fades very rapidly on exposure to HCl vapour, but the dopamine-fluorophore does not. The presence of dopaminergic elements in as-
sociation with the vascular poles of the glomeruli, supports a role for the neuronal control of renal haemodynamics, the release of renin and the related renal changes.\textsuperscript{267}

**IX. HEAVY METALS**

A number of heavy metals are potently nephrotoxic. While autoradiography (see below) has been an important method for studying metal distribution, histochemistry can also be used. Recently the localized renal distribution of mercury to the lysosomes of the proximal tubule has been shown by the silver amplification of the mercury sulphide.\textsuperscript{268} This silver amplification technique could also be applied to other heavy metals, including gold. There are also a number of sensitive chromogenic chelating agents, such as benzothiazolyiazophenol derivatives that bind cadmium very selectively.\textsuperscript{269}

Changes in metallothionein levels are a frequent consequence of heavy metal exposure. The distribution of metallothionein has been shown by immunohistochemical methods\textsuperscript{154} (see above). There are a number of histochemical methods that have been used to demonstrate the presence of macromolecular thiols. Morselt et al.\textsuperscript{270,271} showed a dose- and time-related increase in histochemically stainable macromolecular disulphide granules in the proximal and distal (high dose only) tubules of rats treated with CdCl$_2$. Based on the high disulphide:protein ratio these granules were assumed to be cadmium-thionein. Ultrastructural studies and X-ray microprobe analysis supported high sulphur and cadmium levels and showed that these "granules" were in fact lysosomes.

**X. AUTORADIOGRAPHY**

One of the major advantages of autoradiography is the wide variety of tracer molecules that are available, the ability to study both water-diffusible and "fixed" molecules, and the fact that the technique can be used in tandem with other histochemical methods. Furthermore, the distribution of a host of potentially nephrotoxic radiolabelled molecules can be assessed at both a whole-body and a microscopic level.\textsuperscript{272} The technique can be greatly strengthened by the use of an appropriately labelled precursor, where some degree of certainty can be maintained on the nature of the molecule, and by the selective administration of the label, where localized infusion (rather than the systemic route) will label a tissue of choice. Many of the advantages of autoradiography can be further enhanced by the use of semi-thin sections, particularly because it is then possible to use $^{14}$C-labelled material and still maintain very good localization of distribution at the light microscopic level.\textsuperscript{272a}

The interpretation of autoradiography has been covered in several texts\textsuperscript{273,274}, but may still need careful controls and intelligent consideration. For example the binding of $^{125}$I-insulin to the apical surfaces of the proximal tubule represents the normal handling of filtered peptides and not a hormone receptor.\textsuperscript{275}

Most of the histochemical techniques have the disadvantage of...
dead end-point measurements. In order to obtain kinetic information it is necessary to introduce markers into the animals which will be incorporated into renal cells, i.e. radioactively labelled precursors of DNA (mostly $^3$H-thymidine), and of carbohydrates or proteins. While there are a large number of studies that have used the autoradiographic distribution of nucleic acid precursors, it has not been generally recognized that their incorporation shows some degree of tissue-specificity, that may depend on the distribution of enzymes involved in purine salvaging. For example, the incorporation of uridine (as assessed at the whole-body level) has been reported to be far greater in the kidney than deoxythymidine.$^{276}$

It is possible to obtain information on the duration of S-phase cell cycling in a tissue specimen using the double isotope pulse-labelling technique.$^{277}$ The two DNA-precursors $^3$H- and $^{14}$C-thymidine are pulse-labelled a few hours apart. All the cells in S-phase at the time of both pulses will be dual labelled, while the cells that are at the end of S-phase when the first pulse is given will only be labelled by the first isotope and not the second. Conversely those cells that are in S-phase when the second label is given will only carry that isotope. The duration of the S-phase can be estimated from the time between pulses, and the proportion of cells that have the three different combinations of labelled nuclei. As yet there are no published data on the use of this technique in relation to nephrotoxicity, but it would be appropriate to give an insight into regeneration in different regions of the kidney. Dual labelling has also been used to study the dynamics of metal-protein complex handling by the kidney. Murakami and co-workers$^{278,278a}$ showed that while $^{109}$Cd and $^{125}$I-labelled metallothionein enter the cell together, the $^{109}$Cd is lost at a very early stage.

The effect of some polycyclic aromatic hydrocarbons consists of an inhibition of thymidine incorporation$^{279}$ and that following HgCl$_2$-induced nephrotoxicity the incorporation of amino acids increased, consistent with the morphological criteria of regeneration.$^{280}$ Similarly, an increased incorporation of thymidine into DNA has been described for HgCl$_2$ nephrotoxicity$^{14}$. Using the 1-h $^3$H-thymidine pulse labelling technique it has been possible to show that the proliferative rate of tubular cells is low in adult rats (8 weeks or older), but about twice this rate in 5-week-old rats. In both age groups the area of highest proliferative activity is that of the inner stripe, which is about twice that of the rest of the kidney. Severe proximal tubule damage 24 h after a single dose of cephaloridine is associated with a slight proliferation, and increases up to 4 days with repair (Figure 12). Regeneration is fast, and similar after a single dose or after multiple daily doses. Balazs$^{281}$ has already drawn attention to the resistance of regenerated cells to further toxic insult. Conventional light microscopy of the cephaloridine-induced lesion did not show any dose-dependent abnormalities at the end of the chronic study, but hyperplasia could be assumed on the basis of an increase in organ weight. By contrast chronic dosing with gentamicin produces a dose-related increase in proliferation and severity of the lesion. Thus $^3$H-thymidine pulse labelling allows those nephrotoxic effects where cells develop resistance to multiple insults to be differentiated from toxins that repeatedly damage cells. Similar results were obtained
by Laurent et al.\textsuperscript{282}, using homogenate analyses in a 14-day toxicity studies with gentamicin.

**Figure 12** Autoradiography of the rat kidney 24 h and 48 h after a single 0.8 g/kg i.v. dose of cephaloridine and a 1 h i.p. pulse of \textsuperscript{3}H-thymidine. The number of labelled (proliferating) cells is greatly increased 24 h after the nephrotoxin and is largest in the regenerating tubules by 48 h. Frozen, acetone-fixed sections stained for alkaline phosphatase (APP), Carnoy fixed, PAS-stained autoradiographs in darkfield illumination (R). Arteria arcuata (arrowheads) and some labelled cells (arrows). Note the reduced distribution of FRP in necrotic tubules at 24 h, which is increased in the regenerated tubules at 48 h.
While pulse labelling gives information of the extent of proliferative change at a single time point, it does not give a measure of tissue regeneration. Such information can be obtained by assessing the total number of new cells formed over a period during which $^3$H-thymidine is continuously infused from a mini-osmotic pump, or other device. Using this technique it is possible to show the marked increase in cell labelling within 24 h of a nephrotoxic insult. More importantly, while there is no recognizable renal pathology 5-8 days after a single dose of cephaloridine, the tremendous labelling of cell nuclei shows that the proximal tubule represents almost totally repaired tissue (Figure 13). Other techniques cannot identify the regeneration of relatively resistant cells and there is a need to establish where similar changes escape conventional techniques used in subchronic and chronic toxicity studies.

It is also possible to study the kinetic response of different renal cell types in response to renal injury. Contralateral hypertrophy (in response to uninephrectomy) is up to 4-fold more marked in the cortex compared to the medulla, and this response to the release of renotropic factor is suppressed by water deprivation.

Figure 13 Autoradiography can show the total cell proliferation that has occurred, using $^3$H-thymidine continuously infused s.c. from a mini-osmotic pump 2 to 5 days after the insult, and the rat killed on day 7. There is limited cell proliferation in control rats (A), whereas heavy nuclear labelling occurred after 0.8 g/kg i.v. cephaloridine, (B). Darkfield illumination, G shows glomeruli.
Figure 13 continued (C) Conventional histology fails to identify the presence of regenerated cells (left side) despite the extensive silver grains above regenerated cells (right side). Carnoy fixed, PAS-stained autoradiographs: darkfield photographs of the juxtamedullary junction, showing glomeruli (G), some labelled cells (arrows). Transmitted light for conventional histological evaluation (left side) and the same field in darkfield illumination for demonstration of the labelled nuclei (right side).
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Autoradiography of semi-thin, methacrylate-embedded sections showed that normally the turnover of epithelial cells (infusing \(^{3}\text{H}\)-thymidine at zero order from an implanted mini-osmotic pump for 144 h) differed in the major regions of the kidney. The cell turnover was similar in the proximal and distal tubules, the urothelia covering the papilla and the adjacent pelvic epithelia. A lower labelling index was observed in the ureter and the collecting duct and the lowest was the pelvic fornix. Following an acutely induced papillary necrosis there was a 6-fold increase in the pelvic fornix adjacent to the papillary injury and a 2- to 3-fold increase in the turnover in all other regions.

XI. OTHER HISTOCHEMICAL METHODS FOR ASSESSING THE KIDNEY

A. Renal haemodynamics, glomerular permeability and filtration

Assessment of the renal haemodynamics and glomerular filtration generally includes examination of the whole kidney by blood flow monitoring or gross anatomical distribution of a labelled material. These methods measure regional flow or total clearance and give no data on the distribution within the medulla and cortex. They cannot therefore be used to study the subtle and focal changes that may relate to target-selective injury. Several histochemical methods are available to assess these changes, although these have not been used to follow the time course of those renal injuries where such information would be valuable.

1. Nephron perfusion and microvascular control

The subtle control of kidney microvasculature and the shunting of blood to (or from) different regions of the medulla and cortex present a most fundamental process in normal renal function. This may be altered in nephrotoxic lesions that have been linked to ischaemic injury. The introduction of exogenous particulate material into the renal microvasculature gives some indication of the patency of the vessels and/or the presence of vasoconstriction/occlusion. The morphological methods described below cannot differentiate between stasis and high flow rate areas, nor can they generally identify "leaky" capillaries, an endothelial defect that could play a very important role in disrupting renal compartmentalization.

Colloidal carbon has been used to show the loss of medullary microvascular filling at an advanced stage of ethyleneimine and aspirin-induced renal papillary necrosis. The introduction of this material for assessing vascular filling may present some difficulties. India ink has been used as the common source of colloidal carbon, a variety of additives (phenols, shellac and fish glue) used to enhance its drawing properties, and the colloidal nature of this material imparts a substantial oncotic pressure, both of which may cause artefacts in assessing microvascular filling. These circumvent using India ink that has been dialysed against isotonic saline. Colloidal carbon prepared thus has been used to follow the time course of microvascular changes in animals treated with 2-
bromoethanamine. There was an early shift (2-4 h after dosing) of microvascular filling from the cortex to the outer medulla, after which the filling of the inner medulla was more pronounced, but at the expense of the outer part of the medullary plexus at 8-26 h. These changes coincided with the development of renal papillary necrosis. By 48 h when necrosis was complete the damaged medulla was avascular. During the course of development of RPN, however, the microvasculature was patent in the medullary tissue beyond the regions in which necrosis had occurred. These data were interpreted as showing that an acute medullary necrosis can occur without capillary occlusion\textsuperscript{107}. These observations have also been confirmed by high-resolution microscopy where platelet adherence and microvascular changes did not occur until late in the development of the lesion\textsuperscript{108}.

The inherent difficulties associated with using colloidal carbon as a suitable particulate material for intravascular filling prompted Joris et al.\textsuperscript{231} to use Monastral Blue B (a copper phthalocyanine), the advantages of which are water-insolubility, non-toxicity, uniform size distribution, commercial availability, and high contrast for thin and thick sections. Recently, this method has been used to address the possible role of microvascular occlusion or leakage in the genesis of renal papillary necrosis. The distribution of vascular labelling in semi-thin methacrylate sections showed that glomeruli and pelvic basal epithelia were well labelled (Figure 14). No Monastral Blue B was present in the papillary matrix, data that suggest that the capillary integrity was intact and the leakage of material into the interstitium was not involved in the pathogenesis of renal papillary necrosis\textsuperscript{108}.

**Figure 14** Distribution of Monastral Blue B vascular label throughout kidney. Photomicrograph from en bloc kidney embedded in glycol methacrylate.
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Erythrocytes also offer a natural marker for studying vascular filling, and can be isolated, washed, formaldehyde-fixed and tagged with a fluorescent or radiolabelled marker, but it has been suggested that labelling the erythrocyte impairs its flexibility, and may alter its normal distribution. It is, however, possible to make use of the endogenous erythrocytes, because of several of their unique staining properties. Early studies made use of the benzidine staining technique, and showed the marked blood filling changes in the kidney following 5-hydroxytryptamine (serotonin) exposure, which were consistent with the shunting of blood away from the cortex to the juxtamedullary area. It is generally assumed that this shunting of blood plays an essential role in the development of the ischaemic proximal tubular necrosis that commonly follows high doses of serotonin. The established carcinogenic properties of benzidine preclude its use, but erythrocytes can be contrasted by the presence of enzymes such as esterase, glucose-6-phosphate dehydrogenase, acid or alkaline phosphatase; the high levels of phospholipids shown by the Sudan black method; by a variety of routinely used stains such as Masson's trichrome, Toluidine blue, Giemsa and the high macromolecular thiol levels by the diazotized N-(4-aminophenyl)-maleimide method.

2. Glomerular filtration

Total glomerular filtration may be unchanged in those nephropathies (e.g. renal papillary necrosis) where single nephron glomerular filtration rates are reduced. While it is possible to measure single nephron glomerular filtration rates in superficial nephrons by micropuncture, this technique is very slow and gives no information on the juxtamedullary nephrons. The "Hanssen technique" makes use of the localization of glomerular filtered ferrocyanide to the tubular lumen (which is not reabsorbed or secreted), that has been precipitated by ferric chloride (in the frozen kidney) as Prussian blue. Many workers have then microdissected whole nephron segments and studied the distribution of Prussian blue in relation to nephron length, total glomerular filtration rate and in the diuretic versus normal and/or antidiuretic states. This approach has also been quantitated using radiolabelled ferrocyanide.

There are few reports using this technique to describe the degree of glomerular filtration in nephrons exposed to chemicals. Normally only 75% of the nephrons are actively filtering, an observation that supports the functional reserve that is present in the nephron. The number of non-filtering nephrons increases in animals in which hydronephrosis has been induced by ureter ligation, probably in those nephrons where there is cast formation and tubular dilatation. This does, however, need confirmation using the Hanssen technique and suitable markers for luminal casts. This may be relevant in renal papillary necrosis where the presence of Tamm-Horsfall glycoprotein casts has been linked to tubular dilatation and subsequent glomerular sclerosis and scarring of the cortex.
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There is presently interest in the marked hyaline droplet formation that occurs in male rats exposed to branched-chain hydrocarbons\textsuperscript{189-192}. These can be shown by Mallory’s Heidenhein stain, their eosinophilia or the use of antibodies to $\gamma$-globulin. The heterogeneity of hyaline droplet formation highlights several questions:

(i). which nephrons are filtering, those with or without the protein over-load; and

(ii). is switching off the filtering nephrons a way of giving the resting nephron a chance to remove this material?

Ferrocyanide does, however, bind to protein\textsuperscript{300} and therefore will give higher values in male rats (because of the sex hormone related proteinuria), in those circumstances where glomerular permeability is altered, as a result of glomerular damage and when the proximal tubule protein reabsorbptive capacity has been decreased.

3. Changes in glomerular protein filtering and proximal tubule uptake

Glomerular permeability and the proximal tubular uptake of a number of filtered proteins is a very important indication of normal renal function. The peroxidative activity of several low molecular weight enzymes (such as horseradish peroxidase, myeloperoxidase and myoglobin) have been used to study glomerular permeability and protein uptake by the proximal tubule\textsuperscript{301-303}. The horseradish peroxidase (HRP) is taken up into apical vacuoles or phagosomes in the proximal tubule that merge with lysosomes, and slowly undergoes degradation. The clearance of HRP can be altered by a saline or mannitol diuresis, which decreases cellular uptake. Mannitol in particular produced a large number of vesicles which were assumed to be involved in fluid transport at the expense of HRP uptake\textsuperscript{304}. The administration of these exogenous protein markers is not, however, without the adverse effect of vascular leakage, a response that can be inhibited by histamine and serotonin antagonists\textsuperscript{305}. There is also some evidence to suggest that arachidonic acid metabolites may be involved in the hypotensive effect caused by the administration of HRP, because this can be prevented by indomethacin and aspirin\textsuperscript{306}. Despite the potential value of these methods there appears to be a paucity of published data on their application to studying chemical induced proteinuria.

B. Fluorescence visualization of chemicals

Many chemicals with nephrotoxic potential fluoresce strongly (Table 7), and therefore offer the potential to visualize their distribution at a cellular and subcellular level (an objective most often achieved by autoradiography). Some of these chemicals also show spectral changes as a result of metabolism, a property that ensures that
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more information can be attained from this method than from autoradiography, which measures total drug-derived material only. One of the major problems with the use of fluorescent monitoring is the significant tissue autofluorescence that may be present. Thus it is essential to screen control tissue to ensure that the choice of filter combinations maximizes the visualization of the chemical of interest.

Table 7 Nephrotoxic chemicals with fluorescent properties

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<td>Aflatoxins</td>
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For example, the fluorescence of streptomycin has provided data on its distribution in the inner ear \(^{307}\), an approach that could also be applied to the kidney. The anthracycline antibiotic adriamycin (doxorubicin) and its analogue daunomycin are rapidly taken up by the kidney and cleared from the cytoplasm to leave only the nuclei showing the presence of these chemicals after 60 min \(^{308}\). These data closely paralleled the renal pharmacokinetics of the anthracyclines, but failed to help explain why this compound targets selectively for the glomeruli \(^{101}\).

XII. CONCLUSION AND FUTURE TRENDS IN THE USE OF HISTOCHEMICAL TECHNIQUES

The applications of histochemistry and immunohistochemistry are many, and the topics we have covered represent only a partial overview of how fundamental questions can be addressed. More importantly, despite the problems that may be experienced from time to time in reproducing these techniques they have several very important advantages:

1. They provide the most cost-effective way of bridging the dichotomy between renal structure and function, and may give information that relates directly to a subcellular or molecular level.

2. Even when the bases for histochemical change are empirical (as many still are), they are discriminatory and help visualize renal heterogeneity and focal lesions.
3. Microspectrophotometry and fluorimetry, with computer manipulation of the extensive data that have been generated, and the automation of slide scanning, are increasingly providing objective means of handling large quantities of material.

4. The indication that a "general" histochemical change has occurred (e.g., loss of a carbohydrate matrix or glycoprotein, lipid change, etc.) is a far more logical starting point for specific and specialized studies (e.g., biochemical, immunological assessment, ultrastructural evaluation, etc.) than can currently be rationalized from other experimental protocols.

5. There are instances where histo- and immunohistochemical methods give information that is usually only otherwise available from ultrastructural studies, but provide markedly larger areas of tissue and offer the potential for more rapid assessments.

6. Histochemistry gives data on the kinetics of cell damage and its repair, in relation to associated cell types that may not be damaged. Another approach is the determination of proliferative and regenerative capacity of renal cells by means of either the frequency of mitotic figures or counting labelled cells after infusion with \(^{3}H\)-thymidine. Such techniques may be helpful in the detection of specifically affected sites and in the quantifying of pathological changes, and may even shed light on mechanisms of toxicity.

7. The trend in toxicology has shifted from descriptive pathology to one with a molecular focus that will provide a rational basis for safer drug design, better treatment of disease and more reliable hazard assessment. The transition between "organ toxicity" and the focal lesions of "target cell toxicity" must begin with light microscopic evaluation, and progress to a biochemical level in which the identity of the morphologically damaged cell must be maintained.

Histology is still the basis for defining renal lesions in toxicology, while urine and serum analyses can be of help in determining the sequences of events in individuals. For detection of test compound-related renal lesions in animals with varying background pathology, it is advantageous to supplement the conventional staining techniques. The following problems have to be encountered:

1. extensive cell injury may have caused the loss of the specific characteristic;

2. regenerating cells may not yet have acquired the specific characteristic;

3. the method used may be based on minor differences and may not readily discriminate between cells;
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4. residual compound may interfere with the histochemical reaction;

5. sampling error may be large, e.g. in the case of focal lesions;

6. species differences in morphology and reactivity will be encountered;

7. special investigations have to be arranged as an essential part of the study protocol and can cause logistic problems if used for routine toxicity tests.

ACKNOWLEDGEMENTS

The authors are grateful to Ted Lock for critical comment, and to M. E. van Ek and H. Scott for preparing the manuscript. The authors' research was supported by the Wellcome Trust, the Cancer Research Campaign, the International Agency for Cancer Research, the National Kidney Research Fund, the Commission of the European Communities - Biotechnology Action Programme, and Ciba-Geigy.

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