STUDIES OF CHEMICALLY-INDUCED RENAL PAPILLARY NECROSIS

by

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TO MY DARLING JENNY,
MY STRENGTH
Renal papillary necrosis (RPN) in humans may be a consequence of analgesic abuse. The development of the lesion manifests very few symptoms, but the sequelae may include disruption of normal homoestasis and chronic renal failure.

Attempts to define the molecular pathogenesis of the analgesic associated lesion in experimental animals has not been successful. The lesion can only be induced over many weeks, not all of the test animals are equally affected and experiments are notoriously irreproducible.

2-Bromoethanamine (BEA) hydrobromide has been used as a model papillotoxin in this study on rats. A single dose of BEA caused RPN within 24 to 48h in all treated animals. The lesion could be varied from a apex limited focal papillary necrosis (50mg/kg po) to total medullary ablation (>150mg/kg ip). Pretreating rats with mixed functional oxidase inducers (phenobarbitone, 3-methylcholanthrene) or inhibitors (cobaltous chloride); thiol protectors (acetylcysteine, methionine) and free radical scavengers (zinc sulphate); reserpine or dexamethasone cause no change in the magnitude of the lesion. Anti-rheumatic compounds and their analogues (given before BEA) and dehydration exacerbated the lesion. Some degree of protection against BEA-induced RPN was afforded by anti-oxidants and ethanolamine pretreatment. Animals with a steptozotocin-induced diabetes were refractory to BEA.

Microscopically the earliest changes following BEA insult were a hydropic degeneration (4h) reverting to an apparently normal architecture (8h), followed by necrotic change. The histochemical staining intensity of the medullary proteoglycan (PoG)-
glycosaminoglycan (GAG) interstitial matrix increased dramatically within hours of BEA treatment. The staining was lost only from those parts of the papilla which were necrosed. A marked perturbation of the papillary ground substance was confirmed biochemically by an altered pattern of PoG-GAG polydispersion in urine and by loss of $^{35}$S from the papilla, but not from the cortex or from extra-renal tissue.

Carbon-14 labelled BEA was synthesised. About 70% of the administered dose was excreted in urine, 4% as $\text{CO}_2$, 2% as a volatile base, up to 11% in bile, but only 4% in faeces. The BEA-derived products in urine and bile could each be separated into 6–8 components. The bile products were, in general, of higher molecular weight than those in urine.

A model is proposed in which a primary effect on renal interstitial cell function or a perturbation of the medullary PoG-GAG ground substance leads to necrosis and contributes to the sequelae associated with the lesion.
## CONTENTS

| ABBREVIATIONS | vii |
| ACKNOWLEDGEMENTS | viii |

### CHAPTER 1

| INTRODUCTION | 2 |

### PART I

#### CHAPTER 2

| THE KIDNEY STRUCTURE AND FUNCTION | 8 |

- 2.1 The Renal Blood Supply | 9 |
- 2.2 The Nephron | 14 |
- 2.3 The Medulla | 20 |

#### CHAPTER 3

| RENAL PAPILLARY NECROSIS | 55 |

- 3.1 Renal Papillary Necrosis in Man | 55 |
- 3.2 Experimentally Induced Renal Papillary Necrosis | 68 |

#### CHAPTER 4

| MATERIALS AND METHODS | 95 |

- 4.1 Experimental Animals | 95 |
- 4.2 Histologically Related Techniques | 104 |
- 4.3 Handling and Measuring of Radioactivity | 112 |
- 4.4 The Synthesis of 2-Bromo[1-14C]ethanamine Hydrobromide | 118 |
- 4.5 The Analysis of Urinary, Bladder and Kidney Stones and Crystals. | 125 |
- 4.6 The Polydispersion of Proteoglycans, Glycosaminoglycans and Oligosaccharides in Urine. | 127 |

### PART II

#### CHAPTER 5

| 2-BROMOETHANAMINE HYDROBROMIDE-INDUCED RENAL PAPILLARY NECROSIS: HISTOPATHOLOGICAL AND CYTOCHEMICAL CHANGES, AND FACTORS AFFECTING THE DEVELOPMENT OF THE LESION | 133 |

- 5.1 The Response to Different Doses of 2-Bromoethanamine. | 134 |
- 5.2 The Morphological and Histochemical Changes in the Medulla and Cortex During the Development of 2-Bromoethanamine-Induced Renal Papillary Necrosis. | 153 |
- 5.3 Factors Affecting the Development of 2-Bromoethanamine-induced Renal Papillary Necrosis. | 165 |
- 5.4 The Effect of Dehydration on the Response of the Renal Papilla to 2-Bromoethanamine. | 190 |
- 5.5 The Effect of 2-Bromoethanamine on the filling of the Papillary Microvascular System. | 193 |
- 5.6 Chapter Summary | 200 |
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADH</td>
<td>Anti-diuretic hormone</td>
</tr>
<tr>
<td>BEA</td>
<td>2-Bromoethanamine</td>
</tr>
<tr>
<td>BHA</td>
<td>Butylated hydroxyanisole</td>
</tr>
<tr>
<td>BHT</td>
<td>Butylated hydroxytoluene</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CI</td>
<td>Colour index</td>
</tr>
<tr>
<td>d.p.m</td>
<td>Disintegrations per minute</td>
</tr>
<tr>
<td>EA</td>
<td>Ethan-1-ol-2-amine</td>
</tr>
<tr>
<td>EI</td>
<td>Ethylenimine</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>H &amp; E</td>
<td>Heamatoxylin &amp; Eosin</td>
</tr>
<tr>
<td>Hgb</td>
<td>Heamoglobin</td>
</tr>
<tr>
<td>ID</td>
<td>Internal diameter</td>
</tr>
<tr>
<td>ip</td>
<td>Intra-peritoneally</td>
</tr>
<tr>
<td>IR</td>
<td>Infra red</td>
</tr>
<tr>
<td>iv</td>
<td>Intravenous</td>
</tr>
<tr>
<td>MAP</td>
<td>Magnesium ammonium phosphate</td>
</tr>
<tr>
<td>3-MC</td>
<td>3-Methylcholanthrene</td>
</tr>
<tr>
<td>MPS</td>
<td>Mucopolysaccharide</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>OD</td>
<td>Outside diameter</td>
</tr>
<tr>
<td>PAS</td>
<td>Periodic acid-Schiff reaction</td>
</tr>
<tr>
<td>PB</td>
<td>Phenobarbitone</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>po</td>
<td>Per os</td>
</tr>
<tr>
<td>PoG</td>
<td>Proteoglycan</td>
</tr>
<tr>
<td>PTFE</td>
<td>Polytrifluorochloroethylene (Teflon)</td>
</tr>
<tr>
<td>Rf</td>
<td>Chromatographic position relative to solvent front</td>
</tr>
<tr>
<td>RPN</td>
<td>Renal papillary necrosis</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SKF-525A</td>
<td>2-Diethylaminoethyl-2,2-diphenylvalerate</td>
</tr>
<tr>
<td>THGP</td>
<td>Tamm-Horsfall glycoprotein</td>
</tr>
<tr>
<td>t.l.c.</td>
<td>Thin layer chromatographic</td>
</tr>
<tr>
<td>TXA2</td>
<td>Thromboxane A$_2$</td>
</tr>
<tr>
<td>Vo</td>
<td>Void volume</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

Few fields of human endeavour are achieved solely as the results of the effort of a single person, especially in the area of life sciences research. It would be impossible to name each of the many friends and colleagues who have contributed, directly or indirectly, to this research project and to the final thesis, either in the form of inspiration, assistance, discussion or criticism. Many are cited in the text and the others will recognise their contributions.

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viii
An Indo-Persian fable tells how each of six blind men encountered a different part of an elephant. One thought the ear was a fan, another that the leg was a tree, the third that the tusk was a spear, the fourth felt the trunk and concluded it to be a snake, the next thought that the beast's side was a wall and the last assumed that the tail was a rope. Each, from their own brief encounter and their own one-dimensional viewpoint, then tried to describe the entire elephant and its life history.

The wise, all knowing (and sighted) Rajah resolved the confusion and explained that it was only by considering all of those touched parts together that the whole elephant could be perceived.

This blind man may not yet have touched "the elephant", indeed he may not have even heard the sounds made by the living "animal", but when one is close to it, especially if one is down wind, it is difficult not to be aware of the smell of an elephant!

"Science is built up of successive solutions given to questions of ever increasing sublety, approaching nearer and nearer towards the very essence of the phenomena". Louis Pasteur "Etudes sur la Biere". From this and the accumulated knowledge of other blind men we may yet move closer to understanding a little more about renal papillary necrosis, toxicology and, perhaps, the kidney.
Ehrlich's "magic bullet" concept is the fundamental ideal towards which rational therapeutics has strived; that is the use of an exogenous compound to cause only a desired biological effect. En route man has, however, created a milliard of adverse, sometimes disastrous, effects. Modern toxicology is, in the first instance, attempting to identify these undesirable effects, and thence to perceive the exact sequence of events which lead up to these adverse changes at the molecular level. It is only by developing an understanding of the molecular pathogenesis of chemically-induced lesions that safe rational therapies can be devised and, more importantly, that the risk of exposure to environmental xenobiotica can be fully assessed.

Many chemically-induced adverse effects (Kerr & Ward, 1977) and organ specific lesions have now been described Table 1.1, but it is less frequent that a cogent molecular pathogenesis has been documented. Necrosis of the renal papilla in man has been associated with the abusive consumption of mixed analgesics for almost 30 years. Despite these three decades the lesion ranks as one of the most confused areas in the annals of modern toxicology, (see Chapter 3). Initially the aetiological common denominator was thought to be phenacetin, as a consequence of which it was withdrawn from therapeutic usage. Most analgesic and non-steroidal anti-inflammatory drugs have since been reported to have the potential to induce such a lesion (see 3.2.3). The broadening concept in the aetiology underlying this lesion is reflected in the change from the term "phenacetin-induced papillary necrosis" to "analgesic associated nephropathy", as applied to human sufferers.
Table 1.1. Chemically-induced organ selective damage (thought to be mediated by SO\(^{\cdot}\) radical).

<table>
<thead>
<tr>
<th>Chemical or drug</th>
<th>Organ or part of organ</th>
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<tbody>
<tr>
<td>Alloxan</td>
<td>Pancreatic β-cells</td>
</tr>
<tr>
<td>Adriamycin</td>
<td>Cardiac cells</td>
</tr>
<tr>
<td>Dialuric Acid</td>
<td>Pancreatic β-cells, Haemolysis of vitamin E deficient erythrocytes</td>
</tr>
<tr>
<td>6-Hydroxydopamine</td>
<td>Dopaminergic nerve terminals</td>
</tr>
<tr>
<td>1,4 Napthoquinone-2-Sulphonate</td>
<td>Haemolysis of erythrocytes.</td>
</tr>
<tr>
<td>Paraquat</td>
<td>Lung and kidney</td>
</tr>
</tbody>
</table>

After Bus & Gibson (1979)

The reported incidence of renal papillary necrosis varies from less than 0.2% (USA) up to 20% (Australia) for patients undergoing autopsy, but the true dimensions of the problem has probably been grossly underestimated (see 3.1). Analgesics continue to be freely available, and are possibly abused (at one time or another) by most individuals who self-prescribe them. Judging by the extent of their use, analgesics appear to be regarded by the general population as the panacea for most ills. High dose analgesic abuse (non-therapeutic consumption) may be a secret addiction, the practice may be "acquired" by a child from an elder, it may be associated with other psychiatric disturbances and, in some societies, it has even been accepted as a social grace (3.1.1). The health consequences are far reaching. Necrosis of the renal papilla in man is
difficult to diagnose at an early stage. Indeed, the vague non-specific lower back pains which may be an early manifestation of the lesion are treated with analgesics! Destruction of papilla may lead to compromised renal function, recurrent bacterial infection, and eventually chronic renal failure. Even when compromised renal function is diagnosed the underlying cause may not be identified, because any changes in kidney function may be superimposed on age-related renal deterioration and analgesic addiction is rarely admitted. Even the most sophisticated diagnostic aids will, at present, only identify extensive kidney damage and not the early, possibly reversible stages, which precede necrosis. This problem is further obscured by the fact that many of the "new generation" non-steroidal anti-inflammatory drugs are now prescribed, at high doses, for long periods, to elderly patients, where compromised renal function, and its secondary consequences, might be overlooked or considered to be an acceptable or normal consequence of their years.

The role of analgesic compounds in causing the lesion and precipitating renal failure is supported by the fact that patients show an improved or stabilised renal function if they abstain totally from further analgesic consumption. More often, however, damage has progressed too far, or the addictive pattern cannot be broken, and renal function deteriorates to renal failure (3.1.4).

A large number of easily identifiable symptoms such as recurrent urinary tract infection, hypertension, and urolithiasis and calculi are invariably associated with diagnosed papillary necrosis. It is still to be established if these conditions are inevitably secondary to a papillary lesion, but if (as appears to be the case) they are, it seems possible that a stabilised and undiagnosed papillary necrosis may underly some of these easily diagnosed clinical conditions (i.e. urinary tract infection,
hypertension or calculi).

The epidemiology of renal papillary necrosis remains confused, the full dimensions of the aetiology are unknown and the molecular pathogenesis is enigmatic. Little attention has been accorded to the possibility that drugs other than analgesics cause the lesion (Burry et al., 1977), but a broad array of non-analgesic compounds are known to induce similar pathological changes experimentally (see 3.2.3).

Attempts to produce renal papillary necrosis experimentally in animals which will mirror the situation in man have, in part, been successful. The literature on this subject is, however, contradictory and most confusing. One recent reviewer concluded:--

"...it is hardly conceivable that new animal experimentation will in the future bring the answer to a problem that seems to be specific for humans. Nevertheless in spite of past failures it is most probable that new protocols and experiments will try to force human nephropathy attributed to analgesics to fit the Procrustean bed of animal toxicology." (Rosner, 1976)

The lesion has been induced experimentally in rats with single or with mixed analgesics over a period of many weeks or months. Frequently, these model systems appear to suffer from unidentified variables, as a consequence of which the induction of the lesion is often irreproducible, sometimes even in the same laboratory. The inability to reproduce such lesions seems the most likely explanation for the failure of these models to clarify the molecular pathogenesis of this organ specific lesion.

Another important obstacle in investigating experimentally induced renal papillary necrosis arises from the difficulty associated with identifying the lesion. None of the methods for monitoring or assessing changes in renal function in vivo identify papillary necrosis per se
unless there is also an extensive destruction of the medulla and secondary cortical changes. Histopathological identification of the lesion depends on painstakingly laborious sectioning to find the papilla tip. Failure to follow this exacting procedure may result in the inability to identify partial or focal necrotic lesions. One experimental model (Hardy, 1970a,b and 1974) has been described where the necrosis is limited to the apex of the papilla which subsequently sloughs off, and the remaining medulla stump is re-epithelialised. Once this has occurred the repaired medullary stump could easily be mistaken for a normal, (but peculiarly shaped or obliquely sectioned) papilla on routine examination.

An important direct consequence of these diagnostic shortfalls is that few industrial toxicology screening protocols include an assessment for papillotoxicity unless the compound concerned is to be used as an analgesic "type" drug. Thus the spectrum of chemicals with papillotoxic potential remains unknown. Several workers (see Darmady & Maclver, 1980 and McCormack et al., 1981) have continued to publish on diphenylamine-induced cystic kidney, apparently unaware (either from the literature or their own observations) that this compound induces papillary necrosis as the primary lesion and that cortical nephron dilatation (cystic kidney) only develops as the secondary consequence (Hardy 1970a,b and 1974). This state of ignorance on the full spectrum of chemicals with papillotoxic potential has extensive human and eco-toxicological consequences.

A number of other chemicals have, in fact, been shown to produce renal papillary necrosis, experimentally, several with a fair degree of reproducibility, and some within a matter of days, rather than months. Although the histopathological changes associated with these acutely induced lesions have been described, and certain possible underlying mechanisms have been postulated, the detailed sequence of events defining
the molecular pathogenesis have not been established.

The investigations reported in this dissertation were undertaken with the intention of:-

I) Probing the molecular pathogenesis of papillary necrosis caused by a rapidly acting non-analgesic "model" compound, and

II) Trying to establish a rational, sensitive and specific approach to the non-invasive diagnosis of renal papillary necrosis in an experimental situation.

The thesis is presented in two parts. In part I (Chapters 2 to 4) the literature relating to the structure and function of the kidney, and renal papillary necrosis in man and experimental models has been reviewed. Particular attention has been paid to the non-analgesic papillotoxic compounds. A large number of experimental techniques have been used, and some methods have been applied repeatedly in different parts of the work. This, together with "evolutionary" modifications (these were adopted because of improved facilities or newly published work) prompted a separate chapter to describe the methods and the modifications. It is hoped that this format will serve to streamline the experimental section. Part II (Chapters 5 to 7) will present the experimental work and the inferences that have been drawn from these findings, as they relate to both the acutely induced experimental lesion and as they may be related to the chronically induced analgesic lesion, and its secondary consequences, as they affect experimental animals and man.
An in depth review of kidney structure and function is beyond the scope of this thesis. An attempt will, however, be made to provide a general background against which renal papillary necrosis may be framed. A fuller insight into the complexities of the kidney in health and disease can be found in authoritative works by Valtin (1973) and those edited by Orloff & Berliner (1973) and Brenner & Rector (1981).

The statement attributed to Thomas Addis that "All we know for certain about the Kidney is that it makes urine" (Black, 1980) is a scientifically sound epigram. Research has answered only a few of the questions posed 25 years ago about the kidney - it has also raised more. Many areas of renal research remain confused, conflicting and controversial because researchers have failed to use a dichotomous approach to the kidney: all too often structure and function have been studied as two separate entities. "Function seems not so much to rest on structure, as that each is of the other's essence" (Oliver, 1961). An attempt will, therefore, be made to view the kidney in this light.

Mammals have two kidneys which are situated retro-peritoneally, one on either side of the spinal cord. The kidneys "process" 25% of the resting cardiac output via an arterial blood supply, much of the fluid and solutes from blood are "pushed" into the proximal part of the nephron (the functional unit of the kidney) from which essential "nutrients" and
water are reabsorbed and eventually enter the venuous return. There is some secretion from the 'blood' into the distal part of the nephron, but what leaves it is essentially waste solutes and much of the water in which they are dissolved is subsequently reabsorbed beyond the nephron.

Each kidney is made up of a large number of nephrons, groups of which interconnect to continue as collecting ducts or tubules and these, in turn, combine to make up the ducts of Bellini, several of which exit around the papilla tip. The papilla is situated in the renal pelvis, a funnel shaped area which narrows to the ureter. The continued production of urine, together with peristalsis of the ureter carries excreted waste to the bladder. Figure 2.1 shows a sagittal section through a rat kidney, showing the morphologically identifiable zones. The morphophysiology of the kidney varies markedly between species, therefore, a generalised description will be provided, and only where important differences exist between the rat and man will they be referred to (Moffat, 1975).

2.1 THE RENAL BLOOD SUPPLY

Each kidney is supplied by a branch of the abdominal aorta (the renal arteries) which divides to form several interlobular arteries. These in turn give rise to the arcuate arteries, which run between the cortex and medulla parallel to the kidney surface. A large number of interlobular arteries arise from the arcuate vessel and pass through the cortex where:-

A) a small amount of blood reaches the surface to supply the kidney capsule, but

B) most of the blood flow is directed through branches which form the afferent arterioles to the glomeruli. Each afferent arteriole breaks up to form the capillary plexus of the glomerulus (see 2.2.1); this is drained into the efferent arteriole. The efferent arterioles form two
Fig. 2.1. Sagittal section through a normal rat kidney. H&E, x 9.
types of capillary networks:-

I) In the "mid" and "superficial" cortical regions they form the peritubular capillaries surrounding proximal and distal tubules, (in the superficial regions some peritubular capillary networks interlace the nephron from which they were derived, but such an association appears to be the exception rather than the rule).

II) In the juxta-medullary region (and some mid-cortical areas for man) each efferent arteriole is directed into the medulla, where it branches into the vasa recta bundles. Each bundle consists of up to 30 descending vessels, the peripheral vessels of which give rise to a highly branched capillary network in the outer medulla. The core of the vasa recta bundle continues to the inner medulla where it terminates in a capillary network. The inner medulla, although often thought to be relatively avascular, has a capillary volume fraction more than twice that of the cortex (Beeuwkes, 1980).

The different density of the peritubular capillaries in the cortex and inner and outer medulla may facilitate the identification of each zone. In man, but not the rat, some of the descending vasa recta bundles may be derived directly from the arcuate or interlobular arteries.

The walls of all peritubular capillaries in the kidney are made up of a thin fenestrated endothelium resting on a basal lamina. Generally the capillaries in the cortex open into the interlobular vein and thence to the arcuate vein etc., to the renal vein and finally to the inferior vena cava. The capillary plexuses in the medulla drain into the ascending vasa recta, which joins the arcuate vein.

There is a well defined structural relationship between the vasa recta bundles and the nephrons in the outer medulla. A central core (consisting of descending and ascending vasa recta) is surrounded by a peripheral layer consisting of a closely intermingled ascending vasa
recta and the descending thin limbs of the loops of Henle (see 2.3.1). Between these bundles are the thick ascending limbs of the loops of Henle, some descending limbs and the collecting ducts (see 2.3.2). Within the bundles both ascending and descending vasa recta are in intimate contact with each other (rather than the same type of vessel). There are more ascending vessels, all of larger diameter, than the descending ones, and this increased volume capacity relates to the removal of excess water from the interstitium and the maintaining of the medullary osmotic gradient (see 2.3.2).

Many of the "major" and "minor" blood vessels in the kidney have either smooth muscle cells as an integral part of their structure, or other cells which may have a contractile function, thus most of the intrarenal vascular system is interlaced by both adrenergic and cholinergic innervation. Intrarenal blood flow, the factors which alter it, and its effects on renal function are poorly understood. Although there appear to be the facilities for a direct and effective perfusion of the medulla from the arcuate artery this does not seem to occur. (Moffat, 1975 and Beeuwkes, 1980).

2.1.1 Renal Haemodynamics

The measurement of total blood flow through the kidneys can be measured relatively easily using modern techniques (see Pearson, 1979 and Grunfeld et al., 1977).

Defining the zonal blood flow has given some conflicting results, but assessing regional blood flow within the kidney has provided a central enigma and a mammoth stumbling block to renal physiologists. The techniques used to study this multidimensional problem are fraught with difficulties, and results are always subject to varied interpretations (see review by Aukland, 1980; Grunfeld et al., 1971 and Pearson, 1979).
There is, however, consistent data (derived from a number of fundamentally different techniques) to show that intrarenal blood flow is greatest in the cortex (80-85% of total renal flow) and that it decreases through the juxtamedullary region to less than 10% of the total renal flow in the medulla. A clear dual zonal perfusion was shown angiographically by Daniel et al., 1951. Subsequently Kramer and co-workers in 1960 (cited by Grunfeld et al., 1971) using a photoelectric monitor showed that Evans blue was cleared more slowly from the medulla, compared to the cortex. A similar conclusion was reached by Lilienfield et al. (1961) who measured $^{131}$I-labelled albumin clearance from dog medulla. However, the validity of implanting photoelectric devices in the kidney has been questioned and heterologous albumin causes marked damage to the medulla, especially the papilla (see 3.2.2.3).

Thorburn et al. (1963) monitored the clearance of $^{85}$Kr from the kidney of unanaesthetised dogs (measuring the change in X-rays externally) and showed a multi-exponential curve which could be resolved into four components. Autoradiographic distribution of the $^{85}$Kr suggested a correspondence with the anatomical "compartments" of the cortex; the inner cortex and outer medulla; the inner medulla; and perirenal and hilar fat. Similar investigations by Grandchamp et al., (1971) on the exteriorised kidney of an anaesthetised rat (using $^{135}$Xenon) also produced a four component exponential clearance curve and autoradiography revealed a similar time dependant change in isotope distribution. They estimated 82% of total renal blood flow reached the cortex, 12% the outer medulla, 4% the inner medulla and the rest the hilar fat. The "inert gas washout" method suffers from a number of important theoretical and practical defects (Lameire et al., 1977 and Pearson, 1979), but is still widely used.

The intra-renal distribution of synthetic radiolabelled microspheres
(reviewed by Lameire et al., 1977) has been used over the last decade to assess blood flow within the kidney. Most microspheres were found in the superficial areas of the cortex, and the 10% trapped in the efferent arterioles of the juxtamedullary glomeruli of the dog are assumed to represent the blood flow to their medulla (Stein et al., 1971).

More recently, Rosivall et al., 1979 used $^{99}$Tc-labelled erythrocytes to measure the preglomerular blood distribution within the kidney simultaneously with the distribution of $^{86}$Rb as a measure of postglomerular perfusion of the interstitium. Their data concurred with the results from both the inert gas washout and microsphere techniques at low total renal blood flow rates and in addition, showed an intra-organ shunt when total renal blood flow was increased. Under this condition they found a decreased superficial cortical perfusion (by about 10%) and a commensurate increased medulla blood flow. Stein and co-workers (Stern et al., 1979) have applied laser-Doppler spectroscopy to measuring regional renal blood flow and factors affecting it. Their data suggests that blood flow within both the cortex and medulla have similar auto-regulatory responses to pressure changes and that less than 10% of the total renal flow perfuses the medulla.

It must be stressed that although the medulla is poorly perfused in comparison to the rest of the kidney it is, nonetheless (because of the abundant renal blood flow), a well perfused tissue per se. According to Thurau (1964) the medullary blood flow is about 15 times that of resting muscle and the same as the brain! In addition, its capillary volume fraction of the medulla is more than twice that of the renal cortex (Beauwkes, 1980).

2.2 THE NEPHRON

The nephron is the functional unit of the kidney and consists of a continuous tube of highly specialised cells, which show sub-
specialisation along its length and between nephrons. There are marked structural (Fig. 2.2) and functional differences between the nephrons arising in the cortex and those arising in the juxtamedullary regions (see 2.2.1). The total number of nephrons varies between different species and within any one species as a function of age. Figure 2.2 shows a schematic representation of the two major types of nephrons. The macroscopic differentiation of the kidney into distinct zones arises not only from the regional vascularity (2.1), but also from the way different functional parts of the nephron are arranged within the kidney.


2.2.1 The Glomeruli

The glomeruli (more correctly called the renal corpuscles or Malpighian bodies) forms the apex of the nephron and functions as a relatively poorly selective macro-molecular exclusion filter to the hydrostatic pressure of the blood. The number of glomeruli can, in general, be related to the mass of the species, and the size of each glomerulus depends, among other factors, on the environmental water balance, and it increases with age.

Two anatomically distinct types of glomeruli can be identified: those arising in the cortex are part of the superficial nephron, while those of juxtamedullary origin continue as a nephron which loops down into the medulla (Fig. 2.2).

The structure of the glomerulus is complex and has only been clearly defined using scanning and transmission electron microscopy (Moffat 1981, 1982 and Maunsbach et al., 1980).

The glomerular "tuft" is made up of a number of capillary branches
Fig. 2.2  Schematic representation of the "cortical" and the juxtamedullary nephrons in relation to the site they occupy within the different morphological zones of the kidney (From Moffat, 1975, with permission).
that arise from the afferent arteriole and anastomose to the efferent arteriole. There are also communicating vessels between the branch capillaries. The fenestrated endothelium can not prevent plasma molecules from leaving it, but a negatively charged cell coat may impart some selective permeability.

The capillaries are in direct contact with the glomerular basement membrane (or basal lamina) which when viewed electron micrographically can be divided into three layers - outermost the lamina rara interna, the central lamina densa and the lamina rara externa which is in direct contact with the epithelial cells (the podocytes). There now seems little doubt that the basal lamina contains collagen and sialic acid and is rich in glycosaminoglycans, mainly heparan sulphate (Kanwar & Farquhar, 1979), which provides a strongly anionic macromolecular filtration barrier (see 2.3.4.4).

The capillary tuft (ensheathed in its basal lamina) is surrounded by a number of podocytes and each gives rise to several primary processes, which in turn give rise to secondary processes, and finally to numerous tertiary foot processes which are embedded in the lamina rara externa. The foot processes of one podocyte interdigitate with those of an adjacent epithelial cell. The surface of the podocytes are also covered by a strongly anionic material which extends to the spaces between the foot processes. It is through these spaces that the glomerular filtrate reaches the lumen of the nephron via the Bowman's capsule. Thus the podocyte provides a structural support for the basal lamina and may also serve to provide additional anionic forces to the process of biological ultra-filtration. It has been suggested that podocytes may have phagocytic properties and undergo contraction (see Moffat 1981).

The axial regions of each glomeruli contain mesangial cells in close association with several podocytes. Information on the structure and
possible functions of mesangial cells has been reviewed by Moffat, 1981. In brief they are thought to undergo contraction and may thus control glomerular blood flow via biogenic amine or hormonal control. Of equal importance is the observation that these cells take up large molecules (such as colloids, immune complexes and protein aggregates) which may eventually be disposed of via the renal lymphatic system.

The driving force of filtration is provided by the glomerular capillary hydrostatic pressure (which is controlled mainly by the vascular tone of the afferent and efferent arterioles, see 2.1), minus both the plasma oncotic pressure and the hydrostatic pressure in the Bowman's capsule. The resulting "effective filtration pressure" across basal lamina is about 10 to 15 mm Hg.

Selective filtration is achieved, in part, because of the anionic nature of the basement membrane which blocks or slows the passage of negatively changed or neutral macromolecules, leaving those carrying a cationic charge and small molecules (irrespective of charge) to pass unimpeded.

2.2.2 The Proximal Tubule

The proximal tubule is found only in the cortex or sub-cortical zones of the kidney. Anatomically each proximal tubule can be divided into the convoluted portion (pars convoluta), and the shorter straight descending portion of what remains (the pars recta), which then continues to become the descending limb of the loop of Henle (Fig. 2.2).

The proximal tubule plays a decisive role in maintaining homeostasis. Explained in its most simplistic form this is achieved when sodium and chloride ions flux from the tubule lumen to the peritubular capillaries under the control of a number of processes such as non-specific electrophysiological gradients and selective active transport mechanisms.
Water follows the ions by "solvent drag" and, in addition, hydrostatic pressure (attributable to the presence of both proteins and glycosaminoglycans, Wolgast et al., 1973) contributes to water movement from the epithelial cell to the interstitium and thence, by an oncotic gradient, into the capillaries (Valtin, 1973). The flux of ions within the proximal tubule including the absorption and secretion of \( \text{HCO}_3^- \) and \( \text{H}^+ \), and the "lumen trapping" of \( \text{NH}_3 \) controls renal acid-base regulation (Valtin, 1973).

Those proteins which have passed from the Bowman's capsule (a significant amount of albumin in the case of normal rats, Moffat, 1975) are reabsorbed in the proximal tubule. Consensus supports the pinocytotic removal of proteins from the base of the micro-villi brush border into the epithelial cells. The vesicles thus formed combine, form protein-filled vacuoles and these fuse with lysosomes; from which the digestion products of the protein most likely diffuse, eventually, to the capillary system.

There are, in addition, a multitude of other absorptive and secretory mechanisms. These include the active process which reabsorb glucose, and the secretion of both acidic and basic organic compounds (Valtin, 1973; Orloff & Berliner, 1973 and Brenner & Rector, 1981).

2.2.3. The Distal Tubule

The distal tubule connects the thick ascending limb of the loop of Henle to that part of the collecting ducts which originate in the cortex. The distal tubules are involved in both ion and water reabsorption, but play a much less significant role than that occupied by the proximal tubules. The underlying mechanisms responsible for reabsorption appear, in essence, to be similar to those already outlined (2.2.2). The major differences include a stronger \( \text{Na}^+ \) gradient against which to "pump", the ability to reabsorb sodium without reabsorbing water, the controlling
effects of anti-diuretic hormone (see 2.3.3) and aldosterone (among other mediators) and there is very little, if any, reabsorption of protein.

The secretion of potassium ions appears to be under the control of an active transport mechanism the regulating factors of which are many and complex (Valtin, 1973; Orloff & Berliner, 1973 and Brenner & Rector, 1981).

2.3 THE MEDULLA

The medulla differs from the cortex (Fig. 2.1 and Fig. 2.2) at the macroscopic and at the microscopic levels. This region can be divided into:-

A) The outer medulla, which is made up of the thin descending and the thick ascending limbs of the loops of Henle, collecting ducts, the vasa recta and a dense capillary network, and

B) The inner medulla, the free part of which is referred to as the papilla, although some workers apply that name only to the apex of this region. The two terms will be used synonymously in this thesis. The inner medulla contains the thin limbs of the loops of Henle, collecting ducts, the vasa recta and a diffuse network of capillaries. Packed into the spaces between these structures are interstitial cells embedded in a matrix rich in glycosaminoglycans (see 2.3.3 and 2.3.4.4).

The collecting ducts terminates as the ducts of Belini around the tip of the papilla.

Whereas the mouse, gerbil, rat, guinea pig, rabbit, dog, cat and primate kidney has only a single papilla, the pig and man have multipapillated kidneys. There are between 9 and 20 papilla in each human kidney (Burry et al., 1977) where there are two anatomically distinguishable types:-
I) The non-refluxing papillae where the surface orifices of the ducts of Bellini are slit-like. These close when there is an increase in the 'back-pressure' of urine from the bladder and so prevent vesico-ureteric reflux. These papillae occur predominantly in the mid zone.

II) The 'refluxing' papillae occurs predominantly in the polar regions as wide orifices which are prone to retrograde flow of urine into the tubules during vesico-ureteric reflux (Ransley & Risdon, 1979).

The microscopic and ultrastructural features of the medulla have been described by several workers in the field (see Moffat, 1975, 1981, 1982; Maunsbach et al., 1980 and Bohman, 1980 for references).

2.3.1 The Loops of Henle and Collecting Ducts

Two anatomical populations of loops of Henle may be identified, those arising from:

A) The superficial (or cortical) glomeruli. These have short loops which penetrate no further than the outer medulla. The proximal tubule and thick ascending limb are closely associated in the cortex, but in the medulla the descending limb is intimately related to the ascending vasa recta and the ascending limb to the collecting duct.

The association between the proximal tubule and ascending loop of Henle and the limb with vascular systems or with the collecting ducts provides a multi-dimensional network in which solutes or water may undergo countercurrent exchange. These exchanges may either provide a shut which excludes selected solutes (and water) from the inner medulla or, alternatively, solutes (e.g. NaCl and urea) may be trapped in this zone. This exclusion of water and trapping of NaCl and urea helps maintain the osmotic gradient across the inner medulla (see below 2.3.2).

B) The subcortical (juxtamedullary) glomeruli. These have long loops (the length is proportional to the renal concentrating potential) and
only about a third of ascending and descending limbs lie together; in the other instances the ascending limbs are nearer to collecting ducts than to descending limbs.

2.3.2 The Counter Current Multiplier System and Urine Concentration

Less than 1% of the glomerular filtrate leaves the kidney as urine (unless there is a state of diuresis), the remainder having been reabsorbed. The concentration of urine is complex, and depends (at least in part) on the countercurrent multiplier system which establishes a steep osmotic gradient across the inner medulla. The high osmolality is a consequence of the differential permeability of the limbs of the loops of Henle and the collecting ducts to water and ions. The ascending limb is thought to have an active transport mechanism which pumps sodium out of the lumen and into the interstitium, but remains impermeable to water. As a consequence the osmolality decreases in this part of the tubule. The descending limb, on the other hand, is freely permeable to water, but not sodium ions. The high ion concentration in the interstitium would draw water out of the descending limb, increasing the osmolality towards the turn of the U-loop. This is probably augmented by urea which leaves the collecting ducts, and enters the descending limb via the interstitium. The collecting ducts regulate the final urine concentration by controlling the amount of water that is reabsorbed. The passage of water out of the tubules is thought to be mediated largely by cAMP, the synthesis of which is stimulated by anti-diuretic hormone (ADH) which increases the permeability of the luminal cell membrane to water (see 2.3.4.3 and 2.3.4.4). Hydrostatic pressure forces the water out of the cell (through the basement membrane) into the hyperosmotic interstitium. In the absence of ADH the collecting duct is thought to be impermeable and no water is reabsorbed from it.

The interstitial osmotic gradient is assumed to be maintained by the
effective removal of water via the ascending vasa recta, which have both a greater radius than the descending vasa recta and are about twice as numerous. The counter-current exchange associated with the loops of Henle arising from cortical glomeruli (2.3.1 A) offers an important "barrier" zone which is thought to facilitate solute trapping in and solvent exclusion from the inner medulla, and thus helps to maintain the hyper-osmolality in this "compartment".

There are a number of other factors which control, alter or contribute to urine concentration. Medullary blood flow is complex (as are the factors controlling it, 2.1.1). Increased blood flow rates will decrease the efficiency of counter-current exchange in the outer medulla, as a consequence of which the high osmotic gradient in the inner medullary compartment will be 'washed out'; and urine will not be concentrated. Diuresis is associated with increased blood flow rates (Early & Friedler, 1964, 1965 and Chuang et al., 1978).

A unique feature of the vasa recta is its permeability to macromolecules, as a consequence of which the medulla contains a large pool of albumin. The factors controlling the rapid turnover of this milieu are poorly understood. It is generally assumed that (together with the glycosaminoglycans, see 2.3.4.4) these proteins provide an interstitial oncotic pressure which facilitates water reabsorption (see Brenner & Rector, 1981 for a fuller discussion and list of references).

2.3.3 The Interstitial Cells

Interstitial cells occurs in most organs. There are three types of interstitial cells described in the kidney (Bohman, 1980). Cell types 2 and 3 are sparsely distributed and are often overlooked between the tubules, ducts and blood vessels. In the inner medulla, however, "Type 1" cells are numerous and especially prominent, because they are
2.3.3.1 Medullary interstitial cells. These cells have been described by Abrahams, 1964; Osvaldo & Latta, 1966; Moffat, 1975, 1981, 1982; Bohman, 1980 and Maunsbach et al., 1980.

The number of cells and the amount of matrix substance occupies 10 to 20% of the tissue volume in the outer medulla; and increases to 40% near the apex of the inner medulla (Bohman, 1980). The cells, which are arranged in a regular pattern perpendicular to the tubules and vessels, are irregular in shape with many long slender processes. These come into close contact with adjacent interstitial cells, capillaries and the limbs of the loop of Henle, but there is no such relationship with the collecting ducts. The shape of interstitial cells becomes more regular towards the papilla tip, where they are round.

One of the most characteristic features associated with these "Type 1" cells are the lipid inclusion droplets, which occupy at least 2-4% of the total cell volume. The lipid content is largely triglycerides, with variable amounts of cholesterol esters and phospholipids (see 2.3.4.2). A number of conditions have been described where there are marked changes in the size and number of lipid droplets (for example, there is a decrease in the number of droplets in experimentally-induced hypertension). The state of hydration and indomethacin treatment have given conflicting results with respect to the number and size of droplets. The pathophysiological significance of these changes is difficult to interpret because of varied experimental approaches, species variation and contradictory reports. The data is reviewed by Bohman (1980).
2.3.3.2 Function of medullary interstitial cells. The biological roles played by these cells remain uncertain. Several possible functions have been suggested such as a phagocytic mobility within the medulla and physically supporting the loop of Henle and blood vessels. They might also play a role in regulating homeostasis via various types of receptors and could, for example, monitor the contents of the loop and secrete effector substances into the blood (see below).

It has been argued that the contents of the interstitial lipid droplets are too specialised to be used as a metabolic energy store (Bohman, 1980), although this droplet population undergoes marked and rapid change during the short periods that precede various pathophysiological conditions.

The interstitial cells appear to produce the ground substance matrix which surrounds them. The role of renal glycosaminoglycans is discussed in detail below, see section 2.3.4.4.

Early evidence that the interstitial cells of the renal medulla were only a highly specialised prostaglandin (PG) - producing cell type has become equivocal. The interstitial lipid droplets do not, in fact, provide the sole source of arachidonic acid for prostaglandin synthesis, and only 50% of the medullary capacity to synthesise PG is confined to the interstitial cells; the rest is in the collecting ducts (Bohman, 1980). The significance of PG synthesis (see 2.3.4.3 below) in the medullary cells cannot, however, be overlooked as it may play an important role in modulating blood pressure and other renal functions and it has been suggested to occupy a central position in the pathogenesis of renal papillary necrosis (see 3.2.5.3).

In recent years the importance of the endocrine function of the medullary interstitial cells in regulating blood pressure has been highlighted by several workers (see the monograph edited by Mandal &
Bohman, 1980). An in depth consideration of this topic is beyond the scope of this thesis, but suffice it to say that three different groups of vaso-active compounds have now been isolated from the medulla or cultured interstitial cells. The are:-

- \( \text{PGE}_2 \) and \( \text{PGA}_2 \)
- anti-hypertensive "polar" renomedullary lipid, and
- anti-hypertensive "neutral" renomedullary lipid.

Experimentally-induced, spontaneously-occurring and pathologically-precipitated hypertensive states have been reversed by subcutaneous transplants of renal papillary fragments and by cultured interstitial cells. In addition, the systemic administration of both the polar and the neutral renomedullary lipids reduce arterial blood pressure (see Muirhead & Pitcock, 1980 and references cited therein). Similarly, the absence of renal medullary interstitial cells predispose to hypertension (see Chapter 7).

2.3.4 Biochemistry and Metabolism in the Medulla

The kidney offers an imbroglious puzzle of structural, functional and metabolic heterogeneity. Guder & Ross (1980) have highlighted the biochemical aspects of 'heterogeneity des nephrons' as opposed to 'heterogeneity du nephron' in the introductory remarks to a recent symposium which related to the biochemistry of the cortex. The medulla poses similar biological problems. Much of the published data has been derived from whole medulla or medullary slices, and thus still fails to differentiate between the metabolic contribution from the nephrons (loops of Henle), as opposed to the collecting duct epithelia, versus the interstitial cells.

2.3.4.1 Carbohydrate metabolism in the medulla. The metabolism of carbohydrate in the renal medulla has been reviewed by Cohen (1979).
Some early observations (see Cohen, 1979 for references) suggested that the low oxygen tension in the inner medulla (a $pO_2$ as low as 5 to 15 mm Hg compared to 75 mm Hg in the cortex) would necessitate anaerobic metabolism. However, aerobic metabolism is only limited at an $O_2$ availability of less than 1 mm Hg. This, together with recent metabolic studies, suggests that glycolysis in the inner medulla is not obliged to be anaerobic. Indeed, glucose and other substrates are oxidised to $CO_2$, despite the (relatively) small number of mitochondria. The energy so generated is utilised for tubular transport processes and, presumably, other synthetic metabolism.

All investigations have used medulla slices or homogenates, thus the exact contribution of the different cell types in the inner medulla to functional energy dynamics and the changes that underly, for example, diuresis or anti-diuresis, have yet to be related to the phosphorylation and redox states within these individual cell types. Until these investigations have been undertaken the role of altered intermediate metabolism must remain a matter for speculation.

Carbohydrates are stored in the medulla as either glycogen or as glycosaminoglycan, the former in collecting ducts and epithelia and the latter as an important constituent of the interstitial ground substance. There is evidence to suggest that either can be mobilised to provide an energy source or the glucose units for the synthesis of the other macromolecular carbohydrates (Darnton, 1967, 1969a,b).

2.3.4.2 Lipogenesis in the renal medulla. The numerous lipid droplets in the interstitial cells (2.3.3.1) have been found to contain traces of cholesterol esters, a few percent of phospholipids, mainly phosphatidylcholine and, rarely, trace amounts of phosphatidyl-ethanolamine. A few percent of free fatty acids and tri-acylglycerols
make up the remaining 80-90%; the composition of which varies in different species and is shown for the rat in Table 2.1. The most striking features are the varied types and large amounts of unsaturated fatty acids; most notably those of 20 or more carbon atoms such as arachidonic acid and especially adrenic acid. The large amount of arachidonic acid suggests that the interstitial lipid droplets may be an important pool for PG synthesis (see 2.3.4.3) in the kidney (Bojesen, 1974, 1980a).

Using rat kidney medulla slices and eviscerated anaesthetised rats Bojesen et al. (1976) and Bojesen (1980a) have shown that [1-\(^{14}\)C] acetate was incorporated into tri-acylglycerols and phospholipids (only a few percent was found in free fatty acids) where adrenic acid (docosa-7,10,13,16-tetraenoic acid) accounted for 40% of the tri-acylglycerol and 20% of the phospholipid fatty acids. Acetate was incorporated into myristic, palmitic, stearic and adrenic acids, but only myristic and palmitic acids were synthesised de novo. Fatty acids with 18 or more carbon atoms were found to be labelled predominantly on the carboxyl group, suggesting chain elongation of some prevalent fatty acid, such as arachidonic to adrenic acid. Label was, however, absent from arachidonic acid suggesting that it was not synthesised from linoleic acid in this tissue (see 2.3.4.3).

Radiolabelled glucose has also been shown to be a lipogenic precursor (Bojesen, 1980a, b) which provided 70% and 80% of the glycerol backbone to renal interstitial triacylglycerol and phospholipids respectively. The remaining \(^{14}\)C activity was found in the bound fatty acids, with 40 to 60% in saturated molecules and most of the remainder in adrenic acid, as
Table 2.1  Fatty acid composition of the medullary interstitial cell lipid inclusion bodies isolated from papillary slices and plasma tri-acylglycerols in the rat

<table>
<thead>
<tr>
<th>Saturated fatty acids</th>
<th>Medullary Interstitial Cells</th>
<th>Percentage</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myristic acid (14:0)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt; 5%</td>
<td>&lt; 5%</td>
<td></td>
</tr>
<tr>
<td>Palmitic acid (16:0)</td>
<td>~15%</td>
<td>~30%</td>
<td></td>
</tr>
<tr>
<td>Stearic acid (18:0)</td>
<td>~15%</td>
<td>~ 5%</td>
<td></td>
</tr>
<tr>
<td>Arachidic acid (20:0)</td>
<td>&lt; 5%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Unsaturated fatty acid

<table>
<thead>
<tr>
<th>Unsaturated fatty acid</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitoleic (16:1)</td>
<td>&lt; 5%</td>
<td></td>
</tr>
<tr>
<td>Oleic (18:1)</td>
<td>~15%</td>
<td>~30%</td>
</tr>
<tr>
<td>Linoleic (18:2)</td>
<td>~15%</td>
<td>~30%</td>
</tr>
<tr>
<td>Linolenic (18:3)</td>
<td>&lt; 5%</td>
<td>~ 5%</td>
</tr>
<tr>
<td>cis-11-eicosenoic acid&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(20:1)</td>
<td>&lt; 5%</td>
</tr>
<tr>
<td>cis-11,14-eicosa-dienoic acid&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(20:2)</td>
<td>&lt; 5%</td>
</tr>
<tr>
<td>Homo-γ-linolenic acid&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(20:3)</td>
<td>~ 5%</td>
</tr>
<tr>
<td>Arachidonic acid (20:4)</td>
<td>~15%</td>
<td></td>
</tr>
<tr>
<td>Adrenic acid (22:4)</td>
<td>~15%</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Fatty acids are listed in the form of trivial names (where these are established) and carbon number and number of double bonds in parenthesis.

<sup>b</sup>Non-trivial name

After data published by Bojesen (1974 and 1980a,b)
was the case for acetate lipogenesis. This suggested that both glucose
and acetate shared a common acetyl CoA pool from which lipogenesis
proceeds. Glucose also contributed more to fatty acid chain elongation
than to de novo synthesis, but, in common with acetate, no labelled
arachidonic acid was formed.

Bojesen (1980 a,b) also estimated the rate of renewal of the
different fatty acids in to phospholipids and tri-acylglycerols, and
concluded that the hydrolytic release of fatty acids and their
reincorporation (after de novo synthesis or chain elongation) into
glycerolipids either phospholipid or tri-acylglycerol) was rapid enough
to account for pathophysiological changes in the number and size of lipid
droplets. Bojesen (1980b) estimated a half-life of 25h for membrane
phospholipids and 11h for lipid droplet tri-acylglycerols.

The spectrum of fatty acid synthesis varied in vitro (medulla slices)
compared to the in vivo (anaesthetised, eviscerated rat) situation,
probably due to the contribution of plasma free fatty acids as substrates
for chain elongation (Bojesen 1976, 1980a,b).

An increased osmolality (in vitro) depressed lipogenesis overall from
both acetate and glucose and also altered the distribution of
incorporated label from glucose, providing more to the glycerol backbone
and proportionately less to the fatty acids (Bojesen, 1980a,b,c).

Short term (4 day dietary loading with linoleic acid had pronounced
effects on plasma fatty acids, but caused only a slight change in the
papillary tri-acylglycerol fatty acids. Long term (12 week) feeding
similarly failed to produce dramatic changes in the papillary lipids.
Bojesen, 1980b suggested that the fatty acid profile of the renal papilla
was species- and tissue-specific rather than dependant on diet.
2.3.4.3 The biochemistry of renal prostaglandins (PG)  The PG and endoperoxides are a group of ubiquitously distributed hormones with a broad spectrum of potent biological activity that shows marked receptor specificity. They are synthesised (Fig 2.3) from the C20:4 fatty acid arachidonic acid, by an enzyme system (which includes cyclo-oxygenases, peroxidases, isomerases and reductases) collectively called PG synthetase.

The PG are structurally similar, several are labile and undergo spontaneous chemical changes, and they are only present in minute concentrations. Thus most of the methods (both qualitative and quantitative) needed for their biochemical investigations are fraught with subtle pitfalls (Frolich & Walker, 1980).

The literature on renal PG biology is vast and complex, much of it is contradictory and it is difficult to interpret. The field has been reviewed recently by Dunn & Hood (1977), Dunn & Zambraski (1980), Morrison (1980), Welser (1980) Zusman (1980), Dunn (1981) and a recent conference devoted to the topic is edited by Frolich et al. (1981).

PGs are not stored in renal tissue, but synthesised de novo from arachidonic acid which is released from stored phospholipid or triglyceride pools by the action of phospholipase A₂. The factors which modulate the release of arachidonic acid include both receptor mediated responses (such as vasoactive peptides and biogenic amines), and non-specific stimuli (ischaemia), the prostaglandin precursor may be drawn from different lipid pools. Any arachidonate which is not channelled into prostaglandin synthesis may be re-acylated (as are the de novo
Fig. 2.3. Schematic representation for the bioconversion of arachidonic acid to biologically active substances. From Smith (1981).
synthesised molecules) or disposed of via several other metabolic routes (see 2.3.4.2). Arachidonic acid (the availability of which is rate limiting) is converted to prostaglandin \( \text{G}_2 \) and thence to other prostaglandin related substances (Fig. 2.3).

The anatomically identifiable areas of the kidney, all synthesise a different pattern of prostaglandins in vitro (Table 2.2). The in vivo contributions of each area to prostaglandin synthesis and the function of each prostaglandin remains largely a matter of educated speculation at present. Total prostaglandin synthesis is several times higher in the medulla (where typically it is greater in the papilla) than in the cortex (Dunn & Hood 1977). However, the distribution of, for example \( \text{PGE}_2 \) synthesis reflects a more complex picture, its concentration being lower in the papilla than the rest of the inner medulla (van Dorp, 1971). Furthermore, there are marked sex related differences in the effects of cofactors on medullary PG synthetase activity (Hirafugi et al., 1980). Some PGs break down spontaneously (e.g. \( \text{PGI}_2 \) to 6-keto-\( \text{PGF}_{1\alpha} \)), but the majority are metabolically degraded (Morrison, 1980). The enzymic conversions are mediated by a number of enzymes, including dehydrogenases, reductases, and \( \beta \)-and \( \omega \)-oxidases. The enzymes which degrade prostaglandins are located mainly in the cortex, but there are species differences in corticomedullary ratio of these enzymic activities, (Powell, 1980).

The factors regulating the biosynthesis of each type of PG are only poorly defined (Horrobin, 1980). A large number of endogenous and exogenous substances have been reported to alter renal PG synthesis (Table 2.3) and several pathophysiological conditions have been described in which renal PG synthesis is increased. Most attention has been focussed on the inhibition effects of the anti-inflammatory drugs. The steroidal compounds (e.g. corticosteroids) prevent the release of
Table 2.2  Relative amounts of PG synthesised IN VITRO* by different renal tissue cell types

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Product</th>
<th>Likely Functional Relationship</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomeruli</td>
<td>PGF$_{2\alpha}$ &gt; PGE$_2$ &gt; TxA$_2$ &gt; PGI$_2$ &gt; PGD$_2$</td>
<td>Modulating cortical function:- e.g. renal vascular resistance, renin secretion and glomerular filtration rate</td>
</tr>
<tr>
<td>Arterioles</td>
<td>PGI$_2$</td>
<td></td>
</tr>
<tr>
<td>Cortical Tubules</td>
<td>Trace PGE$<em>2$ &amp; PGF$</em>{2\alpha}$</td>
<td></td>
</tr>
<tr>
<td>Medullary Collecting Ducts</td>
<td>PGE$_2$ &gt; PGI$<em>2$ &gt; PGF$</em>{2\alpha}$ &gt; PGD$_2$</td>
<td>Modulating medullary function:- e.g. medullary blood flow, response to anti-diuretic hormone and ion reabsorption.</td>
</tr>
<tr>
<td>Medullar Interstitial Cells</td>
<td>PGE$<em>2$ &gt;&gt;PGF$</em>{2\alpha}$</td>
<td>Contracting bladder smooth muscle</td>
</tr>
<tr>
<td>Bladder</td>
<td>PGE$_2$</td>
<td></td>
</tr>
</tbody>
</table>

* May bear no relationship to in vivo situation

After Dunn & Zambraski (1980) and Brown et al. (1980)
Table 2.3 Exogenous and endogenous substances modulating the synthesis of renal PG.

Substances Stimulating PG Synthesis
- Peptides:
  Angiotensin II, Bradykinin, Anti-diuretic hormone.
- Diseases:
  Ishaemia, Unilateral ureteral obstruction,
  Cirrhosis with ascites.
- Other:
  Catacholamines, Furosemide

Substances inhibiting PG Synthesis.
- Covalent binding to cyclo-oxygenase
  Acetylsalicylic acid, Salicylic acid
  Phenacetin,
  (Activity inhibited for 24 to 48 hours)
- Reversible binding
  Non-steroidal anti-inflammatory drugs e.g.
  Indomethacin, Meclofenamate,
  Phenylbutazone, Fenoprofen
  Paracetamol
  (Activity inhibited for 8 to 24 hours)

After Dunn & Zambraski (1980)
arachidonic acid from its lipid pools, and the non-steroidal products (e.g. Indomethacin) inhibit cyclo-oxygenase. It is, however, essential to be aware that any factor which perturbates PG synthesis may act differently at different sites in the synthetic (or degradative) pathway.

Indomethacin (one of the most extensively studied cyclo-oxygenase inhibitors) produces several alterations in renal PG dynamics (Table 2.4). Uncertainties in defining PG "related" pathophysiological changes are compounded by the recent observation (Attallah & Stahl, 1980) that PGE$_2$ synthesis in slices from each zone of the kidney had a different dose response to indomethacin inhibition: the cortex was most sensitive and the papilla least sensitive. The cyclo-oxygenase inhibitors are generally grouped as either reversible or irreversible (Table 2.3), but the multiplicity of effects (Table 2.4) and the possibility of enzymic polymorphisim in different regions of the kidney, suggests that such a classification may be an oversimplification.

The exact physiological roles of the PGs in normal renal function and how these are altered in the development of nephropathies is not clear. Firstly, indomethacin has, for example, been shown to cause biochemical changes which may be classified as either related or unrelated to altering prostaglandin dynamics, (Table 2.4). Secondly, many attempts to define renal PG function have been based on the hypothesis that urinary PG excretion reflects de novo renal synthesis (Dunn & Hood, 1977; Dunn & Zambraski, 1980 and Dunn 1981), notwithstanding analytical difficulties of measuring very low levels of various PGs and apparently ignoring the fact that de novo synthesised PGs may have undergone extensive degradation. The measurement of urinary PGs, as an estimate of their de novo renal synthesis, remains equivocal because seminal PGE$_2$ is
### Table 2.4. The Actions of Indomethacin

**Prostaglandin (PG) Related Action**
- Inhibits PG synthesis
- Reduces PG degradation
- Reduces conversion \( \text{PGE}_2 \rightarrow \text{PGF}_{2\alpha} \)
- Reduces arachidonic acid release
- Inhibits renal tubular transport of PG

**PG Unrelated Action**
- Inhibits cAMP degradation
- Decreases cellular efflux of cAMP
- Inhibits cAMP - stimulated protein kinase
- Compete with aldosterone for mineralocorticoid receptors
- Reduces angiotensin II binding to adrenal cells
- Inhibits calcium transport - alters smooth muscle contractility.

*After Dunn & Zambraski (1980)*
an unavoidable and variable contaminant in the urine of males (Suzuki et al., 1980) and recently, Brown et al. (1980) have demonstrated that both rabbit and rat urinary bladder can synthesise PGE\textsubscript{2} from aracidonic acid. Finally, the physiology of renal function is controlled by several hormonal systems the detailed functioning of which are not clearly established. It is known that renal PGs may be altered by (or may alter) the renin-angiotensin II-aldosterone system (Franco-Saenz et al., 1980; Haekenthal et al., 1980; Lee, 1980; Weber, 1980 and Baer, 1981) the kallikrein-kinin system (Fitzgerald et al., 1980; Margolns, 1980 and Rockel & Heidland, 1980) and the regulation of fluid balance and water reabsorption via anti-diuretic hormone (Blair-West et al., 1980). Further, each of these hormonal systems may interact with the others via direct or indirect mechanisms. It seems likely that a full understanding of the pathophysiology of the renal hormonal systems will take some time to crystallise.

Despite the rather abstruse biology there is general consensus (Dunn & Hood 1977; Dunn & Zambraski, 1980 and Morrison, 1980) that PGs have a central role in renal function (Table 2.5). It seems, however, that renal PGs play little, if any, major regulatory role in basal renal blood flow in normal conscious animals. There is evidence that PGs are released in response to ischaemic and vaso-constrictive stress, where their role seems to be to provide a protective effect by maintaining glomerular dynamics. The role of PGs (especially PGE\textsubscript{2}) in preventing experimentally induced acute renal failure is conflicting. Arachidonic acid does stimulate renin release, a response which is blocked by cyclo-oxygenase inhibitors, but it remains uncertain which of the PGs mediate this effect in vivo, and the renal zone from which such mediators are synthesised and released. Renin release may, in turn, affect PG synthesis and the kallikrein-kinin system (which in turn may modulate PG synthesis and the
Table 2.5 The Possible Role for PGs in Renal Function

<table>
<thead>
<tr>
<th>Renal Function</th>
<th>Effect</th>
<th>Prostaglandin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renal Blood Flow</td>
<td>Vasodilatation</td>
<td>PGE₂, PGI₂</td>
</tr>
<tr>
<td></td>
<td>Vasoconstriction</td>
<td>TxA₂, PGF₉₂α (weak)</td>
</tr>
<tr>
<td>Glomerular Filtration Rate</td>
<td>Increase</td>
<td>PGE₂, PGI₂</td>
</tr>
<tr>
<td></td>
<td>Decrease</td>
<td>TxA₂</td>
</tr>
<tr>
<td>Renin Secretion</td>
<td>Increased</td>
<td>PGI₂, PGE₂, PGD₂</td>
</tr>
<tr>
<td></td>
<td>Decreased</td>
<td>? TxA₂</td>
</tr>
<tr>
<td>Natriuretic</td>
<td></td>
<td>PGE₂, PGI₂, PGD₂</td>
</tr>
<tr>
<td>Water diuretic</td>
<td></td>
<td>PGE₂</td>
</tr>
</tbody>
</table>

After Dunn (1981)
renin system). Anti-diuretic hormone is assumed to stimulate PGE₂, but published data on the controlling effects of PGs on salt and water balance are very difficult to interpret. Similarly, the mass of literature on hypertension and PGs favours the concept that the two are related, but fails to propound a unifying hypothesis.

2.3.4.4. Medullary glycosaminoglycan (GAG). An in depth consideration of the biology of GAGs is beyond the scope of this thesis. The topic has been considered in broad perspective in the authoritative monograph by Kennedy (1979).

GAGs are linear polysaccharides, which are made up of repeating disaccharide units, one carbohydrate moiety of which is a hexuronic acid, (or a neutral sugar in one case) and the other a hexosamine. The disaccharide units, and the occurrence of N-acetyl groups, together with the position of O-sulphate groups, defines the species of macromolecule. There are seven basic types of GAG (Table 2.6). These molecules also show molecular weight heterogeneity, (when isolated from the same or different organs, Dietrich et al., 1976 and Toledo & Dietrich, 1977) and the molar ratios of sulphate to hexosamine varies up to twofold for the same type of GAG (Suzuki et al., 1976).

In vivo most of these substances probably occur as proteoglycans (PoGs). These supramolecular structures are composed of a linear protein backbone which carries GAGs covalently bound at intervals along its length. In theory any combination and ratio of GAGs may occur. It is only recently that the concept of PoGs has been accepted; before this the presence of protein was assumed to be a contamination and vigorous steps were taken to remove it. Most of the data presented in Table 2.7 below, thus represent experimentally-induced artefact, but it is the only information available to date.

Despite the ubiquity of PoGs, and their composite GAGs, relatively
Table 2.6 The types and structures of GAGs

<table>
<thead>
<tr>
<th>Type of GAG (abbreviation)</th>
<th>Hexuronic Acid</th>
<th>Bond</th>
<th>N-acetylhexosamine Moiety</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chondroitin 4-Sulphate (Ch4S)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>D-Glucuronic acid</td>
<td>1,3 link</td>
<td>D-Galactosamine (N-acetylated and 4-sulphated)</td>
</tr>
<tr>
<td>Chondroitin 6-Sulphate (Ch6S)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>D-Glucuronic acid</td>
<td>1,3 link</td>
<td>D-Galactosamine (N-acetylated and 6-sulphated)</td>
</tr>
<tr>
<td>Dermatan Sulphate (DS)</td>
<td>L-Iduronic acid&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1,3 link</td>
<td>D-Galactosamine (N-acetylated and 4-sulphated)</td>
</tr>
<tr>
<td>Heparin (Hep)</td>
<td>L-Iduronic acid (2-sulphated)</td>
<td>1,4 link</td>
<td>D-Glucosamine (N- and 6-sulphated)</td>
</tr>
<tr>
<td>Heparan Sulphate (HS)</td>
<td>L-Iduronic acid</td>
<td>1,4 link</td>
<td>D-Glucosamine (N-acetylated and 6-sulphated)</td>
</tr>
<tr>
<td>Hyaluronic Acid (HA)</td>
<td>D-Gluronic acid</td>
<td>1,3 link</td>
<td>D-Glucosamine (N-acetylated)</td>
</tr>
<tr>
<td>Keratan Sulphate (KS)</td>
<td>D-Galactose</td>
<td>1,4 link</td>
<td>D-Glucosamine (N-acetylated and 6-sulphated)</td>
</tr>
</tbody>
</table>

<sup>a</sup> When these have not been resolved on separation they are generally referred to as chondroitin sulphates (ChS).

<sup>b</sup> May be up to 20% D-Gluronic acid

After Kennedy (1979) and Casu (1979)
<table>
<thead>
<tr>
<th>Species</th>
<th>Type and amount of GAG*</th>
<th>Yield</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken</td>
<td>HA 38%, HS 50%, Chs 4%, Hep 8%(^a)</td>
<td>2.1g/100g dry defatted tissue</td>
<td>Ng Kwai Hang &amp; Anastassiadis (1980a,b)</td>
</tr>
<tr>
<td>Rat</td>
<td>Heparin - like and some HA only small amounts of galactosamine thus small amounts of Chs(^a)</td>
<td>-</td>
<td>Allalouf et al., (1964)</td>
</tr>
<tr>
<td></td>
<td>HS 56-64%, HA 13-17%, DS 4-13%, Chs 15%(^b)</td>
<td>-</td>
<td>Barry &amp; Bownes (1975)</td>
</tr>
<tr>
<td></td>
<td>HS 88%, DS 12%, Chs&lt;2%(^a)</td>
<td>65μg/g dry tissue</td>
<td>Dietrich et al., (1976)</td>
</tr>
<tr>
<td></td>
<td>HS 65-70%, HA 20-25%(^c,d)</td>
<td>-</td>
<td>Lis &amp; Morris (1978)</td>
</tr>
<tr>
<td></td>
<td>HS as part of glomerular basement membrane</td>
<td>-</td>
<td>Kanwar &amp; Farquhar (1979)</td>
</tr>
<tr>
<td></td>
<td>HS containing proteoglycan as part of glomerular basement membrane</td>
<td>-</td>
<td>Hassell et al., (1980)</td>
</tr>
<tr>
<td>Guinea Pig</td>
<td>HS 70%, DS 22%, Chs&lt;2%(^a)</td>
<td>192μg/g dry tissue</td>
<td>Toledo &amp; Dietrich (1977)</td>
</tr>
<tr>
<td></td>
<td>HA 20-25%, DS + Chs 50%(^c,d)</td>
<td>-</td>
<td>Lis &amp; Morris (1978)</td>
</tr>
<tr>
<td>Rabbit</td>
<td>HA and Ch4S(^c)</td>
<td>-</td>
<td>Farber et al., (1962)</td>
</tr>
<tr>
<td></td>
<td>HA 20%, Ch4S 15%, Chs6 15%, DS&lt;3%, HS trace(^c)</td>
<td>0.89% of dry defatted tissue</td>
<td>Farber &amp; Van Praag (1970)</td>
</tr>
<tr>
<td></td>
<td>HS 62%, DS 24%, Chs 12%</td>
<td>143 μg/g dry tissue</td>
<td>Toledo &amp; Dietrich (1977)</td>
</tr>
<tr>
<td>Dog</td>
<td>HA 30%, Chs 30%(^b,c)</td>
<td>100μg/100g wet tissue</td>
<td>Dicker &amp; Franklin (1966)</td>
</tr>
<tr>
<td></td>
<td>HS 80%, HA 10%, DS 10%(^b)</td>
<td>1 mg/g dry tissue(^b); 9 mg/g dry tissue(^c)</td>
<td>Castor &amp; Green (1968)</td>
</tr>
<tr>
<td></td>
<td>HS 61%, DS 26%, Chs 13%, Hep&lt;0.5%(^a)</td>
<td>534μg/g dry tissue</td>
<td>Toledo &amp; Dietrich (1977)</td>
</tr>
<tr>
<td>Beef</td>
<td>HA and Chs(^c)</td>
<td>1-2% dry weight tissue</td>
<td>Faber et al. (1962)</td>
</tr>
<tr>
<td>Pig</td>
<td>HA 30%, Chs 30%(^b,c)</td>
<td>100μg/100g wet tissue</td>
<td>Dicker &amp; Franklin (1966)</td>
</tr>
<tr>
<td></td>
<td>HS 73%, DS 22%, Chs 5%, Hep&lt;0.5%</td>
<td>300μg/g dry tissue</td>
<td>Toledo &amp; Dietrich (1977)</td>
</tr>
</tbody>
</table>
Table 2.7 (continued)

<table>
<thead>
<tr>
<th>Species</th>
<th>Type and amount of GAG*</th>
<th>Yield</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep</td>
<td>HA 30%, ChS 30% b,c</td>
<td>100µg/100g wet tissue</td>
<td>Dicker &amp; Franklin (1966)</td>
</tr>
<tr>
<td>Man</td>
<td>HS 55%, ChS 17%, DS 15%, HA 10% b</td>
<td>2.7mg/g dry defatted tissue</td>
<td>Constantopoulos et al. (1973)</td>
</tr>
<tr>
<td></td>
<td>HA 38%, HS 33%, DS 15%, ChS 14% c</td>
<td>6.6mg/g dry defatted tissue</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HS 59%, DS 26%, ChS 15%, Hep&lt;0.5% a</td>
<td>150µg/g dry tissue</td>
<td>Toledo &amp; Dietrich (1977)</td>
</tr>
</tbody>
</table>

Distribution a Whole kidney  bCortex  c Inner Medulla
* For GAG abbreviations see Table 2.6  + No data published
little is positively known about their physiological functions, with the exception of their anti-coagulant and anti-lipemic properties which are best studied in heparin. These molecules are bound to cell surfaces (Kjellen et al., 1977) where they possibly control the access of endogenous and exogenous molecules to cell membrane receptors. Similarly, the function of this intercellular polyanionic matrix most probably extend beyond that of "immobilised anti-coagulants" or "space filling", and includes controlling the micro-environment of cells (by binding either inorganic or organic cations and by their immense water holding capacity) and modulating cell-cell communications. A strong case has been put forward for the involvement of GAGs both in controlling cell recognition and adhesion, and in contributing to the control of cell movement, growth, differentiation and proliferation (Long & Williams, 1979).

The association of GAGs with mitochondria, and nuclear membranes (Dietrich et al., 1976), suggests that these macromolecules may also play a direct role in controlling some intracellular functions.

The distribution of GAGs has been assessed in tissue by either the autoradiographic distribution of precursor carbohydrates or $^{35}S\text{O}_4^{2-}$, or by histochemical staining. It is generally assumed that sulphate radiolabel distribution is relatively specific for GAGs, but most of the staining procedures are non-specific (e.g. Toluidine blue interacts with any polyanion to give a metachromatic colour shift) and depend either on a priori knowledge of distribution or, for example, the use of control sections which have been exposed to selective enzymic digestion. Because of the uncertainties associated with the exact biochemical nature of materials assessed by these histochemical approaches it has been suggested (Kennedy, 1979) that stained material is referred to as "mucopolysaccharide" (MPS), and that the name GAGs are applied only to
Norhagen & Odebald (1955) reported the autoradiographic distribution of $^{35}\text{S}$ in the kidney using relatively crude methods. Subsequently, Monis & Longley (1955) reported that "mucins" lined the epithelial of renal collecting ducts in a number of species. (MPS have since been shown to be deposited exclusively as the lining of collecting ducts in the guinea pig medulla, Mowry & Morard, 1957). Longley et al., (1963) demonstrated that the renal incorporation of $^{35}\text{SO}_4$ coincided with the histochemical distribution of "acidic MPS" material.

Few ultrastructural studies have referred to the abundant matrix surrounding the medullary interstitial cells. Furusato (1977) showed that failure to take the special precaution of insolubilising GAG in situ (with cetylpyridinium chloride) during tissue fixations resulted in gross morphological changes. Adequately fixed tissue showed a matrix having diffuse reticular structure, consisting of thick microfibrils enmeshing a dense granular material. Digestion with GAG-specific enzymes removed both the microfibrils and the granules. The granules were also lost when "normal" fixation procedures were followed, suggesting the water solubility of these macromolecules.

The amount and types of GAGs have been reported for the kidneys of various species (Table 2.7). The quantity of polyanionic macromolecule has been confirmed to be greater in the medulla than in the cortex by 2 to 4 fold in the rat (Jacobsen et al., 1964 and Kresse & Grossmann, 1970), 3 fold in the pig (Kresse & Grossmann 1970), 4 to 7 fold in the dog (Castor & Green, 1968 and Kresse & Grossmann, 1970) and by 2 to 13 fold in the normal human kidney (Inoue et al., 1970 and Constantopoulos et al., 1973), where the ratio was age related, increasing rapidly to a maximum in the fourth decade and then declining slowly (Inoue et al., 1970). The heterogeneous distribution of the types of GAG in the kidney
is supported by the data of Constantopoulos et al. (1973) for the human kidney and Castor & Green (1968) who reported that hyaluronic acid was of high molecular weight in the medulla, but low molecular weight in the cortex of dogs. Data on the dog, pig and sheep (Dicker & Franklin, 1966) and the rat (Barry & Bowness, 1975), however, suggests that the types and quantities of GAG are the same in both the cortex and medulla.

The processes underlying and controlling the biosynthesis of PoGs are complex and incompletely documented (Kennedy, 1979). Pitcock et al., 1978 have reported that medullary interstitial cells synthesis PoGs (both in situ and in culture) and that these macromolecules are associated with the cellular cisterns (dilated rough endoplasmic reticulum). Darnton (1967, 1969) presented data to show that glycogen associated with the epithelial cells of the collecting duct in the rabbit were mobilised and incorporated into GAGs. In addition, exogenous sulphate, glucose (Barry & Bowness, 1975), glucosamine and galactosamine (Ng Kwai Hang & Anastassiadus, 1980a,b) are incorporated into de novo synthesised GAGs. The pools from which these precursors are drawn, and the inter-conversion of carbohydrates between the pools is very complex. Ng Kwai Hang & Anastassiadias (1980b) reported a greater incorporation of galactosamine (compared to glucosamine) into fowl kidney GAGs. The time course of radioactivity incorporation differed in the four major types of GAG isolated and there were also subtle differences depending on the type of hexosamine precursor administered.

Barry & Bowness (1975) showed that the specific activity of incorporated radiolabelled sulphate in both the cortex and medullary total polyanion decreased bi-exponentially, with an alpha-phase half-life of 2 days for the cortex and 2.5 days for the medulla and a beta-phase (attained after 5 days) of about 5 to 6 days for both regions. The apex of the medulla (papilla) showed a mono-exponential decay curve
with a half-life of 4 days. Up to 80% of the radioactivity from both the $^{14}$C and $^{35}$S precursors, was incorporated in heparan sulphate during the first 24h. During this early time period (0-24h) there were changes in individual GAG specific activity consistent with the concept that more than one metabolic pool of precursor molecules exists. The turnover rates of individual GAGs were similar to those studied in other tissues, except for hyaluronic acid which was more rapid.

The functions of the medullary GAGs have been the centre of a controversy since Ginetzinsky (1959) suggested that the action of antidiuretic hormone was mediated by the release of hyaluronidase. This would depolymerise medullary GAG and (so it was argued) allow greater water reabsorption from the tubules into the interstitium and thence to the blood supply. This hypothesis was based on two observations:-

a) urinary hyaluronidase increased during anti-diuresis, and

b) during diuresis the interstitial matrix stained intensely for MPS material, but not during anti-diuresis (see the critique below).

Table 2.8 lists the conflicting experimental findings which have been used to support this theory and those which favour its rejection. Jacobson et al., (1964) used papillary hexosamine contents (per mg dry renal papilla) as a direct means of measuring changes in the GAG. They showed that hydration (water loading) increased the amount of hexosamine in dehydrated and in normally hydrated rats. The 'resting' hexosamine contents was lower in dehydrated rats. Anti-diuretic hormone had the opposite effect and decreased hexosamine contents in both normal and 'hydrated' animals. All changes were obvious within 30 to 60 min, the effects were maximal in 1 to 2h, but had returned to normal in 3 to 4 h. These changes were confirmed histochemically. Similarly, Farber et al., 1971 showed that the incorporation of $^{35}$SO$_4^2-$ into slices of rabbit medulla GAG was inhibited by hypertonic incubation medium. It was suggested
Table 2.8  The changes in the staining properties and amount of medullary glycosaminoglycans in response to diuresis and anti-diuresis

<table>
<thead>
<tr>
<th></th>
<th>Diuresis</th>
<th>Anti-diuresis</th>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>↑matrix staining</td>
<td></td>
<td>↑matrix staining ↑urinary hyaluronidase</td>
<td>rat</td>
<td>Ginetzinsky (1958)</td>
</tr>
<tr>
<td>No related changes</td>
<td></td>
<td>No related changes</td>
<td>rat</td>
<td>Boss et al. (1961)</td>
</tr>
<tr>
<td>No related changes</td>
<td></td>
<td>No related changes</td>
<td>rat</td>
<td>Breddy et al. (1961)</td>
</tr>
<tr>
<td>↑matrix staining</td>
<td></td>
<td>↑matrix staining (highly polymerized)</td>
<td>rat</td>
<td>Ivanova &amp; Vinogradov (1962)</td>
</tr>
<tr>
<td>↑matrix staining</td>
<td></td>
<td>↑matrix staining (highly polymerized)</td>
<td>rat</td>
<td>Jacobsen et al. (1964)</td>
</tr>
<tr>
<td>↑matrix staining</td>
<td></td>
<td>↑matrix staining (highly polymerized)</td>
<td>guinea-pig</td>
<td>Vanhegan (1967)</td>
</tr>
<tr>
<td>↑matrix staining</td>
<td></td>
<td>Open fibular appearance (depolymerized, masking of charge?)</td>
<td>rat</td>
<td></td>
</tr>
<tr>
<td>↑amount of matrix</td>
<td></td>
<td>↑amount of matrix of increased acidity</td>
<td>rabbit</td>
<td>Dalton (1967, 1969a,b)</td>
</tr>
<tr>
<td>↑amount of matrix</td>
<td></td>
<td>(more glycogen in collecting duct epithelia)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>↑amount of matrix</td>
<td></td>
<td>(at expense of glycogen in collecting duct epithelia)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\[ a = \text{water loaded} \quad b = \text{mannitol loaded} \quad c = \text{anti-diuretic hormone} \quad d = \text{water deprivation} \quad e = \text{deoxycorticosterone acetate} \]

+ Assessed autoradiographically by the amount of $^3$H-glucose incorporated in mucopolysaccharide staining material.
* Assayed biochemically.
that this inhibition reflected the \textit{in vivo} response to anti-diuresis and thereby lends support to the hypothesis of Ginetzinsky (1959).

On the other hand several workers have failed to reproduce Ginetzinsky's observations. Recently, conflicting data has been generated from Sun et al. (1972) who reported on the histochemical staining of the medullary interstitial matrix of polydipsic and polyuric rats with inherited hypothalamic diabetes insipidus (DI). Compared to normal controls the MPS matrix stained slightly less intensely for heterozygous rats, but only faintly for homozygous DI rats. This has been confirmed by McAuliffe (1978, 1980) and by Sun (1980) who both showed that 3 weeks of daily anti-diuretic hormone administration to homozygous DI rats

a) stimulated the 'synthesis' of histochemically stainable medullary interstitial MPS matrix, and

b) caused Type I interstitial cells (2.3.3.1) to align perpendicularly to the medullary tubular elements. McAuliffe failed to comment on the effects of this anti-diuretic treatment on either urine osmolality, or daily water consumption and urine volume. Unpublished data was, however, quoted to support the view that poor concentrating ability in the homozygous DI rats was associated with only 20% of normal papillary GAGs. Heterozygotes were quoted as having 75% of normal GAGs, but produced a urine having less than half the osmolality of normal rats.

These conflicting data are difficult to resolve into a single unifying theory relating the physiological function of GAGs to the urine concentrating process. It seems reasonable to assume that some of the conflicting data may have arisen because of failure to realise that the histochemical staining of the renal papillary MPS is fraught with difficulties. Firstly, most of their methods are non-specific (see above) and do not differentiate the type of GAG. Secondly, many of the
Dyes used for these procedures have often been adulterated and staining properties are notoriously variable, and may give irreproducible results; standards for their purity have only been established recently (Horobin, 1980). Thirdly the intensity of histochemical staining measures the availability of dye "interactive sites" rather than the quantity of MPS. Fourthly, failure to fix MPS material in situ may give an artefactual histochemical distribution. Fifthly, the relationship between the changes in the interstitial MPS and those lining the collecting ducts during altered states of hydration is uncertain. Vanhegan (1967) reported that the staining intensity of the collecting duct MPS increased during anti-diuresis, but as has been explained the distribution of these macromolecules is peculiar in the guinea pig kidney. Sixthly, the intense diuresis in DI rats (McAuliffe, 1978, 1980) may wash the water soluble (Furusato, 1977) MPS from their less water soluble reticular matrix. Finally, the physiological function(s) of renal glycosaminoglycans may depend on one or more physicochemical features (for example, chain length, or the functionally available -NH₂, -COOH, N-acetyl, N-sulphate or O-sulphate groups), factors which have not yet been investigated. Further, the types of GAG may be heterogeneously distributed within the kidney. Relatively subtle changes or interconversions may obviously alter the interstitial milieu enough to cause major functional changes which will go undetected by inappropriate means of assessment.

2.2.4.5 The metabolism of xenobiotic molecules in the kidney.
Chemically-induced lesions may depend to varying extents on the metabolic capacity of tissues to deal with "insults". The metabolism of xenobiotic molecules may either prevent lesions (by deactivation), or be directly responsible for damage (by activation). The renal
metabolism of chemicals (and its consequences) has been reviewed by Hook et al. (1979), Anders (1980), Connelly & Bridges (1980), Kluwe & Hook (1980), Davis et al. (1981), Rush & Hook (1982), and Ormstadt (1982).

It is likely that the liver meets the challenge of metabolising a major proportion of exogenous compounds, in vivo before they reach the systemic circulation. The very large blood volumes perfusing the kidneys (2.1.1) and the concentration processes that are part of normal renal function (2.3.2) demand, on purely teleological grounds, an effective xenobiotic metabolising function. Indeed most fundamental types of bioconversion have been described for the perfused kidney of several species (Szefler & Acara, 1979; Elbers et al., 1980; Ross et al., 1980 and Emslie et al., 1981) and isolated renal cells and tubular fragments (Fry et al., 1978; Jones et al., 1979; Szinicz & Weger, 1981 and Ormstadt, 1982). Similarly, kidney microsomes have been shown to have most of the enzymic and cytochrome mediated metabolic activities that have been described in other tissues (see reviews and references cited therein). The xenobiotic transformation capacity in the kidney is about 3 to 50% (depending on the system, species and source of data) of that found in the liver (Litterst et al., 1975a, Navran & Louis-Ferdinand 1975, and Fry et al., 1978) but may be much higher than the liver under certain circumstances (see Anders, 1980). There are marked qualitative differences between the hepatic and renal xenobiotic metabolism. Renal enzymes are stable during the Ca$^{2+}$ aggregation method of preparing microsomes (Litterst et al., 1975b), enzymic kinetic constants vary between microsomes isolated from the two organs (Navran & Louis-Ferdinand, 1975) and where as there are marked sex-related differences in hepatic metabolism there are few in the kidney (Litterst et al., 1977). There is evidence to suggest that the cytochrome P-450's from the liver and kidney are similar based on electrophoretic, and electron
paramagnetic resonance studies (Armbrecht et al., 1979), immunological
criteria (Guengerich & Mason, 1979) and on immunometabolic studies
(Kaminsky et al., 1979), but these data are most difficult to interpret
in "absolute" terms, because the P-450's were from organs exposed to
different inducing agents. There is now substantial evidence that
hepatic and renal tissue may respond differently (both quantitatively
and qualitatively) to the various inducers of cytochrome P-450 (Litterst
et al., 1977; Zenser et al., 1978 and Kaminsky et al., 1979). Ascorbic
acid deficiency (Sikic et al., 1977) and carbon tetrachloride
pretreatment (Litterst et al., 1977) alters the metabolism of
xenobiotics differently in the liver and kidney, and the inhibitory
effects of 2-diethylaminoethyl-2,2-diphenylvalerate (SKF-525A) are
similar, but not identical, for renal and hepatic microsomes studied in
vitro (Litterst et al., 1977).

There is now substantial evidence to support the concept that the
metabolism of xenobiotics often produces biologically reactive
intermediates, which mediate their toxic lesions by binding to cellular
macromolecules and blocking normal functional processes (see Jollow et
al., 1977 and Snyder et al., 1981). Similar mechanisms have been proposed
to explain various types of chemically-induced renal lesions, including
papillary necrosis (See 3.2.5.4). The metabolically generated reactive
intermediates have a relatively short life and are most likely formed in
the organ or anatomical area in which they induce damage. Recently, it
has been shown that there are marked quantitative and qualitative
differences in the regional distribution of microsomal mixed functional
oxidase activity within the rabbit kidney (Zenser et al., 1978 and
Armbrecht et al., 1979). Most mixed functional oxidase activity was
located in the cortex and least in the inner medulla in control tissue
and that taken from animals induced with 3-methylcholanthrene.
Cytochrome P-450 was not detected in the medulla of controls and absent from the inner medulla even after induction. In addition, laurate hydroxylase activity (the only mixed functional oxidase activity found in the inner medulla) showed marked differences in the pattern of inhibition by carbon monoxide, α-napthoflavone and metyrapone in the cortex, the outer, and the inner medulla. This suggests some differences in the genetic expression of the same type of enzymic activity in different zones of the kidney.

More recently Zenser and co-workers (Davis et al., 1981) have focussed their attention on the oxidative metabolism in the medulla which is mediated in the absence of spectrophotometrically measurable cytochrome P-450. Zenser et al. (1979a) reported that cortex microsomes metabolised 1,3-diphenylisobenzofuran to O-dibezoylbenzene largely via a cytochrome P-450-like system (it was NADPH dependent and inhibited by carbon monoxide and metyrapone). The inner medulla microsomes had the same metabolic capacity, but largely in the presence of arachidonic acid (the system was independent of NADPH, and inhibited by non-steroidal anti-inflammatory compounds such as indomethacin and not by carbon monoxide or, metyrapone). The outer medulla microsomes had both types of activity. The anti-oxidant ethoxyquin inhibited the arachidonic acid and the NADPH dependant metabolic processes.

The specific arachidonic acid dependent metabolism (assumed to be via prostaglandin cyclo-oxygenase) of benzidine (a potent bladder carcinogen) was shown to be absent from hepatic and cortex microsomes, but active in medullary microsomes, especially inner medulla microsomes. The metabolism was inhibited by non-steroidal anti-inflammatory drugs, ethoxyquin, and arachidonic acid analogues. Approximately 75% of metabolised benzidine was covalently bound to macromolecules, presumably via a reactive intermediate. Addition of sulphydryl protectors, such as
glutathione, reduce the amount of covalently bound metabolite to 25% (Zenser et al., 1979b). Using rabbit renal inner medullary slices Rapp et al. (1980) have confirmed the arachidonic acid dependent co-oxidative activation of low concentration of benzidine, and its covalent binding to tissue. Benzidine caused a dose dependent reversible inhibition of PGE₂ synthesis and non-steroidal anti-inflammatory drugs, ethoxyquin and arachidonic and analogues (that are not cyclo-oxygenase substrates) inhibited benzidine metabolism.

Recently, Mohandas et al. (1981) have shown that paracetamol is activated by an arachidonic dependent pathway predominantly in the inner medulla and minimally in the cortex.
The first reported cases of renal papillary necrosis (RPN) in man were published by von Friedreich (1877) and by Turner (1885, 1886 and 1888). One hundred and sixty cases, involving one or both kidneys, had been reported by 1950. These were reviewed by Mandel (1952) who concluded that diabetes or urinary obstruction (or both) were the most frequently diagnosed conditions associated with the lesion and suggested that the local release of bacterial toxins might also contribute to cell death in the necrotic area.

RPN was first recognised as an iatrogenic disease when Spuhler and Zollinger (1953) drew attention to the apparent increase in the incidence of "chronic interstitial nephritis" (caused by an underlying papillary necrosis) and highlighted the association between this condition and an abusive intake of analgesics. The mixed analgesics which were consumed by these patients all contained phenacetin. Similar observations were reported over the next decade and phenacetin was assumed to be the solitary aetiological common denominator. The condition was soon dubbed "phenacetin kidney", but the aetiological role of phenacetin has since been disputed, and current evidence favours the idea that most, if not all, analgesics and anti-inflammatory drugs may give rise to the lesion (see 3.1.2).

3.1 RENAL PAPILLARY NECROSIS IN MAN

RPN has many possible underlying causes (Table 3.1). The lesion is most difficult to diagnose (except at autopsy, and even here it can easily be overlooked—see 3.1.1), thus the true incidence of drug-induced papillary necrosis can only be a poor estimate. Murray &
Table 3.1 Factors Relating to the Development of Renal Papillary Necrosis -

Frequently Reported:-

Diabetes Mellitus
Analgesic Abuse
High Dose Non-Steroidal Anti-Inflammatory Drug Therapy (?)
Upper Urinary Tract Obstructive Uropathy (consequence?)
Recurrent Urinary Tract Infection (consequence?)
Sickle Cell Haemoglobinopathy
Acute Pyelonephritis (superimposed?)
Dehydrated Newborn Infants (frequently jaundiced)

Less Frequently Reported:-

Renal Vein Thromboses
Chronic Alcoholism
Dehydration in Children (Diarrhoea)
Severe Jaundice
Calyceal Artheritis
Glomerulonephritis
Renal Transplant
Systemic Candidosis
Trauma
Prolonged Hypotension
Dapsone

From Data Cited by:-

Harrow (1967); Knepshield et al. (1968);
Watanabe & Sakaguchi (1969); Hoffbrand (1978); Greenlaw (1979);
Shah & Kisilevshy (1980)
Goldberg (1978) suggest that the low incidence in the USA is, in part, under-diagnosis, and Cove-Smith & Knapp (1978) found that there was a 30% under-estimate in the prevalence of the lesion in the UK. Up to 1973 about 3200 cases had been reported (Rosner, 1976). The literature relating to the topic of analgesic associated renal papillary necrosis is extensive, and has been reviewed by Shelley, 1967; Gault et al., 1968; Abel, 1971; Nanra & Kincaid-Smith, 1972; Murray & Goldberg, 1975; Stewart & Gallery, 1976; Duggin, 1977; Kerr & Ward, 1977; Burry et al., 1977; Nanra et al., 1978; Shelley, 1978; Cove-Smith & Knapp, 1978; Hook et al., 1979; Kincaid-Smith, 1979; Duggin, 1980; Nanra, 1980 and Stuygles & Iuliucci, 1981.

3.1.1 Diagnosis of RPN in Man

The clinical symptoms now known to be associated with the early development of analgesic associated renal papillary necrosis (Table 3.2), are unlikely to draw attention to themselves. When they do, it is only the observed presence of a papilla in the urine (a rare and oft overlooked occurrence, Burry et al., 1977) that is likely to pinpoint the lesion. The progression of renal damage is insidious and as much as 60 to 85% of renal function may be compromised before symptoms become obvious. These will not, however, in themselves, be indicative of the underlying cause.

Radiological examination may not identify papillary necrosis (if the necrosed papilla remains in situ), and even when loss of the papilla is obvious (in the presence or absence of other degenerative renal changes) the underlying cause might be any of those listed in Table 3.1 (Harrow, 1967 and Lindvall, 1978).

The only definitive criteria for confirming the underlying cause of a diagnosed papillary lesion is excessive analgesic intake in the absence of other possible clinical causes (Table 3.1) and:-
Table 3.2 Clinical Features Associated with Renal Papillary Necrosis.

"Early Symptoms"

Females predominate 3:1 to 8:1. Most common 40 to 60 years of age

Psychiatric abnormalities: immaturity, dependence, emotional instability, anxiety, headaches, introversion and neurosis. (Incidence very high)

Upper gastrointestinal disease: Peptic ulceration of Stomach* or Duodenum*, "Dyspepsia" (Incidence up to 86%)

Anaemia: GIT bleeding*, haemolysis, iron deficiency, Cyanosis* (Incidence up to 60%)

"Intermediate Symptoms"

Urinary Tract Disease: Bacteriuria, Sterile pyuria, nocturia, dysuria, microscopic haematuria, ureteral colic, lower back pains. (Incidence up to 100%)

Urinalysis: Defect in ability to concentrate and acidify urine, Proteinuria, Nocturia, Azotemia

"Late Symptoms"

Hypertension (Incidence up to 70%)

Cardiovascular manifestations, Ischaemic heart disease, Peripheral vascular disease

Renal calculi and bladder stones (Incidence high)

Renal Malfunction, Decreased glomerular filtration rate, Increased blood urea nitrogen, Renal tubular acidosis.

Renal osteodystrophy and bone pain

Carcinoma of Renal Tract.

Acute Renal Failure: Especially common after surgical procedures (Clark & Linton 1973).

From data cited by: Gault et al. (1968); Clark & Linton (1973); Murray (1974); Duggin (1977); Stewart (1978); Kincaid-Smith (1980) and Nanra (1980).

+ Possibly caffeine withdrawal related. * Direct secondary consequence of high dose analgesic intake.
A) the identification of abnormally high plasma or urine levels of these compounds (or their metabolites) over long periods, or

B) a history of high dose, long term analgesic usage, or both (Dubach et al., 1978).

Patient histories are, however, notoriously unreliable, especially if (as is the case) there is a social stigma associated with abusive drug consumption. Further, the question of what quantity, and which analgesic(s) has been abused have been obscured by failure to identify the causative agent(s) (3.1.2) and hence the lack of a definition of what constitutes "an abusive consumption".

The inappropriate reasons underlying excessive analgesic use have been evaluated by many research reports. These have been reviewed by Murray, 1974; Murray & Goldsberg, 1975; Rosner, 1976 and Murray, 1978. They include such factors as the mood altering effects of analgesic constituents such as codeine, caffeine and other sedatives or stimulants; use to "overcome" caffeine withdrawal headaches that have developed as a consequence of abusing caffeine containing analgesics; personality traits which predispose to addiction; accepted social or working environment customs, and possibly other unidentified factors.

The criteria for abuse of "analgesics" is poorly defined, and covers the broad range of 2 to 35kg (of phenacetin) over 3 to 45 years (Goldberg & Talner, 1975). Other reports suggest an accumulative estimate of each of the separate analgesic intakes, or the total mixed analgesic consumption (Gault et al., 1968 and Cove-Smith & Knapp, 1978).

3.1.2 The Epidemiology, Geographical Distribution and Incidence of RPN in Man.

A number of reports (see Shelley, 1967; Abel, 1971; Murray & Goldberg, 1975; Duggin, 1977; Shelley, 1978 and Dubach et al., 1978 for
A) the increased incidence of renal lesions in those patients who had consumed excessive amounts (see 3.1.1) of analgesic(s),

B) a proportionality relationship between the amount of analgesic (phenacetin) consumed and the extent of renal damage, and

C) the stabilization of (or improved) renal function that developed in patients who ceased to abuse analgesics; while those who continued to consume excessive amounts showed a steady deterioration in renal function.

Much of the early data supported the theory that phenacetin was the solitary underlying cause of RPN. Gilman (1964), however, questioned the validity of this theory, and suggested that each or all of the constituents (in mixed analgesics) may contribute. The question has still not been resolved, but most analgesic and nonsteroidal anti-inflammatory drugs (NSAID) have (at one time or another) been shown to induce the lesion experimentally, if not in humans (see below). There have been no reports of the development of papillary necrosis in patients who took only phenacetin (but the prescribing of phenacetin on its own was very uncommon). There is some evidence to support the view that salicylates can cause the lesion in rheumatic patients, but this has been questioned (on the basis that the data was derived retrospectively), and refuted by prospective surveys (see Goldberg & Talner, 1975; Duggin, 1977; Cove-Smith & Knapp, 1978 and Kincaid-Smith, 1980 for discussions).

The dangers of "therapeutically induced" papillary damage from mixed analgesics has been highlighted by Cove-Smith & Knapp (1978) who reported that 42% of their patients with "analgesic nephropathy" were taking prescribed high dose, longterm mixed analgesic therapies for either rheumatoid arthritis or osteoarthritis. In 88% of the patients in this group the cause of death was chronic or acute renal failure. The
analgesics taken were either aspirin-phenacetin or aspirin-paracetamol mixtures.

Recently, there have been a number of reports suggesting that the "second generation" NSAID may induce papillary necrosis at commonly prescribed doses. The NSAID so far linked to this lesion include propoxyphene (Murray & Goldberg, 1978), fenoprofen and naproxen (Husseri et al., 1979), phenylbutazone, indomethacin, antipyrine, ibuprofen and aloclofenac (see Prescott, 1979 for references) and recently Robertson et al. (1980) have drawn attention to several cases of papillary necrosis following the use of mefanamic acid (see Section 3.2.3.1).

No workers have looked beyond the anti-inflammatory, or analgesic groups of drugs as possible papillotoxic compounds in man, but one unsubstantiated report cites therapeutic doses of dapsone as having induced the lesion (Hoffbrand, 1978). Burry (Burry et al., 1977, Burry 1978) and Gloor (1978) appear to be the only workers who have speculated that other drugs (or chemicals) may also contribute to, or even cause RPN in man.

Some workers have ignored these findings and still insist that phenacetin (or one of the impurities associated with its synthesis - p-chloracetanilide) was the only cause of the lesion (see Shelley, 1967; Rosner 1976 and Shelley, 1978). Phenacetin was withdrawn from most commercially available analgesics between 1967-1975 and replaced by either salicylamide or paracetamol. The Australian experience suggests that these substitutions have not (contrary to expectations) decreased the incidence of the lesion - in fact several reports suggest an increase (see Kincaid-Smith, 1979).

Over the last few years increasing attention has been focused on the supposed importance of paracetamol (acetaminophen) in the underlying aetiology of analgesic associated renal disease. Many attempts to link
paracetamol and RPN have been speculative, some of them perhaps wildly so, especially those that draw inferences on the chronic nephrotoxicity of paracetamol from its acute effects. Rosner, 1976 is of the opinion that the initial "link" between papillary necrosis and paracetamol was forged by the observations that paracetamol (a major metabolite of phenacetin) was concentrated across the medulla, but that phenacetin was not (see 3.2.5.1). This tenuous association has most probably been strengthened by:-

A) The uncompromised blind dogma that phenacetin is the original and only cause(!) of papillary necrosis.

B) Failure to decrease the frequency of analgesic nephropathy when phenacetin was replaced by paracetamol in mixed analgesics.

C) Mitchell's group (see Mitchell et al., 1977 for references) have described a mechanism to explain the acute hepatic necrosis that develops as a consequence of high doses of paracetamol. Only renal proximal tubule necrosis has been associated with acute paracetamol intoxication, nevertheless some workers have transposed this mechanism (see 3.2.5.4) to "fit" chronically induced renal papillary necrosis.

D) Dubach has reported a prospective study where urinary paracetamol was used as the distinguishing criteria of patient analgesic abuse, (Dubach, 1978; Dubach et al., 1978). However, these investigations fail to differentiate between patients who took analgesic mixtures which contain paracetamol as opposed to those containing phenacetin (which was still available in Switzerland, Gloor, 1978).

E) Recently, Zollinger has published a series of papers on his lifes work in the field of "phenacetin abuse". It concludes that phenacetin and paracetamol containing analgesics cause an expensive abuse problem,
varied renal lesions (including malignant changes) and suggested that both drugs should be replaced by other analgesics (Zollinger, 1980; Mihatsch et al., 1980a,b,c, and Dalquen et al., 1980). The incrimination of paracetamol and phenacetin are not, however, based on sound reasoning because the vast majority of patients had abused MIXED analgesics.

One of the other puzzling features underlying the aetiology of RPN is the varying incidence between countries (Table 3.3) and especially within countries (Table 3.4). Some of these variations may relate to a lack of awareness and consequently failure to diagnose the condition. It is equally possible that the extent of abuse and nature of the offending chemicals varies between and within countries. The per capita consumption of phenacetin (still assumed by many to be representative of total analgesic intake) shown in Table 3.3 has been used to support this hypothesis. A number of workers have questioned the origins of these figures, the validity of the assumptions underlying the calculations (Gault et al., 1968; Rosner, 1976 and Murray & Goldberg, 1978) and the fact that "consumptions" are similar in Canada, South Africa, the United Kingdom and the USA, but there are markedly different incidences of analgesic associated nephropathies.

The higher incidence of papillary necrosis in sub-tropical Queensland, together with the greater frequency of diagnosed papillary necrosis (not frequency per se as is often suggested) in the summer months in Australia, is generally assumed to substantiate the role of "climatic factors" in the geographic variability and possibly the pathogenesis of the lesion (see 3.2.5.1), (Burry et al., 1977, and Burry, 1978).

3.1.3 The Pathological Course of RPN in Man

The evolution of papillary necrosis reported by Burry et al., 1977; Burry, 1978 and Gloor, 1978 shows that the lesion is primarily one of the
<table>
<thead>
<tr>
<th>Country</th>
<th>Patients Entering Dialysis Programme as Consequence of Analgesic Usage</th>
<th>Autopsy Surveys</th>
<th>Per capita consumption of Phenacetin (g/head/year)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australia</td>
<td>15 - 30%</td>
<td>3.7 - 20%(^a)</td>
<td>40</td>
</tr>
<tr>
<td>Canada</td>
<td>5.5%</td>
<td></td>
<td>6 - 7</td>
</tr>
<tr>
<td>Denmark</td>
<td>-0.3 to 4%(^c)</td>
<td></td>
<td>25(^c)</td>
</tr>
<tr>
<td>Finland</td>
<td>0.3 to 4%(^c)</td>
<td>12 - 25%(^c)</td>
<td>25(^c)</td>
</tr>
<tr>
<td>France</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Germany</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>South Africa</td>
<td>20(^b)</td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>Sweden</td>
<td>0.3 - 4%(^c)</td>
<td>0.3 - 4%(^c)</td>
<td>25(^c)</td>
</tr>
<tr>
<td>Switzerland</td>
<td>0.3 - 4%(^c)</td>
<td>1.1 - 1.3(^a)</td>
<td>22</td>
</tr>
<tr>
<td>United Kingdom</td>
<td>12%</td>
<td>0.16(^a)</td>
<td>8(^a)</td>
</tr>
<tr>
<td>United States</td>
<td>0.2%</td>
<td>0.41 - 0.54(^b)</td>
<td>(Scotland 12)</td>
</tr>
</tbody>
</table>

\(^a\) Kincaid-Smith (1978)

\(^b\) Cove-Smith & Knapp (1978)

\(^c\) Gault et al. (1968)

\(\dagger\) Figure for Europe

\(^*\) Figure for Scandinavia
Table 3.4  Variation in the Incidence of Renal Papillary Necrosis within Countries.

Australia\textsuperscript{a}  (Data for period 1971 - 1976)

<table>
<thead>
<tr>
<th></th>
<th>Percentage population abusing analgesics</th>
<th>Cases of papillary necrosis per million population</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>Queensland</td>
<td>16</td>
<td>11</td>
</tr>
<tr>
<td>Northern Territory</td>
<td>No data</td>
<td></td>
</tr>
<tr>
<td>New South Wales</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>South Australia</td>
<td>No data</td>
<td></td>
</tr>
<tr>
<td>Tasmania</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Victoria</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Western Australia</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

United Kingdom\textsuperscript{b}  

<table>
<thead>
<tr>
<th></th>
<th>Reported Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Midlands</td>
<td>low</td>
</tr>
<tr>
<td>North-West England</td>
<td>low</td>
</tr>
<tr>
<td>Newcastle</td>
<td>moderate</td>
</tr>
<tr>
<td>South East England</td>
<td>moderate</td>
</tr>
<tr>
<td>Glasgow</td>
<td>high</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Stewart (1978)  
\textsuperscript{b} Murray (1973 & 1978)
apex of the papilla and this then progresses slowly to include secondary cortical changes.

3.1.3.1 Early papillary necrosis. The earliest reported changes related to papillary necrosis include necrosis of the limbs of Henle, the capillaries and interstitial cells (with or without calcification). PAS staining shows a thickening of the basement membrane. Whereas Burry et al., 1977 and Burry, 1978 report a more intense staining of mucopolysaccharide, Gloor (1978) suggested loss of this ground substance from the interstitial matrix. Cryostat sections stained with oil red-O may show an accumulation of lipid material deposited in the necrosed tissue, which constitute columns progressing up the medulla, but leaving the vasa recta, some limbs of Henle and the collecting ducts intact. Needle shaped crystals have been noted in lipid rich necrosed areas. These were reported to be cholesterol esters and possibly arachidonic acid (Burry, 1978).

3.1.3.2 Intermediate papillary necrosis. Those anatomical elements in the medulla which were previously unaffected, with the exception of collecting ducts and occasional vasa recta, are necrosed. Calcium deposits are more extensive in the necrosed area and the outer medulla may show atrophy and sclerosis, and varying degrees of fibrosis and inflammatory response. Bone formation may also occur in the necrosed papillae.

The necrosed papilla ghost may remain attached or separate from the medulla. The separated papillae may:-

a) remain in situ, or
b) leave the minor calyces either whole or fragmented.

The medulla above the line of sequestration is never histologically normal, but, nevertheless, is coated by transitional epithelium. Bacterial infection may be superimposed up on these changes.
3.1.3.3 **Total papillary necrosis.** Total papillary necrosis develops from the progressive destruction of the inner medulla, following either a crescent or a central course.

3.3.3.4 **Cortical changes in papillary necrosis.** Microscopically the cortex appears normal until the papillae which "functionally underlies it" is totally necrosed. The form of cortical changes then depend on the degree of sclerosis at the line of sequestration (if the papilla is cast off). The cortical changes are essentially characterised as "chronic interstitial nephritis", and include tubular atrophy, interstitial fibrosis, inflammatory infiltration, glomerular changes, hyalinization within collecting ducts and destruction of vascularity.

Burry et al., 1977 stress that confusing similar histological changes may arise from factors such as age, amyloidosis, and 'idopathic' papillary calcium lesion. Further histopathological changes that may develop in the medulla as a consequence of diabetes, ureteral obstruction, sickle cell anaemia, alcoholic cirrhosis or chronic dehydration have many similarities which are strikingly common with those associated with abusive analgesic intake.

3.1.3.5 **Changes in the pelvis, ureter and bladder in papillary necrosis.** An association between analgesic abuse and carcinoma of the pelvis, ureter and bladder transitional epithelia has been clearly established (Bengtsson et al., 1978; Burry, 1978; Gloor, 1978; Gonwa et al., 1980 and Lomax-Smith & Seymour, 1980 a,b). The underlying pathogenesis is not clear, but has been assumed to be induced via biologically reactive intermediates formed from phenacetin (Bengtsson et al., 1978). There appears to be little experimental work published in this area, but recent studies on cyclo-oxygenase mediated xenobiotic metabolism suggest this may be a key factor (see 2.3.4.5).

In addition, non-malignant histopathological changes in the pelvis,
ureter and bladder (such as thickening of capillary walls, sclerosis of lamina propria, and changed fat and collagen deposition) have been reported (Burry, 1978 and Gloor, 1978).

3.1.4 The Prognosis for Patients Suffering from Papillary Necrosis.

The prognosis for patients with RPN depends on an early diagnosis. Depending on the degree of renal damage the patient may progress along three clinically distinct channels. If the patient stops abusing the offending compounds then kidney function may either:-

A) improve dramatically, or
B) it may stabilise.

If, however, (as more often seems to be the case) large doses of the drugs are still consumed the kidney function will:-

C) deteriorate and eventually the patient will suffer renal failure.

Even if patients are part of a sophisticated dialysis or transplant programme their prognosis is poor. Kingsley et al., 1972 have reported a 51% death rate in a 5 year period, while Cove-Smith & Knapp (1978) reported a mean survival of 31.3 months from the time when patients were diagnosed to be suffering from RPN.

3.2 EXPERIMENTALLY INDUCED RPN

Investigations into the pathogenesis of experimentally induced nephrotoxicity is hampered by species and sex variation in renal morphology (see Symposium editored by Stolte & Alt, 1980), the complexities of renal function (see Chapter 2) and the inherent limitations of the available means of assessing renal malfunction (Snell, 1967 and Zbinden, 1969). These difficulties are further compounded by the many and varied degenerative changes that are often encountered in untreated animals. These are either genetically linked spontaneous lesions, or occur with increasing frequency in aging animals - a problem that is impossible to overcome in chronic toxicity.
3.2.1 Spontaneously Occurring RPN.

Several workers have reported spontaneously occurring RPN in different murine strains and hybrids (Gover, 1940; Dunn, 1944 and Cornelius, 1970). This was invariably associated with amyloid deposits in the kidney. The lesion was age related in that it occurred with increasing frequency and severity after 12 months. The necrosis was preceded by amyloid infiltration of the interstitium at the papillary apex. The necrosis extended to other functional areas, but was limited to the medulla. The changes in the cortex were secondary to papillary necrosis and included amyloid deposits, and cystic dilatation.

There appears to be no published research into the molecular pathogenesis of these age related degenerative changes.

Papillary necrosis has also been reported to occur in adult homogenous Gunn rats (Schmid et al., 1959, Blanc & Johnson, 1959 and Axelsen, 1973). The necrotic lesion developed from the papilla tip and was always associated with a deposit of bilirubin-like crystals in the interstitial matrix. The lesion may be comparable with that observed in jaundiced infants and adults (Table 3.1), but the pathogenesis has not been explored.

3.2.2 Non-chemically-Induced Renal Papillary Necrosis

There are also a number of model systems which can be induced by non-chemical means.

3.2.2.1 Dietary induced renal papillary necrosis. Renal papillary necrosis can be induced by maintaining rats on a fat-free diet for up to 43 weeks (Burr & Burr, 1929, 1930; Boyland & Jackson, 1931 and Molland 1978a). The morphology has been described in detail (Molland, 1978a) and includes degenerative changes in the interstitial cells and tubular
epithelium, hyperplasia of pelvic epithelium, loss of MPS staining from the interstitial matrix and fatty deposits in the cells of the medulla and cortex. McAmis et al. (1929), have also reported the increased incidence of calculi in the renal pelvis, the ureters and the bladder, but this may have been due to a concomitant vitamin A deficiency (Boyland & Jackson, 1931).

The molecular pathogenesis has generally been explained on the basis of an essential fatty acid deficiency limiting the availability of prostaglandin precursors (2.3.4.3 and 3.2.5.3). The interstitial cells are very rich in lipid materials (2.3.4.2), and dietary perturbation of this most complex metabolism might equally explain cellular necrosis.

3.2.2.2 Renal papillary necrosis induced by "manipulating" renal haemodynamics. Muirhead et al. (1950) demonstrated that ligating the ureter caused both hydronephrosis and papillary necrosis in 64% of dogs thus treated. They suggested that intrapelvic pressure had altered the medullary haemodynamics and so caused the lesion. Similar results have been reported in uninephrectomized rabbits by Sheehan & Davis (1959a). Necrosis was not, however, induced in rats by ureter ligation (Edmondson et al., 1947).

Occluding the major renal blood vessels may also cause papillary necrosis. Beswick & Schatzki (1960) reported that total renal vein occlusion caused a complete medullary necrosis, with some cortical lesions. Chronic partial renal vein occlusion, however, caused necrosis (of varying complexity), but only in the medulla. Similar observations have been reported by Sheehan & Davis (1959b), Baum et al. (1969), and Davies (1967) who showed, in addition, that occluding the renal artery caused necrosis of the cortex with only minimal changes in the medulla.

3.2.2.3 Heterologous serum-induced renal papillary necrosis. Patrick et al., 1964 were the first to report that papillary necrosis developed
in rats within 72h of injecting human serum intravenously. If, however, the heterologous serum was incubated with rat erythrocytes it caused haemolysis and agglutination, but the necrotic effect was lost when the incubated serum was subsequently administered. Serum from other species caused only minor changes, if any.

It seems likely that agglutinated rat erythrocytes could disrupt medullary blood flow (3.2.2.2), but Patrick et al., 1964 also suggested a direct antigenic effect of human serum components on the rat kidney. This is supported by the fact that the rabbit kidney shows a tremendously violent and rapid (within 30s) fixation of human antibodies, and of complement, when xenoperfused with fresh human blood (Rossmann & Matousovic, 1979). Recently, Rudofsky et al., 1980 reported (without comment) that SJL mice were found to have papillary necrosis 56 days after immunization with rabbit tubular basement membrane. The possibility that this was a spontaneous lesion (3.2.1) cannot, however, be excluded until similar nephroimmune responses have been induced in other species.

3.2.3 Chemically-induced Renal Papillary Necrosis.

A diverse array of chemicals (mostly analgesics or NSAID or their structural analogues) are now known to have papillotoxic potential, Table 3.5. However, the induction of renal papillary necrosis is disturbingly irreproducible for many of these agents, presumably because of unrecognised and uncontrolled variables.

A detailed review of the extensive literature on the topic is beyond the scope of this thesis; it has been reviewed or discussed by Shelly, 1967; Abel, 1971; Rosner, 1976; Duggin, 1977; Burry et al., 1977; Shelley 1978; Molland, 1978a,b; Duggin, 1980 and Nanra 1980 among others.

The pathological course (3.2.4) and possible molecular pathogenesis
From data published by Bruckner et al. (1974), Wiseman & Reinert (1975), Todd et al. (1975), Shelley (1978), Husserl et al. (1979) and Prescott (1979)
of the experimentally induced lesions (3.2.5) are considered below. Several important points must be highlighted.

The diagnosis of chemically-induced papillary necrosis is not easy. The behaviour and appearance of test and control animals is apparently normal and weight gains are similar (Molland, 1978a). Changes in blood biochemistry have been described, but they are either transitory and non-specific, or they reflect secondary cortical changes (Hardy 1970a; Ellis et al., 1973 and Ellis & Price, 1975).

Intravenous urograms appear normal in rats with an acutely induced total papillary necrosis (Sherwood et al., 1971). Urine analysis also fails to give a clear cut diagnosis of papillary necrosis. The exfoliation of renal tubular cells follows the administration of many analgesics (see Shelly, 1967), but it is not indicative of papillary necrosis per se (Davies et al., 1968) and most likely reflects a response to an irritant compound. The pattern of enzymuria is not specific for papillary lesions (Ellis et al., 1973 and Ellis & Price, 1975) and, in common with proteinuria, probably reflects secondary cortical changes. Decreased urinary osmolality (due to the destruction of the anatomical elements responsible for concentration), lack of concentrating capacity and failure to acidify the urine after oral ammonium ion loading are the only features ascribed to papillary necrosis, but other nephrotoxins may produce the same response (Ellis et al., 1973; Ellis & Price, 1975; Hardy, 1970a and Nanra, 1980).

Thus none of these functional changes are specific indications of papillary necrosis, and there are circumstances where apparently normal function is recognised (Hardy, 1970a), although it is likely that the renal functional reserve capacity would fail under stress such as infection or dehydration.

There are no known specific biochemical markers for the diagnosis of
papillary necrosis, thus the lesion can only be established by histopathology. The papilla tip may, however, be missed unless painstakingly careful sectioning is undertaken. This difficulty is compounded by the re-epithelialisation of the truncated medulla when the necrosed area sloughs off. Thus a section that appears to be an obliquely cut medulla may, in fact, be adjacent to the remnants of a necrosed area (Hardy, 1970a).

3.2.3 The papillotoxicity of analgesic and anti-inflammatory compounds and their structural analogues.

Much of the published data is conflicting, often experimental details have been omitted (such as strain, dose regimens, routes of dosing, sex, age, weight etc), many of the findings are irreproducible (sometimes even in the same laboratory) and only a proportion of test animals ever suffer from the lesion after a protracted period of dosing.

3.2.3.2 The papillotoxicity of non-analgesic compounds. Renal papillary necrosis has been induced by two related compounds which are structurally dissimilar to the "analgesic group" of chemicals.

Ethylenimine (EI, originally called vinylamine) was reported to cause renal papillary necrosis in several species by (Levaditi, 1901). Oka (1913) reported a histologically indistinguishable lesion that was induced by 2-bromoethanamine (BEA) hydrobromide, and suggested that the lesion was mediated via the cyclization of BEA to EI, which was assumed to be the proximate papillotoxin.

Tables 3.6 and 3.7 gives the chronology of investigations into the papillotoxic effects of EI and BEA hydrobromide (respectively) and gives the most important conclusions regarding the pathogenesis of the lesion, and its consequences.

EI is a powerful alkylating agent, with established mutagenic activity (Ninan & Wilson, 1969), that has found extensive industrial
Table 3.6  Summary of the findings from the publications in which the mechanisms or consequences of EI - induced RPN was studied. Papers in chronological order.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Most important findings or conclusion</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Levaditi (1901)</td>
<td>Coagulation type necrosis.</td>
<td>Rats, mice, rabbit, guinea-pig and goat</td>
</tr>
<tr>
<td>Mandel &amp; Popper (1951)</td>
<td>Anuric response followed by urine of low specific gravity, proteinuria, alkaline pH and increasing azotemia.</td>
<td>Rabbit and dog</td>
</tr>
<tr>
<td></td>
<td>Suggested lesion developed in three stages:-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stage I  Vascular shunt from cortex to medulla causes medullary hyperemia and cortical ishaemia. Degeneration of convoluted tubular epithelium.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stage II  Medullary necrosis caused by anoxia due to hyperemia, vasoparalysis and multiple thrombus formation. Few cortical changes.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stage III  Fibroblast infiltration of necrotic tissue and calcification. Obstruction of lower nephron caused secondary dilatation of convoluted tubules.</td>
<td></td>
</tr>
<tr>
<td>Davies (1967, 1968)</td>
<td>Reported that the primary lesion was in the medulla. Cortical changes always secondary to damaged medulla.</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Davies et al. (1968)</td>
<td>Showed that exfoliation of renal tubular cells was not an indication of papillary necrosis per se.</td>
<td>Rats</td>
</tr>
<tr>
<td>Davies (1970)</td>
<td>Cortical changes only developed where &quot;underlying&quot; medulla was damaged. Suggested that counter current concentration caused an accumulation of the compound to cytotoxic levels which affected the vascularity of the medulla.</td>
<td></td>
</tr>
</tbody>
</table>
Ham & Tange (1969)  
Final lesion was independent of route of administration. Necrosis started at tip of the papilla and spread to include all elements of medulla. Earliest changes in interstitial cells. Degenerative changes in first 24 hours included intra- and extra-cellular oedema, endothelial damage and necrosis of interstitial and epithelial cells. The clearance of iv injected colloidal carbon showed that the microvascularity was functional up to and beyond (in some cases) the time when necrosis developed. By 3 weeks the truncated medulla had re-epithelialised with 'open' collecting ducts.

Davies (1970)  
Pronounced degenerative changes in the medullary ultra structure and microvascular system preceded the arrest of medullary circulation and favoured ischaemic injury as the underlying cause of necrosis.

Sherwood et al. (1971)  
Intravenous urographs of kidneys with papillary necrosis appear normal.

Ellis et al. (1973)  
Urine of low specific gravity. Transient proteinuria and multiphasic enzmuria together with other criteria of renal malfunction which normalised. Changes in cortex secondary to medullary necrosis.

Ellis & Price (1975)  
Caused a multiphasic diuresis, proteinuria and enzmuria which approached normality before they reflected secondary cortical damage. Suggested pattern of enzmuria possible criteria for differential diagnosis of medulla or cortex damage.

Axelsen (1978a)  
Differentiated several types of effect - a) Low doses where no lesion was induced. b) Low dose 'subtotal' necrosis where only interstitial cells, thin limbs of the loop of Henle and vasa recta were necrosed and collecting ducts were spared. c) High dose 'total' necrosis where all these elements had been destroyed. There was a substantial degree of overlapping between the three dose ranges.
Table 3.7  Summary of the findings from the publications in which the mechanism or consequences of BEA-induced RPN was studied.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Most important findings and conclusions</th>
<th>Sex, Species, Dose Route &amp; Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oka (1913)</td>
<td>Reported capillary thromboses and oedema.</td>
<td>Rat</td>
</tr>
<tr>
<td>Fuwa &amp; Waugh (1968)</td>
<td>Papillary necrosis induced in almost 100% of treated animals. Diuresis (5% glucose po or 20% mannitol iv) ameliorates the lesion, whereas anti-diuretic hormone caused a lesion of greater severity. Supported the concept that increased concentration in medulla cytotoxic effect via counter current concentration.</td>
<td>Female Holtzman rat 250 mg/kg iv Single dose</td>
</tr>
<tr>
<td>Murray et al. (1972)</td>
<td>Immediate drop in urine osmolality which was sustained. Before 24 h only mild dilatation of collecting ducts and gorging of vasa recta. Progressive degeneration of epithelia and thin limbs, filling of collecting ducts with eosinophilic material, and papillary tip necrosis developed between 4 and 7 days. The dead papillae had sequestered by 21 days. Secondary cortical changes only in area which 'fed' necrosed medulla.</td>
<td>Female Holtzman rat 250 mg/kg iv Single dose</td>
</tr>
<tr>
<td>Hill et al. (1972)</td>
<td>First cytochemical changes were the loss of ATPase activity from vasa recta at 6 hours, but this was normal by 12 hours. Loss of lactate dehydrogenase activity from anatomical elements of papilla tip. Earliest ultrastructural changes at 3 hours, included degenerative changes in vasa recta and limbs. Minor changes in interstitial and collecting duct cells. The degenerative changes were more marked by 6 hours. By 12 to 24 hours it was difficult to distinguish between cells from the vasa recta and limb, and by 48 hours the collecting duct was the remaining element of architecture. The damage to the vascular system paralleled rather than preceded the necrosis. Cell regeneration started at an early stage in those thin limbs of the loops of Henle which had not been necrosed.</td>
<td></td>
</tr>
</tbody>
</table>
Shimamura (1972) The area of medullary necrosis did not extend beyond the cortico-medullary junction (after 2 doses) even after six daily doses of bromoethanamine. Interstitial cells showed a variable increase in rough endoplasmic reticula and Golgi apparatus.

Solez et al. (1974) Medullary blood flow was normal up to 1 hour after bromoethanamine and increased slightly after 6 hours. Plasma prostaglandin levels were unaltered. Suggested the data did not support vasoconstrictive mechanism of necrosis.

Thiele (1974) A reproducible renal infection was easily induced in mice provided the papillae were necrosed 72 hours before.

Shimamura et al. (1974) Post necrotic alterations in the interstitium ground substance, such as collagen fibers and vesicular bodies on the basement membranes act as nucleation sites for calcification.

Hepinstall et al. (1975) Mild hypertension developed 3 to 4 weeks after papillary necrosis. Renal artery clipping caused a rapidly developing hypertension that was more severe.

Cuppage & Tate (1975) Early lesion included platelet and erythrocyte aggregates in the capillaries associated with necrosed loops of Henle. The collecting ducts were the last anatomical element to be necrosed. Repair and re-epithelisation took place via proliferation and migration of cells from functional nephron and collecting duct segments and lining epithelium. Cortical changes were secondary.

Shimamura (1975) Progressive increase in the weight of the adrenal glands due to increase in width of zona fasciculata. The increase in urinary volume following induction of papillary necrosis was greatest in animals drinking 0.9% NaCl and less in animals drinking distilled water. Suggested a profound derangement of renal homeostatic functions.

Wyllie et al. 1972 Pretreatment with reserpine ameliorated the necrotic lesion caused by bromoethanamine and delayed the otherwise immediate increase in urinary output. The protective effect was assumed to be mediated by preventing vasoconstrictive ischaemic injury.
<table>
<thead>
<tr>
<th>Author(s)</th>
<th>Summary</th>
<th>Species/Details</th>
</tr>
</thead>
</table>
| Murray & Von Stowasser (1976)   | Secondary glomerular changes from 7 days associated with granular immuno-deposits of gamma-globulin.                                                                                                     | Female Donrju rats  
|                                 | Single 50mg dose iv to 200-250g animal.                                                                                                                                                                 |                 |
| Shimamura & Bonk (1976)         | The repair of papillary necrosis after the lesion had developed was enhanced in rats drinking 0.9% (m/v) NaCl and urine concentrating capacity reverted to near normal. Successful repair included collecting ducts, thin loops of Henle and capillaries. Interstitial cells failed to repopulate the papillae and there was loss of mucopolysaccharide stain from the medulla matrix. | Young adult male Fischer 344 rats given single doses 50mg iv |
| Shimamura (1976)                | Glomerular filtration rate depressed to 30% of control on day 2 and 50% of control after 16 weeks. Systolic blood pressure only increased if rats loaded with NaCl.                                               | Young adult male Fischer 344 rats 50mg iv |
| Mehta et al. (1977) [Abstract]  | Decreased urine osmolality, glomerular filtration rate and free water reabsorption; increased urine flow but no change in free water clearance in treated animals. Sodium wastage, intact urinary acidification and K⁺ excretion. | 2 rat  
|                                 | Single iv dose 50mg                                                                                                                                                                                   |                 |
| Axelsen (1978b)                 | Depending on dose lesion varied from minor necrotic foci to entire medullary ablation. After 5 months kidney mass of one kidney reduced but contralateral organ showed compensating hypertrophy. Kidneys severely scarred if papillae totally necrosed. Otherwise lesion only apparent microscopically. | Female Sprague-Dawley  
|                                 | 80mg/kg to 25mg/kg sc                                                                                                                                                                                |                 |
| Sabatini et al. (1978a) [Abstract] | Papillary necrosis caused Na⁺, Cl⁺, and Ca²⁺ wastage and K⁺ wastage if K⁺ intake was limiting. Minimal NH₄⁺ excretion and pH unaffected during acidosis by the papillary lesion.                            | ? rat  
|                                 | Single iv dose 50mg                                                                                                                                                                                   |                 |
| Sabatini et al. (1978b)         | Urea recycling unaltered, PO₄²⁻ wastage suggests deep nephron damage. Decreased medullary Na⁺,K⁺-ATPase 72 hours after dosing                                                                              | ? rat  
|                                 | Single iv dose 50mg                                                                                                                                                                                   |                 |
| Arruda et al. (1979)            | Twenty-four hours after dosing glomerular filtration rate was normal.                                                                                                                                   | Single iv dose 50mg per rat |
Reinick et al. (1980) The glomerular filtration rate in the superficial nephrons was unchanged, but the total glomerular filtration rate was decreased by 50% between 18 and 24 hours after treatment. Suggested almost total lack of function of juxta-medullary nephron.

Bach et al. (1980) Radiolabelled bromoethanamine distributed largely to kidney, bladder stomach and liver. Most of the compound excreted to the urine (up to 70%) and up to 7% via faeces and breath. Several uncharacterised metabolites in urine and different metabolites in bile. (See section 6.5)

Sabatini et al. (1981a) [Abstract] Inherited or induced diuresis protected against the formation of the lesion but normal urine concentrating capacity (per se or achieved in the homogeneous Brattleboro rat after anti-diuretic hormone treatment) was associated with the lesion. Suggested bromoethanamine concentrated in the medulla.

Sabatini et al. (1981b) Marked decrease in percentage of filtering juxtamedullary nephrons and an increased excretion of Na⁺, Cl⁻, P³⁻, and Ca²⁺. The rats could not adapt to low P³⁻ and Mg²⁺. Suggests a profound impairment of juxtamedullary nephrons.

Cuttino et al. (1981) Injected micropulverized barium sulphate (in a gelatin vehicle) as a contrast agent to assess the time course vascular changes using microangiographic technique. Suggested reduced flow to vasa recta within 2h and total medullary vascular obliteration by 24-48h.

Vanholden et al. (1981) Measured papillary plasma flow (using ¹²⁵I-labelled albumin) 24h after BEA in dehydrated and volume expanded rats. Volume expansion did not ameliorate the BEA-induced lesion. Plasma flow was increased by BEA treatment in the dehydrated rats. Volume expansion also increased plasma flow which was unaffected by BEA.

? Munich Wistar. Single dose 200mg/kg iv.

Male Wistar rats single dose 50mg/kg ip and 100 mg/kg po and sc.

? Homozygous and heterozygous Brattleboro rats and Sprague Dawley ? iv.

Male ? rats, single dose, 50 mg iv - weight 200-250g.

Female Holtzman (Sprague-Dawley) rats, single dose, 250mg/kg ip.

Male Wistar rats, single dose 150 mg/kg iv.
applications. The chemistry and toxicology of El have been reviewed in a monograph by Dermer & Ham, 1969. The toxicological effects parallel those of the alkylating compounds. Papillotoxicity has only been described for El, although aziridine alkylating agents (which break down to release El) have been reported to induce a "sustained diuresis" which may have been caused by papillary necrosis (Jackson & James, 1963, 1965). The metabolism and distribution of $^{14}$C-El has been reported by Wright & Rowe (1967) and will be discussed in Chapter 7.

BEA has a number of industrial and commercial applications and is used in organic syntheses. This compound cyclizes to form El especially rapidly under aqueous alkaline conditions (e.g. $k = 3.7 \times 10^{-4}$ sec$^{-1}$ in aqueous 0.3M pH 8 phosphate buffer at 35°C), Dermer & Ham, 1969. The pharmacological and toxicological effects of BEA (with the exception of papillotoxicity Table 3.7) have not been studied. Nothing has been published on the metabolism of BEA although extensive data has been compiled on alkylhalide metabolism (Jones, 1973 and Plotrick et al., 1979) and the conjugate which forms in vitro between the sulphydryl containing molecules (glutathione) and BEA has been investigated (Raftery & Cole, 1963 for fuller discussion).

3.2.4 The Pathological Course of Experimentally-induced Papillary Necrosis.

A clear definition of the development of a lesion that may be induced by such a variety of agents, either acutely or chronically, especially with such a diffuse literature would be expected to be difficult. This is compounded by biological variability, especially in chronically induced systems.

Surprisingly there are many similar features between the acute and chronic lesions in model systems, and between these lesions and that reported in humans. Table 3.8 shows the pathological changes that have
Table 3.8  Comparison of pathological changes in experimentally induced renal papillary necrosis and the lesions found in man.

<table>
<thead>
<tr>
<th>Morphological and functional Changes</th>
<th>Acutely induced (DEA and E1)</th>
<th>Chronically induced (Analgesic and fat-free diet)</th>
<th>Analgesic associated (Man)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Early:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Papillae apex primary site of lesion</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Extends to include medulla but not beyond corticomedullary junction</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Loss of thin loop of Henle and interstitial cells</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Loss of vasa recta</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Microvascular occlusion</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Intermediate:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Loss of collecting ducts and covering epithelia</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Changed staining of MPS matrix</td>
<td>increase then loss</td>
<td>loss</td>
<td>increase or loss</td>
</tr>
<tr>
<td>Epithelial hyperplasia</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Inflammatory response</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Regeneration and re-epithelialisation</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Fatty changes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Calcification</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Renal and bladder calculi</td>
<td>?</td>
<td>?</td>
<td>Yes</td>
</tr>
<tr>
<td>Urinary tract infection</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Hyperplasia of transitional urothelia of pelvis, ureter and bladder</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Late:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secondary cortical changes overlying necrosed papilla</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Cystic dilatation</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Hypertension</td>
<td>After salt loading</td>
<td>Yes</td>
<td>Common</td>
</tr>
<tr>
<td>Fatty changes in papilla</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Carcinoma of transitional urothelia of pelvis, ureter and bladder</td>
<td>No</td>
<td>No</td>
<td>Commonly associated with analgesic abuse</td>
</tr>
</tbody>
</table>
Table 3.9 Time scale upon which different types of RPN can be compared.

<table>
<thead>
<tr>
<th>Type</th>
<th>Early</th>
<th>Intermediate</th>
<th>Late</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental Acute</td>
<td>8-36 hours</td>
<td>2-6 days</td>
<td>7-30 days</td>
</tr>
<tr>
<td>Experimental Chronic</td>
<td>2-30 weeks</td>
<td>4-8 months</td>
<td>6-12 months</td>
</tr>
<tr>
<td>Analgesic Associated* (Humans)</td>
<td>1-5 years</td>
<td>2-15 years</td>
<td>5-30 years</td>
</tr>
</tbody>
</table>

* Estimates
been reported to develop in the acute and chronic experimental models and in humans who abuse analgesics, and Table 3.9 provides a time-base upon which to compare these changes.

The similar sequence of pathological changes in the development of papillary necrosis (be it acutely or chronically induced, experimental or not) suggests that a common set of molecular events could underly these lesions.

3.2.5 The Molecular Pathogenesis of Renal Papillary Necrosis.

Several theories have been postulated to explain the sequence of events which precedes papillary necrosis in man and experimentally induced lesions in animals.

Several different molecular events may give rise to the same end effect, but mechanistic differences may exist between the acutely (EI and BEA) and chronically (analgesic) precipitated lesions, although the pathological courses are remarkably similar (3.2.4). In discussing these theories it is probably desirable to establish artificial boundaries between what may be similar or inter-related mechanisms.

3.2.5.1 The Counter-current concentration mechanisms. One of the most oft cited mechanisms to explain papillary necrosis is based on the supposition that a concentration gradient of the offending compound (increasing from the cortex to the papilla) develops as a consequence of the counter-current concentration mechanism -2.3.2). This concentration gradient is assumed to be high enough to exert a "direct" (although never defined) toxic effect (see 3.2.5.5.).

Such a concentration gradient (of 10 to 1 for conjugated paracetamol and 19 to 1 for free paracetamol) was found in dehydrated dogs by Bluemle & Goldberg (1968) and confirmed by Duggin (1975) and Duggin & Mudge (1976). Bluemle & Goldberg (1968) reported that neither phenacetin (confirmed by Duggin, 1976) nor aspirin was concentrated, but Gault
(1971) found modest increases in medullary concentrations for phenacetin, aspirin and paracetamol. More recently, Molland (1978a) showed the distribution of aspirin (assessed by autoradiography at the light microscopic level) was greater in the medulla, where it was confined largely to the epithelium of the proximal tubule, principally near the pars recta.

The discrepancies between the findings of Bluemle & Goldberg (1968) and Duggin & Mudge (1976), as opposed to those of Gault (1971) and Molland (1978a) may relate to the state of hydration of test animals, species differences, or the methods used (chemical assay versus radiolabel distribution, respectively), but all are of questionable relevance because they were single dose studies. The autoradiographic distribution of aspirin described by Molland (1978a) is inconsistent with the degenerative changes seen in the interstitial cells (Molland, 1967, 1978a, b). More meaningful results may be derived from autoradiographic studies during the course of chronic dosing.

Antipyrine has been shown (in common with other pyralazones) to produce RPN (Brown & Hardy, 1968). This compound is, however, present in higher concentrations in the cortex than the medulla (Moffat, 1975, 1982) although the data needs to be confirmed by autoradiographic techniques.

The fact that dehydration favours the experimental induction of the lesion together with the suggested contribution of "hot climates" to human papillary necrosis (3.1.2) is thought to strengthen the importance of this mechanism. Dehydration (in common with uninephrectomization) may, however, put an added stress factor on the kidney. It seems likely, although it appears never to have been tested, that dehydration (or other "stress") may sensitise the kidney to all chemical insults and not only medullary lesions. The argument does not hold for man, who is at liberty
to maintain his state of hydration, even if over-night water deprivation is more severe in hot climates. Further its role in the high incidence of papillary necrosis in cold climates such as Switzerland and the Scandinavian countries must be questioned (Rosner, 1976).

This mechanism does not in itself explain the molecular pathogenesis underlying the lesion, but favours the concept that active counter current concentration exacerbates the lesion.

3.2.5.2 Medullary Ischaemia The medulla is poorly perfused (compared to the cortex) and functions with a relatively low oxygen tension (2.1.1). This is assumed to predispose the papilla to "anoxic injury" and several possible mechanisms have been proposed by which a localised ischaemic injury might be induced chemically (see Fig. 3.1 and review by Shelley, 1978 for discussion).

The induction of medullary lesions by vascular occlusion (3.2.2.2) is assumed to support this concept, but stopping the blood supply to any organ for several hours will cause profound changes, especially in the kidney where vascular flow is the quintessence of renal function.

Attempts to resolve the contribution of microvascular changes to papillary necrosis have given conflicting data. There is ample evidence (Ham & Tange, 1969; Murray et al., 1972; Hill et al., 1972, Wyllie et al., 1972 and Solez et al., 1974) to show that vascular changes parallel (if not lag behind) cell necrosis in the acutely induced models of RPN. Thus vasoconstriction does not appear to be a prerequisite. It is possible, however, that vasodilatation of the vasa recta might cause haemostasis and ischaemic injury.

Extensive vascular changes have been described for both the chronically-induced lesions in the rat and for human kidneys (Molland, 1976, 1978a,b; Shelley 1978; Burry et al., 1977; Zollinger, 1980 and Mihatsch, 1980a), but it is impossible to be certain if these are the
Fig. 3.1 Schematic representation of the possible pathogenic factors which may give rise to medullary ischaemia and papillary necrosis. Data from Shelley (1978) and other sources.
primary cause, parallel changes, secondary effects or unrelated. The question may be resolved when chronically-induced model lesions are adequately controlled (to make them reproducible) and fully investigated.

3.2.5.3 The role of renal PG in papillary necrosis  Attention has been focussed on the fact that most analgesic and NSAID (and their analogues) inhibit PG synthesis, by blocking the enzyme cyclo-oxygenase (See Dunn, 1981 for review). PG play an essential, but still poorly understood role in renal function (see 2.3.4.3).

a) It has been argued (Shelley, 1978 and Nanra, 1980) that perturbation of PG synthesis might cause a reduced synthesis of PGE$_2$, or an increased synthesis of TXA$_2$, either or both effects would give rise to vaso-constriction and hence local ischaemic injury (3.2.5.2). Fig.3.1.

b) The analgesic compounds may cause profound changes in renal haemodynamics (2.2.2) which could induce ischaemic injury (3.2.5.2). Similarly, the depression of PG synthesis might alter haemodynamics by perturbing the renin or kallikrein systems (2.3.4.3).

c) Perturbation of prostaglandin synthesis might alter the trans-renal osmotic compartments (2.3.4.3).

d) One of the theories offered to explain the papillary necrosis that develops after prolonged fat-free diet (Molland, 1978a) is that the limited ingestion of arachidonic acid, or its precursors, would lead to depletion of prostaglandin precursors.

e) Recently, Bray (1980) has suggested that PG (particularly PGE$_2$) plays an essential role in modulating the immune response. The possible contribution of immunological changes to the pathogenesis of papillary necrosis is considered below (3.2.5.6).

The role of altered prostaglandin metabolism in the pathogenesis of renal papillary necrosis is difficult, if not impossible, to assess on
Table 3.10. A comparison of the in vivo papillotoxic potential of biphenyl analogues and analgesics and the relative amount of PGE inhibition

<table>
<thead>
<tr>
<th>Biphenyl Compounds</th>
<th>Inhibition of PGE Synthesis</th>
<th>RPN induced in rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biphenyl Compounds (Analgesic papillotoxin)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diphenylamine</td>
<td></td>
<td>+++</td>
</tr>
<tr>
<td>Flufenamic acid</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>(Indomethacin)</td>
<td></td>
<td>+++</td>
</tr>
<tr>
<td>(Aspirin)</td>
<td></td>
<td>++</td>
</tr>
<tr>
<td>N-Phenylanthanilic acid</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Diphenyl ether</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Diphenyl</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Diphenyl methyl alcohol</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>(Aminopyrine)</td>
<td></td>
<td>++</td>
</tr>
<tr>
<td>Diphenyl-2-carboxylic acid</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

(After Hardy, T.L., personal communication).
The dynamics of papillary lipid materials is complex and probably involves several metabolic pools (2.3.4.2). Van Dorp (1971) has reported that essential fatty acid deficiency in the rabbit depressed inner medullary PGE\(_2\), but increased it in the outer medulla. Kaa (1976) reported that prostaglandin synthesis was enhanced when medullary slices (from rats with essential fatty acid deficiency) when incubated with exogenous arachidonic acid. More recently Dunham et al., (1978) have confirmed this enhanced enzymic activity, but showed that both endogenous linoleic and arachidonic acids were reduced in both the triglyceride and the phospholipid pools. Thus greatly reduced pools of these precursors would be the likely rate limiting factor in prostaglandin synthesis. There were, however, (as might be expected) major changes in other lipid constituents including elevated palmitoleic, oleic and homo-\(\gamma\)-linolenic acids, decreased adrenic acid and a large concentration of a fatty acid thought to be derived from 20:3\(\omega9\), 22:3 chain elongation. It is thus equally possible that loading or depletion of lipid constituents other than arachidonic acid might perturbate medullary function and cause papillary necrosis.

Similarly, those substances which alter PG synthesis may disrupt
medullary lipid metabolism. The contribution of PGs and other lipid components to the molecular pathogenesis of this lesion must remain speculative until methods have been developed to specifically assess their effects in the medulla during both the acutely and chronically induced renal papillary necrosis.

3.2.5.4 Biologically reactive intermediates. In recent years the concept of metabolically generated electrophilic molecules disrupting cellular function by binding to "essential" macro- (or micro-) molecules has served to explain many toxicological enigmas (see symposiums edited by Jollow et al., 1977 and Synder et al., 1981). This approach has clarified much of the genesis of paracetamol-induced hepatic necrosis (Mitchel et al., 1977). The mechanism underlying this acute response in the liver has been extrapolated to explain a chronically induced lesion in the medulla (see Mitchell et al., 1977). Mudge et al. (1978) reported a marked depletion of renal glutathione and extensive covalently binding of paracetamol (to renal proteins) in both the cortex and the papilla. The papilla had lower endogenous glutathione concentrations, and a slower turnover of covalently bound label than the cortex. These were both single high dose studies, however, where the only apparent renal lesion was an acute proximal tubular necrosis in the cortex (see 3.2.5.7). The evidence from both Mitchell et al. (1977) and Mudge et al. (1978) supports the view that metabolic activation takes place in the kidney, although relatively low levels of xenobiotic metabolism occur compared to the liver (see 2.3.4.5).

The view that active metabolites may contribute to renal papillary necrosis is given some support by the fact that aspirin provides the acetyl group for trans-acetylating macromolecules (most notably prostaglandin cyclo-oxygenase, Caterson, 1978); that BEA may cyclizes to ethylenimine (Dermér & Ham, 1969), a potent alkylating agent
(3.2.3.2), and that cyclophosphamide has been reported to cause papillary necrosis (Solez et al., 1974).

The generation of a reactive intermediate and its covalent binding to macromolecules does not, in itself, predestine cell necrosis. Nor does covalent binding explain the molecular pathogenesis per se: it only suggests that some essential molecular function is altered.

A single high dose of paracetamol only induces necrosis of the proximal tubule. Thus the significance of covalent binding of (a single acute dose) paracetamol to the medulla must remain a matter for dispute, because papillary necrosis has only ever been induced over long periods with difficulty. The binding of paracetamol to various kidney zones might be more meaningful if repeated on chronically dosed animals.

Recently, Mohandas et al., (1981) have shown that paracetamol undergoes co-oxygenation with arachidonic acid to form an active intermediate which binds to macromolecules. This binding was most prominent in the medulla.

3.2.5.5. **Chemically induced changes in intermediate metabolism as the underlying cause of papillary necrosis.** A number of workers have probed the possibility that the amount of papillotoxin is increased by counter current concentration to a level high enough to inhibit normal medullary metabolic functions. Davidson et al. (1973 a,b) studied the incorporation of amino acids into medullary proteins and showed that paracetamol caused no change on its own, but enhanced the inhibition of de novo protein synthesis by salicylic acid.

Goldberg et al., 1971 reported that high concentrations of salicylate inhibited the pentose phosphate shunt, caused a decreased cellular concentrations of reduced glutathione. This might predispose to the covalent binding of electrophilic molecules (see 3.2.5.4). The effects of various compounds on key aspects of anaerobic glucose metabolism have
given conflicting data (see Shelley, 1978) probably because some studies
were on medulla tissue and others on outer medulla and cortex tubules.
Thus the observations of Dawson, 1972, 1975, and Szinicz et al., 1979 are
probably inapplicable to the medulla because in the former case
medullary cells were absent from the in vitro preparations and in the
latter mixed medulla and cortex cell types were used. Even the use of
medulla slices or cell suspensions would not be appropriate to
differentiate between the metabolic effects on different cell types
unless additional techniques are applied to solve the problem of
heterogeniety (see 7.3).

3.2.5.6. The contribution of immunological changes to the
reported the first immunological changes to occur in the cortex 7 days
after an acute papillary necrosis had been induced. No
immunopathological changes were reported in the medulla, which might
suggest that an immune response had played no part in the pathogenesis of
the BEA-induced lesion. The occurrence of a hyperacute immune response
by the kidney has, however, been described (3.2.2.3).

A number of workers have speculated on the contribution of
immunological changes to the chronically induced lesion, but evidence is
conflicting. Hook et al., 1979 raised the possibility that failure of all
human analgesic abusers to develop papillary necrosis might indicate an
immunological basis for the disease. The possible contributions of the
immunogenicity of salicylate and several phenacetin metabolites (e.g.
phenetidine) has been discussed by Rosner, 1976 and Dubach, 1978. Gault
et al. (1971), however, found no immunodeposits in the medulla or cortex
of patients with early analgesic nephropathy.

It seems possible that immunological changes that take place after
papillary necrosis, or as a consequence of it, may mediate some of the
secondary cortical changes (See Chapter 7)

3.2.5.7. Functional changes in the cortex as an underlying cause of renal papillary necrosis. It is commonly assumed that papillary necrosis is caused primarily by a direct effect on one or more of the elements of the medulla (see 3.2.5.1 to 3.2.5.6). The nephron is, however, a highly structured and ordered array of cell types, each of which shows inter-dependance on the functioning of other cell types. It is thus possible that an altered function (without lethal consequences) in one cell type in the proximal part of a nephron could have profound secondary effects on a more distal cell population. This might manifest as a pathological lesion.

The concept appears never to have been probed, but would, for example, explain many of the anomalies associated with this lesion. A precedent for this is the previous failure to recognise papillary necrosis (Darmady & McIver, 1980 and McCormack et al., 1981) as the underlying cause of diphenylamine-induced cystic kidney (Hardy 1970a,b and 1974). Recent, unpublished, data to support the possible inter-relationship between lesion-free nephron functional changes and this nephrotoxic lesion is presented in Chapter 7.
MATERIALS AND METHODS

The reagents employed for these studies were commercially available grades of the highest purity, generally analytical or equivalent. Details relating to additional purification steps will be provided where applicable. The source of specialised chemicals will be given in the relevant section the first time each compound is referred to.

Distilled water was not passed through a mixed bed deioniser.

4.1 EXPERIMENTAL ANIMALS

Animals used in these investigations were Wistar rats from the University of Surrey stock, (unless otherwise stated). Weight range and sex will be given under the relevant experimental sections.

Animals were randomly assigned to treatment groups, and were allowed a period of 4 to 7 days to acclimatize to their new environment. The rats were housed in translucent "shoe box" type cages and bedded on ethylene oxide-sterilized soft wood shavings (Lee & Son, Chertsey) which were changed weekly. The cage density did not exceed 10 rats of up to 250g for a 14"W x 24"L x 11"D cage and 8 weanling rats (up to 80g) per 10"W x 16"L x 6"D cage. The rats were handled regularly during the course of the acclimatization period to minimise stress responses during investigations (see Gartner et al., 1980).

Animals were kept in a strict 12 hour light/dark cycle (light cycle starting at 0700h GMT) and all metabolic collection studies were conducted under the same conditions except where the experimental protocol demanded that specimen containers were changed in the "dark period". One 100 watt lamp was used only for the duration of such
manipulations (5 to 10 min). It is, however, standard animal house practice that cages of animals treated with carcinogenic (e.g. 3-methylcholangiathrene) or radioactive materials be left in an area with a continuous 24 h light period.

Animals were given tap water ad libitum (unless otherwise stated) and maintained on Lab Diet 1 (Spratts, Barking) rat cubes or Lab Diet 2 (Spratts, Barking) powdered diet (unless otherwise stated).

4.1.1 The Marking and Dosing of Animals

The methods used for marking rats depended on the type of investigation and its duration. Waterproof marking pens were used to provide unambiguous identification on the tails for short-term investigations of up to 72h. For experiments of longer duration the groups or individual rats were marked, normally during the acclimatization period, by "ear-notching" with scissors or a punch (Junior punch; Brookwick, Ward & Co., London).

The BEA (BDH, Poole) used for dosing rats was made up freshly (dissolved in 0.9% (m/v) NaCl) and used within 5 to 10 minutes. Animals were dosed between 0900 and 1100 h; unless otherwise stated. Details for the preparation and dosing of other compounds will be given in the relevant experimental section.

4.1.2 Metabolic Studies

Two types of metabolic studies were undertaken.

4.1.2.1 Total metabolite collections. Urine, faeces and exhaled air containing $^{14}$C radioactivity were collected from individual adult rats, or pairs of weanling rats, housed in Metabowls (Jencons, Hemel Hempstead) where food cubes, and water were available ad libitum throughout. The animals were acclimatized for 24h before the experiment with the vacuum air pumps running. No pre-cage CO$_2$ traps containing soda lime were used in any of the experiments. Air was drawn through each
Metabowl at the rate of 350-400mL/min, and through three traps, the first containing 1N-HCl (for exhaled basic substances) and the second and third containing 2N-NaOH for trapping CO₂. Spontaneously voided urine was collected at 4, 8 and 12h, then at 24, 48 and 72h, unless otherwise stated. Volumes were measured, recorded and aliquots taken for analysis, (see 4.3.1) and the remainder stored at -20°C until analysed, (see 4.3.2). Both urine and faeces were collected in glass containers cooled to 0°C for the duration of the study.

Exhaled metabolic products were drawn through three Dreschel bottles in series. The total basic metabolite exhaled in 24h was trapped in 100mL of 1N-HCl (a preliminary experiment showed that there was no carry over into a second HCl trap). At the end of each 24-hour collection period the volume was reconstituted to 100 mL with distilled water (to compensate for evaporation) mixed and an aliquot taken for analysis (see 4.3.1). The first Dreschel bottle was recharged with 100mL 1N-HCl for the next collection period.

The total CO₂ was similarly assessed in the second Dreschel bottle containing 2N-NaOH. The volume was re-constituted to 100 mL, mixed and an aliquot taken for analysis. (see 4.3.1). The third bottle of NaOH was moved to position "two" and fresh alkaline (100mL) added to the Dreschel bottle in the third position.

4.1.2.2 Selective metabolic studies. Urine and faeces collection was undertaken either in a "Metabowl" as described above (4.1.2.1), where the "bowl" was covered with a mesh lid or, similarly, in a Techniplast metabolic cage (code 1700872 Forth-Tech Services, Dalkeith, Scotland). Water and food (unless otherwise stated) were available throughout ad libitum.

Spontaneously voided urine was collected at 4, 8, 12, 24, 48 and 72h and faeces 24-hourly for 72h as described above (4.2.2.1), unless
otherwise stated. Urine (4.3.1) and faeces (4.3.2) were analysed as described.

4.1.3 Surgical Procedures

Surgical manipulation of experimental animals has provided an essential tool in life-sciences research. In accordance with Home Office requirements these were either:

A) conducted under "licence alone", in which case animals were maintained under 'surgical' anaesthesia for the duration of the procedure, after which they were killed by an excess of anaesthetic or a massive iv air embolism, or

B) under one of several "B Certificates", in which case animals were allowed to recover from an aseptic surgical procedure.

4.1.3.1 Anaesthetisation and preparation. Rats were anaesthetised by the ip administration of 1.0mL/kg Sagital (Hexobarbitone BP for injection 60 mg/mL; May & Baker, Dagenham). Experience made allowances for variations from this dose depending on pretreatment. Before surgical anaesthesia was reached animals were shaved, if applicable, using a number 40 blade on an Oster Clipper (Brookwick, Ward & Co., London). Once a surgical plane of anaesthesia had been reached animals were positioned on a surgical platform, the site of incision cleaned with 70% (v/v) ethanol, and the first incision made. Surgical anaesthesia was assessed by absence of a twitch or withdrawal response to painful stimuli (pin prick or pinch with forceps to foot or tail). All surgical instruments used under certificate B procedures had been sterilised in boiling water for at least 30 min or autoclaved prior to use.

The duration of anaesthesia was normally 2 to 3 hours, and, as such, adequate for surgery from which animals were to recover. The depth of anaesthesia was tested every 10 to 15 min in acute experiments, and between 0.05 and 0.10 mL of Sagital added to the peritoneal cavity (at
intervals based on experience) to maintain anaesthesia for up to 5 hours. The estimated fluid loss was replaced intraperitoneally with sterile pyrogen-free 0.9% (m/v) NaCl.

4.1.3.2 Acute Bile Duct and Bladder Cannulation. Animals prepared as described above were opened by a mid-line incision to expose the bladder and liver. The bile duct was ligated near its junction with the duodenum, and pancreatic tissue was removed by blunt dissection, taking care to cause as little bleeding as possible.

The bladder was cannulated through a small midline incision between the major blood vessel systems. The cannula consisted of a length of polythene tubing (800/100/300; Portex, Hythe) tubing fitted into a 4.0 mm OD, 2.0 mm ID Tygon tube. The Tygon tubing made it easier to secure the cannula into the bladder with a double silk ligation around the walls. The bladder cannula was positioned so that flow was unimpeded, and the open end placed in a receiving vessel on one side of the animal. The now distended bile duct was cannulated using a polythene tubing (800/100/100 or 800/100/160; Portex, Hythe) tubing described by Ross (1972). The cannula was positioned so that bile flow was unimpeded and led to a suitable receptacle. Both the urine and bile cannulae were kept in position by Cellotaping. The abdominal cavity was covered by surgical grade cotton wool soaked in 0.9%(m/v) NaCl, and finally by Parafilm. Intravenous injection and withdrawals were made via the right external jugular exposed as described below (4.1.3.5). The body temperature was maintained by positioning a 100 Watt lamp about 15 to 20 cm above the rats.

4.1.3.3 Unilateral Partial Papillectomy. A number of techniques have been described for undertaking a partial or complete papillectomy. Most interrupt the blood supply to the kidney and then remove the papilla (or
medulla) via an incision in the cortex, which is stitched before the flow of blood is resumed. These techniques have always caused ischaemia, blood loss and scarring of the renal cortex and medulla. Hardy (1970b) avoided these drastic consequences by taking advantage of the fact that the papilla protrudes into the renal pelvic cavity in young rats, and developed a technique which is simple, rapid and atraumatic.

Rats weighing less than 80g were anaesthetised (4.1.3.1) and the left kidney exposed through a paralumbar incision. The kidney was supported in 0.9%(m/v) NaCl soaked gauze throughout the procedure. The rest of the operation was undertaken with the aid of a stereo-microscope. The peritubular fat was removed by blunt dissection (Fig. 4.1a) to expose the renal pelvis leading into the ureter. The papillary tip was exposed through a 1-2mm longitudinal incision made in the ureter (Fig. 4.1b) using a razor blade fragment held in a Dixey Hoskins holder (Dixey & Co., London). The papilla was exteriorised (Fig. 4.1c) using gentle pressure from the supporting face of a modified Allen's conjunctival fixation forceps (John Weiss, London) and the papilla was resected by closing the sharpened cutting face on it (Fig. 4.1d). Sham operation animals had the papilla exposed and manipulated, but not resected. The release of gentle pressure allowed the medulla stump to withdraw back into the pelvis and the two cut faces of which come into close proximity, Hardy has found (personal communication) that the incision heals rapidly without suturing. The kidney was returned to the abdominal cavity, approximately 2mL of 0.9% (m/v) NaCl was placed in the peritoneal cavity, the peritoneal walls were sutured with 4/0 catgut (Chromic; Ethicon, Edinburgh) and the body wall closed with 12mm Michel clips (Down Surgical, London). The whole procedure can be completed in 5 to 7 min. The wound and area around it was sprayed with Nobecutane (BDH Pharmaceuticals, London). No antibiotic coverage was given.
Fig. 4.1  Schematic representation of the surgical procedure for unilateral partial papillectomy. Shown are a: clearing of the renal pelvic and ureter of tissue, b: exposing the papilla through an incision in the ureter; c: the exteriorisation, and d: resection of the papilla.
4.1.3.4 **Uninephrectomization**  
Unilateral nephrectomization was undertaken on partially papillectomized animals (4.2.3.3) after a 21 days recovery period. Rats were prepared as described and either the left (normal) or right (partially papillectomized, or sham operated) kidney exposed through a paralumbar incision, freed from the adjacent tissue and the venous and arterial supply, together with the ureter, ligated with 2/0 silk (Mersilk; Ethicon, Edinburgh) close to the kidney. A second ligation was undertaken a few millimetre away from the kidney and vessels between the two ligatures cut. The whole kidney and its adrenal gland was removed. The surgical procedure took less than 5 minutes.

The incision was closed and the wound treated as described above, (4.1.3.3). Animals were allowed a 7 to 9 day recovery period before they were used for subsequent investigations.

4.1.3.5 **Chronic Venous Cannulation.** One of the most fundamental drawbacks to in vivo studies on rats is the inaccessibility of the vascular supply for multiple additions or withdrawals (Migdalof, 1976). Chronic cannulation is, in principle, a straight-forward procedure, but suffers from a bad reputation because of high failure rates caused by a host of minor manipulative and post operative difficulties. These can, however, be minimised and many 'simple' approaches have been described. The method chosen was a modification of that used by Steffens (1969) and that described by Gellai & Valtin (1979).

Animals were prepared as described (section 4.1.3.1). An incision was made over the right external jugular vein to expose it in the region of the clavicle. A 16 gauge blunt-tipped trochar was passed under the skin, behind the neck and exteriorised, through a small incision, midline about 5mm behind the base of the skull. The cannula (described below) was threaded through the trochar which was then removed. Two small dumb-
bells (made from polythene tubing, 800/100/360; Portex, Hythe) flared at both ends by flaming, were threaded onto the cannula. These serve, to prevent kinking of the tubing by allowing the cannula to be tied to adjacent fascia (without running the risk of occluding the tubing).

The implanted portion of the cannula was made from silastic medical grade tubing (0.25in ID, 0.47in OD, Cat. No. 602-155; Dow Corning Corp., Michigan) with the tip cut at an angle of about 45°. This was joined to a length of Tygon Micro-Bore tubing (0.51mm ID 1.52mm OD; Norton Co., Ohio). The "join" was made by softening and expanding the silastic tubing in toluene for 10 min, carefully sliding the Tygon tubing into it and allowing the silastic tubing to shrink to normal diameter when toluene evaporated. At least 72h elapsed before the cannula was used. It was sterilised in 70% (v/v) ethanol before use, and stored in sterile 0.9% (m/v) NaCl.

The cannula was filled with saline and the rat strapped supine to the operating platform. A small wad of cotton wool was placed behind the head (to arch the neck upwards, and make entry of the cannulae easier) and the external jugular vein was cleared of overlying connective tissue by wet, blunt dissection. Two silk ligatures were positioned around the vessel. The anterior one was used to ligate the jugular vein, and the cannulae inserted through a small incision cut in the vessel with microscissors, the tubing was advanced and positioned in the right atrium and secured with the posterior tie. The anterior ligature was used to secure one dumb-bell, the second dumb-bell being secured to the fascia of the neck.

The free flow of blood from the cannulation was checked before the incision on the neck was sutured (see 4.1.3.3), and again after the exteriorised Tygon tubing had been secured to the fascia at the back of the neck, and finally after the dorsal incision had been sutured.
The exteriorised cannula was cut short enough to prevent the rat from gripping it, but long enough to handle (about 2 cms) and plugged with a small "g" shaped stainless steel wire.

Rats were housed individually, and the effectiveness of the cannula maintained by flushing with 0.05mL sterile 0.9% (m/v) NaCl every 24 to 48h. The wounds were inspected daily for 5-7 days.

4.2 HISTOLOGICALLY RELATED TECHNIQUES

4.2.1 Routine Histology.

Rats were anaesthetised using ether and killed by exsanguination, unless otherwise stated. The kidneys were dissected free and trimmed sagitally above the plane of the papilla to give a "slice" of tissue 4 to 6 mm thick for fixing. Similarly slices of tissue 4 to 6mm thick from other organs were fixed in phosphate buffered neutral 10% (v/v) formaldehyde for at least 18h and up to 7 days. The fixed tissue was dehydrated in increasing concentrations of ethanol, transferred to toluene and thence to wax (Fibrowax; Lamb, London) in a Histokinette (British American Optical, Slough). Sections of 7 μm thickness were cut on a Spencer 820 microtome (British American Optical, Slough) and processed for haematoxylin and eosin (H&E) according to Pearse (1972). Other staining procedures undertaken according to Pearse (1972) were PAS, Diasterase - PAS, and Schmoels. All sections were mounted under coverslips with DPX (Lamb, London).

4.2.2 Mucopolysaccharide Staining

Prolonged fixation was found to cause leaching of mucopolysaccharide material from the renal papilla. The staining distribution was only similar to cryostat sections if the kidneys were fixed in formal (4.2.1) for 18 to 26h.

4.2.2.1 Toluidine blue metachromasia. The highly charged nature of muco-polysaccharides cause a metachromatic shift from blue to either
red, pink or purple.

The Toluidine blue (CI 52040, Lamb, London) was made up as a 0.1% (w/v) solution in 0.1 M-acetate buffer, pH 3.5.

Cryostat sections were fixed in acetic acid ethanol and wax sections were taken to water, the sections were stained for 30s, rinsed in distilled water, blotted dry, cleaned in xylene and finally mounted under coverslips with DPX.

4.2.2.2. Alcian blue staining. Wax sections were taken to water, stained in 0.5% (m/v) Alcian blue (CI 74240, Lamb, London) in 3% (v/v) aqueous glacial acetic acid for 30 to 60min, rinsed in running tap water, counterstained in 1% (m/v) aqueous Neutral red (CI 50040; Lamb, London) for 1 min, washed in tap water, taken rapidly to 100% (v/v) alcohol, cleared and mounted, as above (4.2.2.1). Acid mucopolysaccharide stains green-blue and the cell nuclei stain red.

4.2.2.3. Safranin O metachromasia. The metachromasis of Toluidine blue is largely lost in non-aqueous synthetic mounting resins and Toluidine blue, Alcian blue and Neutral red are very alcohol soluble, thus staining may be substantially reduced during dehydration.

Shepard & Mitchell (1976) have described the advantages of using Safranin O for proteoglycan staining. Wax sections were taken to water, stained in 0.1% (m/v) Safranin O (CI 50240; Lamb, London) for 2 min, rinsed, dehydrated, cleared and mounted (4.2.2.1). Proteoglycan stain orange and non-polyanionic material appears red.

4.2.2.4. Hale's colloidal iron staining. Colloidal iron is chelated by polyanionic groups and this is demonstrated by the Prussian blue reaction (Hale, 1946).

Wax sections were taken to distilled water, flooded with dialysed iron (BDH, Poole): 2M-acetic acid::1:1(v/v), for 10 min, rinsed in several changes of distilled water, flooded with equal parts of 2% (m/v)
potassium ferrocyanide and 2% (v/v) hydrochloric acid (both made in distilled water) for 10 min and rinsed in several changes of distilled water. The sections were then counter stained with 1% (m/v) Neutral red, washed, dehydrated, cleared and mounted (4.2.2.2). Acid mucopolysaccharide stained blue and cell nuclei stain red.

4.2.3 Cryostat Sections of Tissue

Animals were killed by cervical fracture, opened by a midline incision and the kidneys rapidly but carefully removed. The mass of tissue was reduced by removing both perihilar poles and sagittal face above the place of the papilla with a blade. The tissue was "snap frozen" in petroleum-free hexane which had been pre-cooled to -70° C in a solid CO₂-methanol slurry (Chayen et al., 1973). Segments of liver about 5-8mm cube were similarly "snap frozen". Tissue samples were removed from the hexane (with insulated forceps pre-cooled to -70° C) and stored on solid CO₂ in airtight glass containers (lined with absorbent paper) precooled to -70° C. Tissue blocks were mounted on metal chucks (so that the cutting place was parallel to plane of the papilla) using Tissue-Tek II (Lamb, London) and left for 4 to 6 hours (or overnight in air tight containers) to equilibrate with the cryostat cabinet temperature (-20 to -30° C). Sections were cut in a Bright Cryostat cabinet on Cambridge rocker microtome with a blade angle of 12° and the anti-roll plate set optimally. The blade was not cooled with solid CO₂. The kidney blocks were trimmed to the region of the papilla (determined by intermittent microscopic examination of Toluidine blue stained sections, (see 4.2.2.1), then 10 to 20 sections of 10 µm were cut and "flash evaporated" onto microscope slides at room temperature. Slides were stored below - 20° C until used.

4.2.4. Assessment of renal microvascularity

The functional integrity of kidney micro-vascularity was assessed
using the iv colloidal carbon technique. Drawing ink provides a convenient source of colloidal carbon, but contains several undesirable constituents such as phenols (Hardy, T.L., personal communication). These were removed by the dialysis of 20 mL Rotring drawing ink (Rotring Werke, Hamburg) against four changes of at least 400 volumes of 0.9% (m/v) NaCl over a 96-120h period at 5 to 8°C. After the dialysis period the volume had increased substantially because of the oncotic pressure of the colloidal carbon.

The resulting solution was centrifuged at 3500 rev./min. for 20min. (to remove the larger particles of carbon or aggregates) and stored at 5 to 8°C for no longer than 72h. Rats that had been chronically cannulated (4.2.3.5) 72h before the administration of BEA were injected with 2.0mL of the colloidal carbon solution at 37°C over a 2 min. period) at 3, 6, 12 and 24h and killed immediately by cervical dislocation. The bladder and kidneys were removed, trimmed and fixed for 24h in phosphate buffered formal.

One kidney and the bladder was processed for routine histology (4.2.1) and the other kidney was prepared for thick section cutting in celloidin (4.2.5.).

4.2.5. Celloidin Embedding, Blocking and Sectioning

This procedure was adapted from Culling (1974) the kidneys to be mounted in celloidin were fixed for 24h, dehydrated and transferred to ethanol (abs.): diethyl ether ::1:1 (v/v) for 24h. The tissue was taken through a graded series of celloidin (dissolved in ethanol:diethyl ether) at concentrations of 2%, 4% and 8% (m/v); 7 days were allowed for impregnation at each concentration.

Following the impregnation period the tissue was placed in silicon rubber moulds and covered with fresh 8% (m/v) celloidin, which was "topped-up" as necessary. The hardening of these blocks was undertaken
in a desiccator (housed in a fume cupboard), where controlled and even evaporation of the ether was accomplished by removing the top in the lid during the day, but closing the system overnight to allow the celloidin to re-equilibrate. This process was continued until the celloidin was a hard rubbery consistency, at which stage a wooden block with a roughened face (to be held in the microtome chuck) was stuck to the block with 8% celloidin. After a few days the celloidin blocks were hardened in 70% (v/v) ethanol overnight. Blocks and sections were subsequently stored in 70% ethanol.

Sections of 100 μm were cut on a sledge microtome (Riechert, Austria) with the knife at an angle of 90° to the horizontal, and the cutting edge set to an angle of about 45° to the direction of movement. The block was positioned so that one corner was first to meet the blade. The block, knife and cut section were kept flooded with 70% ethanol at all times.

Suitable sections for mounting were selected under a stereomicroscope, softened in absolute alcohol for 30s, placed on a slide, the edges "teased out" (to prevent kinking) and the slide flooded several times with xylene saturated with phenol, to clear the section. The remaining phenol was washed out of the section with xylene, and the section mounted under a cover slip with a large quantity of DPX.

4.2.6. Autoradiography at the Macroscopic and Microscopic Levels

These studies were undertaken at Ciba-Geigy Research Laboratories with the assistance and co-operation of Robin Christian and John Baker.

Rationale The distribution of water soluble diffusible materials can be assessed (provided they are radiolabelled) by rapidly freezing tissue (to prevent or reduce translocation), cutting sections of the frozen material which are then placed in intimate contact with a photographic emulsion. The radiation from the immobilised material produces a
distribution pattern of silver grains.

Despite this simple principle autoradiography is a most complex technique, the theory and practice of which have been described by Roth & Stumpf (1969) and Rogers (1979 a,b).

4.2.6.1 Whole body autoradiography. Whole body autoradiography was undertaken using the approach developed by Ullberg (See Larsson & Ullberg, 1981). Animals were killed (by cervical dislocation) and frozen in a large volume of hexane-solid CO$_2$ mixture at -70°C for 30 minutes. The legs and tail were removed and the animal mounted in a thick slurry of carboxymethylcellulose which was allowed to freeze overnight. The mounted carcass was trimmed down to approximately the level required for sectioning (using a rough knife) in a Brights Model OT whole-body cryostat (Brights, Huntingdon) at -15°C. Final sections were cut with a tungsten-carbide tipped knife. Before each section was cut Scotch tape No 810 (3M) was firmly applied to the surface, to support the section and to facilitate autoradiography. The tape was attached to a metal frame and the sections left to freeze dry at -15°C to -30°C (normally overnight).

The dry sections were applied to sheets of Ultrafilm (LKB, Croydon) in a dark room. The intimacy of contact between the emulsion and section was increased by the use of a "pressure device" and the autoradiograph was exposed at -10°C to -20°C for 7 to 20 days.

The adhesive mounted section was separated from the film (in the dark) under water, so as not to generate static electricity. The film was developed in D19 (Kodak, Hemel Hempstead) at 20°C for 4 min, passed through a 1% (v/v) acetic acid stopbath for 1 min, fixed in Hypam (Ilford, Mobberly, Cheshire) for 10 min, washed thoroughly and dried. The sections were placed in phosphate buffered formalin (pH 7.4) for 4 min, washed and dried.
4.2.6.2 Autoradiography of diffusible substances at the microscopic level. The technique used was essentially that of Appleton (1964). Clean dry microscopic slides were prepared (see Rogers, 1979b) with either "dipping film" emulsion (Type K2 or Type K5, Ilford, Mobberley, Cheshire) on one side, or covered with "stripping film" (Type AR.10; Kodak, Hemel Hempstead), with the emulsion upwards, in the dark room. Slides were left to dry and packed into light-proof boxes, each containing a small amount of silica gel dessicant.

Rats were dosed with radioactive labelled or non-labelled material and killed at the prescribed times. The kidneys and portions of liver were removed, prepared and frozen (as described above 4.2.3) and mounted on microtome chucks, which were left to equilibrate at -30°C while the knife angle, anti-roll bar position and temperature of the knife were optimised.

The frozen kidney blocks were trimmed down to that level which included the papilla tip, and a section of 10 μm was flash evaporated onto a slide, fixed in absolute ethanol, stained with Toluidine blue and assessed for:

a) morphological integrity (lack of ice crystal damage), and

b) suitability for autoradiography (i.e. ensuring that the section included the papilla tip).

Sections for autoradiography were cut in the dark, aided only by a safelight positioned to reflect off the knife blade. The slides, each with preformed emulsion layers (pre-cooled to -20°C) were removed from their light-proof box, and touched gently on the section hanging from the knife edge. The section was thus transferred from the knife to the slide, where it associated closely with the emulsion layer. At this point two precautions are essential:

a) the emulsion layer is very sensitive to mechanical impact which
may cause artefactual silver deposits, and

b) the slides must be at a temperature approximately the same as the tissue to ensure that there is no thawing (and hence translocation of water soluble substances) during the mounting phase as is the case for "flash evaporation" (see 4.2.3.). The success of the mounting is apparent by the absence of a "condensation frost" on the knife after a section has been transferred to an adequately pre-cooled slide.

Slides carrying serially cut sections were distributed between 3 or 4 light-proof boxes; thus only one box was opened at the end of each exposure interval (see below). The procedure for cutting and mounting sections was repeated for each tissue, ensuring that all slides were protected from accidental exposure to light. The chemical under investigation or its metabolites, or other endogenous chemicals in the tissue may cause two types of artefacts, the occurrence of which can only be assessed using tissue that is free of radioactivity:-

a) Positive chemography occurs when a chemical in the section causes silver deposits (which would be assumed to be caused by radioactivity) and,

b) negative chemography is apparent when a chemical in the section prevents or removes radionucleotide-induced silver grain deposits. Therefore sections from tissue treated identically, but with no label were employed as positive chemography controls and negative chemography was assessed by mounting non-radioactive sections on emulsion that had been uniformly fogged by exposure to light.

Radiolabelled and control sections were stored in a light-proof box (containing silica gel) sealed with black plastic tape and exposed at -20°C for 10 to 40 days.

At the end of each exposure period boxes were allowed to warm to room
temperature and the tissue fixed in 5% (v/v) acetic acid in ethanol, for 2 min, washed in running tap water for 4 min. and processed as shown below:-

   a) developed using D19 (Kodak, Hemel Hempstead), 5 min at 18°C,
   b) stopped using 1% (v/v) acetic-acid, 1 min at 18°C,
   c) fixed in 30% (m/v), sodium thiosulphate, 10 min at 18°C,
   d) washed in running tap water, 10-15 min at 18°C,
   e) air dried in a dust free place

The sections were strained with either Toluidine blue or Haematoxylin and Eosin as described above (see 4.2.1). All reagents had been pre-filtered to prevent 'grot' which might be confused with silver grain deposits. The stained sections were mounted under cover slips with DPX.

The distribution of silver grains was assessed under transmitted and incident dark-field illumination.

4.3 HANDLING AND MEASUREMENT OF RADIOACTIVITY

The two radio-isotopes used for these investigations were $^{14}$C and $^{35}$S. Departmental safety rules and good laboratory practice were adhered to throughout.

Radioactivity was quantitated by liquid scintillation counting using either:-

   A) Ultrabeta Model 1210 (LKB, Croydon)
   B) Rackbeta Model 1216 (LKB, Croydon), (both refrigerated)
   C) a Packard PRIAS (Packard, Caversham).

Polyethylene mini-vials (Beta-Vials; Hughes & Hughes, Romford) were used for counting injected label, acid and alkaline breath trap aliquots, urine, bile and column eluents. Mini-vials were also used for counting eluted samples from t.l.c. separations. Glass vials (standard volume, low potassium, Beckman, High Wycombe) with polyethylene inserts were used for digesting samples of tissue and faeces for subsequent
The counting efficiency was determined for each liquid scintillation counter, type of vial, liquid scintillation cocktail (see below) and each type of homogeneous system using commercially prepared $^{14}$C internal standards described by Reunanen & Soini (1974) and Gordon (1980) containing water soluble material (LKB-Wallac, cat 12/0-123; LKB, Croydon). The counting efficiency for $^{35}$S was determined using 50 μL aliquots of calibrated dioctyl sulphide (Amersham International, Amersham).

Two detergent based liquid scintillation cocktails were used. The Synperonic NXP:toluene::1:2 (v/v) cocktail described by Wood et al. (1975) was satisfactory for most samples. The cocktail consisted of 5.5g of 2,5-diphenyloxazole (PPO; Packard, Caversham) per 1L toluene (sulphur free) mixed with 500mL Synperonic NXP (ICI, Billingham). Phase separation of scintillant and solutions of high molarity (e.g. 0.1-M phosphate and 2N-NaOH) necessitated special treatment. Increasing the detergent ratio in the Synperonic NXP:toluene cocktail to 2:1 (v/v) facilitated the counting of 0.1M-phosphate buffer (pH 6.0). The counting of small volumes of 2N-NaOH (100 μL) was accomplished by diluting with 1.4mL water: methanol (15:1,v/v) and the use of Pico-Fluor 30 (Packard, Caversham) -see below.

The counting efficiency was determined at the start and end of all series of experiments where samples (same type of vial, homogeneous make-up, liquid scintillation counter etc.) were counted daily for an unbroken period. Otherwise the efficiency was checked for each batch of counting. Vials (6 to 12) containing no radioactivity were included in each of counting series to measure background.

Colour quenching was assessed by the two counting method. Samples were prepared in duplicate and counted, then an internal standard of
Table 4.3  Sample preparation of different radioactive liquids for scintillation counting.

<table>
<thead>
<tr>
<th>Type of Liquid Sample</th>
<th>Total Volume</th>
<th>Volume Used</th>
<th>Other Additions Type</th>
<th>Volume</th>
<th>Scintillant Cocktail Volume</th>
<th>Counting Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEA used for Dosing and BEA metabolites</td>
<td>Variable</td>
<td>10 - 50µL Depending</td>
<td>Water</td>
<td>300µL</td>
<td>Synperonic 1:2,5mL</td>
<td>52 - 80%</td>
</tr>
<tr>
<td>for assessing recoveries from column</td>
<td></td>
<td>on total activity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>separation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acid Breath Trap</td>
<td>100mL</td>
<td>100µL</td>
<td>Water</td>
<td>200µL</td>
<td>Synperonic 1:2,5mL</td>
<td>63 - 80%</td>
</tr>
<tr>
<td>Alkaline Breath Trap</td>
<td>100mL</td>
<td>100µL</td>
<td>Water: Methanol (15:1,v/v)</td>
<td>1.4mL</td>
<td>Pico-Fluor 30, 3.5mL</td>
<td>63 - 80%</td>
</tr>
<tr>
<td>Urine</td>
<td>Variable</td>
<td>200µL or 200µL*</td>
<td>Water</td>
<td>100µL</td>
<td>Synperonic 1:2,5mL</td>
<td>52 - 80%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>or 300µL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bile</td>
<td>Variable</td>
<td>100µL</td>
<td>Water</td>
<td>200µL</td>
<td>Synperonic 1:2, 5mL</td>
<td>48 - 62%</td>
</tr>
<tr>
<td>Column Effluent</td>
<td>Continuous</td>
<td>1.2</td>
<td>None</td>
<td>-</td>
<td>Synperonic 2:1,4,8mL</td>
<td>60 - 70%</td>
</tr>
</tbody>
</table>

*(Acute cannulation experiments)*
approximately 110,000 d.p.m of $^{14}C$ or 100,000 d.p.m of $^{35}S$ in 20 µL, was added from a 1000 µL gastight microsyringe using a repeating dispenser – model PB6000-1 (Hamilton, Whittier, USA). The $^{14}C$ standard was methyl-$[^{14}C]$toluene (Amersham International, Amersham) and the $^{35}S$ was carrier free SO$_2$ (Amersham International, Amersham) diluted to the required activity. The coefficient of variance of internal standard additions was less than 3.8% and 2.5% for $^{14}C$ and $^{35}S$ respectively.

4.3.1. Preparation of Liquid samples for counting

Table 4.3 shows the methods used for sample preparation of different radioactive liquids for counting.

4.3.2. Preparation of faeces for counting

A weighted 24-hour faeces sample was homogenized in 10mL water using a Ultra Turrax (Scientific Supplies, London), diluted to a final volume of 5 times the weight, re-homogenized and duplicate 0.2mL aliquots transferred to glass vials for digestion and counting. Samples were digested in 1mL Soluene-350, (Packard, Caversham) according to the manufacturers instructions, using 10mL of Synperonic 1:2 'cocktail'. Colour quenching was corrected by internal standardisation.

4.3.3. Preparation of tissue for counting

Up to 100mg of tissue was added directly to 1mL soluene-350, (Packard, Caversham) digested and counted as described above. Colour quenching was corrected by internal standardisation.

4.3.4. Thin Layer Chromatographic Separation of Radio-labelled Substances

T.l.c. was used to assess the purity of radio-labelled BEA and, subsequently, to provide some initial insight into urinary and bile metabolites formed from this compound. The methods used for spotting and running t.l.c. plates will be detailed below (4.3.5). The distribution of radio-labelled material was assessed using either a gas-phase thin-
layer scanner, autoradiography or by liquid scintillation counting of eluted material from zones of adsorbent support. The pros and cons of each method have been reviewed (Touchstone & Dobbins, 1978–Chapter 10) and will not be considered here.

4.3.4.1 Gas-phase thin-layer scanning. Preliminary assessment of t.l.c. radiolabel separations was undertaken on a Thin layer-scanner II, Model LB2723 (Berthold, Wildbad, Germany F.R.) using methane (BOC, Croydon) as the gas counting phase. The instrument was optimised for $^{14}$C counting.

This method of assessing t.l.c. separation is relatively rapid, but counting efficiency is low (less than 30% for $^{14}$C) and resolution is poor because of high electronic noise.

4.3.4.2 Autoradiography. This method gives the best resolution and can, if exposure times are long enough, locate trace components.

Dry t.l.c. plates were placed (in a pressure box) against Blue-brand X-ray film (Kodak, Hemel Hempstead) for periods of up to three months, in the dark. The plates were developed in Teknol 1:19(v/v), (May & Baker, Dagenham) for 5 min, stopped in 1% glacial acetic acid and fixed for 10 min in Amfix (May & Baker, Dagenham) 1:4 (v/v). The plates were washed for 2-3 hours and dried in a dust-free place.

T.l.c. plates were kept for spraying or quantitation.

4.3.4.3 Quantitation of labelled separated components. The quantitative measurement of separated components was undertaken by scraping adjacent areas of support material (1.0cm wide and 0.5cm long) off t.l.c. plates from the origin to beyond the solvent front. Each zone of scraping was transferred to a mini-vial for counting.

Initial experience with a thixotropic-toluene based scintillant gel (Cab-O-Sil; Packard, Caversham) gave a variable high background and poor reproducibility. The most favourable results were achieved by eluting
the support material in the vial with 300 \( \mu \)L of water and adding 5mL of scintillant cocktail. Tubes were left overnight (to allow the scrapings to settle) and counted. Backgrounds were in the normal range, and recoveries were in excess of 97%.

4.3.5 Thin-Layer Chromatographic Procedures

The recommendations of Touchstone & Dobbins (1978) were followed for preparing and applying samples (Chapter 4), preparation of mobile phases and development (Chapters 5 and 6), visualisation (Chapter 7) and improving reproducibility (Chapter 11).

4.3.5.1 Absorbant and solvent systems Chromatographic separations were carried out on one of two types of support material either:-

a) Silica gel 60 (0.2mm) on glass or aluminium sheets (Merck, Damstadt) or

b) Cellulose MN-300 (0.5mm) on glass (Anachem, Luton).

Neither support was heat activated, and both were free of fluorescence indicators.

The solvent systems were:-

i) propan-2-ol : ammonium hydroxide (33% m/v): water:: 90:8:2 (v/v).

ii) methanol saturated with \( \text{NH}_3 \) vapour,

iii) chloroform: methanol:: 9:1 (v/v),


Chromatographic separation was undertaken in air-tight glass tanks, placed on a stable surface free from temperature fluctuations. Solvent systems were replaced for each separation and at least 2 hours was allowed for the liquid and vapour-phase to equilibrate before the t.l.c. plates were developed by ascending chromatography.

4.3.5.2 Sample preparation and spotting. Samples were dissolved in absolute methanol and carefully spotted onto the t.l.c. plates, using 5
or 10 μL Drummond Microcaps (Shandon, London) under a gentle stream of pure dry nitrogen.

4.3.5.3. Two-dimensional development of t.l.c. plates. Two-dimensional t.l.c. was carried out in the same solvent systems to assess if multiple radio-labelled components were formed as a result of chemical instability during chromatographic separation (Sheppard, 1972).

4.3.5.4 Visualisation of functional amine groups. Functional amine groups were visualised by spray t.l.c. plates with fluorescamine (Sigma, Poole), 25mg of which was dissolved in 10mL acetone.

All t.l.c. plates were sprayed in a fume cupboard.

4.4 THE SYNTHESIS OF 14C-BROMOETHANAMINE HYDROBROMIDE

None of the described methods for the laboratory synthesis of BEA (Cortese, 1943) are amenable to a micro-scale, high yield and high purity radiosynthesis, from a commercially available starting product. Further, the purification of BEA is hampered by its cyclization to ethylenimine in neutral and basic aqueous solutions (Dermer & Ham, 1969) and handling may be hazardous because ethylenimine is toxic, being a powerful alkylating agent known to be mutagenic and possibly carcinogenic (Ninan & Wilson, 1969).

The industrially patented method of Groves (1937), used to prepare megagram quantities, was scaled down to a micro-synthesis (less than 100mg) of high yield in a pilot study. A special reaction chamber was designed to facilitate the synthesis of BEA and then its purification in situ (by fractional sublimation), thus avoiding losses that would otherwise have occurred during recrystallisation and preventing unnecessary handling hazards.

The method has been described (Bach & Bridges, 1982) but will be presented again as an integral part of this dissertation.

4.4.1 Synthesis of Labelled BEA
The apparatus is shown in Fig 4.4a. The borosilicate reaction chambers (f) was constructed as shown in Fig 4.4b. The chamber was loaded with 500 µCi (18.5 MBq) 44 mCi/mmol of [2-¹⁴C]ethan-1-ol-2-amine HCl (stated radiochemical purity 99%, Amersham International, Amersham) dissolved in 0.5 mL of water, containing 52 mg of carrier ethan-1-ol-2-amine HCl (Aldrich Chemicals, Gillingham) through a pasteur pipette inserted through the opening at v. The label and carrier were evaporated in a gentle stream of dry nitrogen at 25°C. The opening (v) was flame sealed and the chamber incorporated into the apparatus housed in a fume cupboard.

HBr was generated from freshly redistilled dry tetralin [tetrahydro-naphthalene] (BDH, Poole) in a flask (a) by the addition of a slow stream of Br₂ from a dropping funnel (b). Sodium thiosulphate solution (to quench unreacted bromine) or additional tetralin could be added through the dropping funnel (c). The gas was passed through a splash head (d) then a washbottle (e) containing tetralin to remove any unreacted Br₂ vapour, and thence to the reaction vessel (f) which was immersed in a continuously stirred, heated oil bath, fitted with a contact thermometer (g). A water-cooled coil between e and f served to condense any tetralin vapour in the HBr flow. The pressure was kept slightly greater than atmospheric (0.5 cm s water) using a gas absorption trap (i), the outflow of which was cowled by a funnel (j) connected to a 'fast-flowing' water vacuum line, to remove and dissolve the unreacted HBr. The empty washbottle (h) served to prevent water 'suck-backs' into the reaction chamber. All fittings were Quick-fit or Tygon (Norton Co., Ohio).

The complete system was flushed with pure dry N₂ at room temperature for 10 minutes though the dropping funnel c, then purged with HBr for 10 minutes.

The slow addition of Br₂ through a constricted tip in the dropping
Fig. 4.4a Schematic representation of apparatus used for the microscale synthesis of BEA.

a Round bottom flask  f Micro-reaction chamber
b Dropping funnel, PTFE tap  g Heated oil bath
c Dropping funnel, PTFE tap  h Drechsel bottle
d Splash head  i Gas absorption trap
e Drechsel bottle  j Funnel connected to water vacuum line

Fig. 4.4.6 Micro-reaction chamber used for the synthesis and purification of BEA. Side elevation shown as Z-Z.
funnel below the surface of the tetralin gave a steady stream of HBr for the duration of the synthesis. The oil bath was heated to 140°C and increased in increments of 10°C every hour to 190°C at which it was maintained for 1 hour.

Once all the Br₂ had reacted the system was purged with pure dry N₂ for 1 hour. The reaction vessel was removed from the oil bath, cooled to room temperature and the adhering oil washed off with methanol:chloroform 1:1 (v/v). During this time the liquid in the reaction chamber solidified to an off white material.

4.4.2 Purification by Fractional Vacuum Sublimation

The reaction vessel was flame sealed at w, and after cooling the purification vessel was returned to the oil bath submerged to y (Figure 4.4b). A water-cooled copper coil was placed on the non-immersed portion and a vacuum line connected to the outlet via a trap immersed in solid CO₂-acetone. The vessel was evacuated to 0.05 mm Hg and heated at 100°C for 16 hours. After cooling, washing etc., the limb was broken at y'. This was to remove trace amounts of unreacted ethan-1-ol-2-amine HCl and other low temperature sublimates. The low temperature fraction contained a small amount of BEA and an unidentified trace component. BEA was sublimed as a white solid at a vacuum of 0.05 mm Hg and heated at 140°C for 24 hours, with the vessel immersed to x and a cooling coil on the limb out of the oil. After cooling, washing etc., the limb was broken at x and the BEA removed from it and stored at 0-5°C. The reaction vessel contained a brown residue of unknown composition.

There was no detectable radioactivity carried over from the reaction vessel during synthesis or beyond the cooling coils used in any of the purification steps. This suggests that ethylenimine (which is relatively volatile) was not formed at any of these stages.

The authenticity of non-labelled synthesised material was confirmed
by its IR spectrum, (measured in a KBr disc using a Perkin Elmer model-577 grating spectrophotometer - see 4.5.2), melting point (171-172°C, uncorrected) and mixed melting point and by its behaviour on the four t.l.c. systems listed above (4.3.5.) visualised with either ninhydrin or fluorescamine (4.3.5.4) and compared to commercially available BEA (BDH, Poole).

4.4.3 Yield, Specific Activity and Purity

The weight of BEA synthesised was determined twice using 'cold' ethan-1-ol-2-amine HCl, and yields of 74% and 71% were obtained. Based on the incorporation of radioactivity, however, the yield was in excess of 89%. The discrepancy between the chemical and radiochemical yields most likely arose from using a sublimation time of 16 hours for the purification of non-labelled BEA. Using these conditions, however, the unsublimed residue in the reaction chamber contained BEA. Impurities in the carrier ethan-1-ol-2-amine HCl would also contribute to such differences.

The specific activity was 210.16 kBq/mg; 44.4 kBq/mmol (5.68 μCi/mg; 1.2mCi/mmol).

BEA purity was assessed by t.l.c. radiochromatography (4.3.5.1) after co-chromatographing with 4 mg/mL of carrier BEA (4.3.5.2) which had been synthesised (4.4.1) and purified (4.4.2) as described (Sheppard 1972). The distribution of radiolabelled components was assessed autoradiographically (4.3.4.2) for 20 days after preliminary scanning (4.3.4.1). The R_f values for BEA and ethan-1-ol-2-amine HCl are shown in Table 4.2.

Neither the low temperature sublimate nor the reaction product contained ethan-1-ol-2-amine. Purified BEA chromatographed as a single component in systems aiv and biv and as a major component (with five trace components) in system aiii. The alkaline systems gave one major
### Table 4.2 The $R_f$ for 2-bromoethanamine and ethanolamine.

<table>
<thead>
<tr>
<th>Solid Support</th>
<th>Chromatographic Support System</th>
<th>2-Bromoethanamine $R_f$</th>
<th>Ethanolamine $R_f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silica Gel</td>
<td>i</td>
<td>0.15</td>
<td>0.16</td>
</tr>
<tr>
<td>(support a)</td>
<td>ii</td>
<td>0.67</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>iii</td>
<td>0.08</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>iv</td>
<td>0.67</td>
<td>0.67</td>
</tr>
<tr>
<td>Cellulose</td>
<td>i</td>
<td>0.90</td>
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component, with a trace substance leading and tailing it (systems ai and aii). The two-way elution technique (4.3.5.3) suggested that both the tailing and leading component associated with the alkaline chromatographic systems were due to chemical instability of BEA at an elevated pH (Dermer & Ham, 1969). Attempts to prevent cyclization by derivatizing the BEA-amine group before chromatography, with either fluorescamine or dansyl chloride (Aldrich Chemicals, Gillingham), were not successful. The fluorescamine derivate hydrolysed under alkaline conditions during the chromatographic separation. The alkaline conditions required to promote dansylation favoured BEA instability, cyclization and possibly polymerisation.

Purity was assessed by liquid scintillation counting of transverse areas of support material (4.3.5) and found to be 95% of the alkaline chromatographic system (where the BEA was apparently unstable) and 98% on the other systems.

4.4.4 Discussion

The synthesis of a pure, high specific activity radiolabelled BEA was of paramount importance in order to probe the molecular pathogenesis of this acutely induced RPN. A radiolabelled molecule would make it possible to:

A) Produce data on the absorption and excretion of BEA,

B) Study the distribution of the parent molecule (and its metabolites) in the whole animal and, more importantly across the kidney from the cortex to the medulla tip, and

C) Start answering some of the critical questions relating to the metabolism of BEA such as:

I) What are the structures of BEA metabolites, with a view to identifying the "proximate" papillotoxin?

II) What metabolic processes (e.g. Cytochrome P-450 versus cyclo-
oxygenase) are responsible for these bioconversions?

III) Does the generation of biologically reactive intermediates play any role in the formation of BEA metabolites? and

IV) If so:-

a) Are these formed via ethylenimine (which was formed from BEA, and

b) could the papillary necrosis be caused by these unstable intermediates binding covalently to micro- or macro-molecules, and, if so, what is the nature of the electrophiles and how does the binding of the activated product mediate the pathophysiological changes?

This micro-modification of a patented method (for the industrial synthesis of BEA) has been applied to a radiochemical synthesis (using a commercially available starting material). The synthesis is manipulatively easy, as is the subsequent purification, and both yield and purity of the final product are high.

It is also likely that the method could, for example, be used for the synthesis of other members of the halogen alkylamine group of their N- or C- substituted products subject to suitable modification being introduced. This 'synthetic' approach could obviously be applied to molecules labelled on either or both carbon atoms, and to a carrier-free synthesis or to other isotopically labelled starting material.

The containerised approach may also find other applications where either the starting material or the final product is toxic or carcinogenic. Furthermore, fractional vacuum sublimation as a means of purifying radiochemicals has a greater potential than has hitherto been reported.

4.5 THE ANALYSIS OF URINARY, BLADDER AND KIDNEY STONES AND CRYSTALS

Calculi in the urinary tract may be homogeneous or heterogeneous and might consist of any of a vast number of complex inorganic mineral salts
and various organic components (see Nordin et al., 1979 and Cheng, 1980) set in a protein, glycoprotein or glycosaminoglycan matrix.

The nature of these deposits can be assessed by several well established methods which are outlined below.

4.5.1 Crystal Shape and Deposit Solubility

The shape and solubility of urinary sediments offers a simple means of assessing the major chemical composition for the most commonly encountered urinary sediments. (see Diem & Lentner, 1970).

4.5.2 Infra-red Analysis of Urinary Tract Deposits

Detailed methods for infra-red analysis of renal tract crystals and calculi and their assessment, have been described by Weissman et al., 1959; Oliver & Sweet, 1976; Gault et al., 1980 and Modlin & Davies, 1981.

In brief 1 to 2 mg of crystalline deposits or calculi were finely ground in a micro-agar ball mill with about 200mg of oven dried infra-red quality KBr (Merck, Darmstadt) for 15 min. KBr discs were made in a KBr press (Beckman-RIIC, Glenrothes) using a pressure of 7 to 10 tons for 20 min under vacuum. Reference spectra were prepared similarly from magnesium ammonium phosphate (kindly donated by BDH, Poole).

The discs were scanned on a Perkin Elmer Model-577 Infra-red Grating Spectrophotometer (Perkin Elmer, Beaconsfield ). Where necessary an alternator AT-02 (Beckman-RIIC, Glenrothes) was placed in the reference path to optimise the trace. The sample scanning time was 15 min, with the slit set to normal and the time constant set on auto. The calibration of the instrument wavelength was checked with a polystyrene film at 1583 and 1601 cm\(^{-1}\).

The chemical composition of the scanned material was assessed

a) Using the flow chart of Oliver & Sweet (1976).

b) The published spectra of Weismann et al., 1959; Oliver & Sweet, 1976; Gault et al., 1980 and Modlin & Davies, 1981, and, finally,
c) From a reference spectra of authentic material.

4.5.3. X-ray micro-analysis

Although it is the most widely used method of analysing calculi, infra-red, suffers from certain limitations in that:-

a) traces may be difficult to interpret if the deposits are heterogeneous,

b) the trace may be altered substantially by the type and amount of matrix material, and

c) the trace gives no data on the type of matrix material. X-ray microanalysis can supplement infra-red data.

Micro-X-ray analysis was undertaken by the Structural Studies Unit at the University of Surrey. The calculi or crystal deposits were stuck to a perspex base with double-sided adhesive tape (Cellotape) vacuum carbon coated and viewed in a Joel model JXA-50A scanning electron microscope. Spot x-ray analysis was undertaken using a Link 860 System (Link, High Wycombe, Bucks). The emitted x-rays were monitored for 100s. Heterogeneity was assessed by analysing up to 20 different crystals or 5 different areas on up to 10 different calculi, and comparing each analysis to the first. The final accumulated analysis (from homogeneous deposits) were plotted from the multichannel analyser of the Link system.

4.6 THE POLYDISPERSION OF PROTEOGLYCANS, GLYCOSAMINOGLYCANS AND OLIGOSACCHARIDES IN URINE.

4.6.1 Urinary Macromolecules and Middle Molecules

Intense proteinurias are associated with many types of renal lesions (see symposium edited by Pollak, 1974) most often, however, only total protein or albumin have been measured. The electrophoretic distribution of urinary proteins of different molecular weights has served to distinguish the site of renal lesion: (viz. tubular, glomerular or mixed
damage) in kidney disease in man (Balant et al., 1974 and Alt et al.,
1979).

The use of this approach to assessing experimentally induced lesions
in the rat is complicated because proteinuria is physiologically normal
in this species. The proteinuria is more intense in the male than in the
female (it appears to be dependent on androgen synthesis) and increases
with age. The electrophoretic pattern differs between the sexes, with
age and between strains of the same age and sex (Galaske et al., 1980 and
Alt et al., 1980).

In recent years an important group of relatively low molecular weight
dialysable proteins (300-2000 daltons), the 'middle molecules' have been
associated with uremia (Furst et al., 1975; Bergstrom & Furst, 1976 and
Furst et al., 1976). This group of proteins has been resolved into at
least 10 components, one of which was present in the plasma of uremic
patients. No clear role has yet been defined for this component,
although it has been suggested that it may contribute to those systemic
complications during uremia which are ameliorated by maintenance
dialysis.

The excretion of urinary proteoglycans (PoG) and glycosaminoglycans
(GAG) has been most studied in the inherited hyperglycosaminoglycanurias
(see Kennedy, 1969). PoG-GAG are also excreted by normal humans, where
there are age related changes (Taniguchi, 1972), sex differences, and
possibly circadian rhythms (Scott and Newton, 1975 and Newton et al.,
1979) in the types and quantities. Most of the 'classical' methods used
for PoG-GAG isolation depend on cetylpyridinium chloride precipitation
and dialysis. White & Kennedy, 1979 reported that over 90% of urinary
carbohydrate and proteinaceous "macro-molecules" were dialysable and
thus lost by these "common" techniques.

The high PoG-GAG concentration in the medulla (2.3.4.4), especially
the papilla, and the loss of histochemical staining of mucopolysaccharides from those areas of BEA-induced necrosis (5.2), suggested that the monitoring of urinary PoG-GAG (and their polydispersion) could be a rational selective and sensitive approach to non-invasive assessment of renal papillary necrosis.

4.6.2 Column Chromatographic Separation of Urinary Components.

The method was essentially that of White & Kennedy (1979), where urinary molecules were separated using a gel filtration column and the eluent monitored for uronic acid or fractions containing radiolabelled material counted.

4.6.2.1 Preparation of gel filtration column Bio-Gel P-2 (200-400 mesh, Bio-Rad Laboratories, Watford) was swollen in a sodium phosphate buffer (0.1 mol/L, pH 6.0) over night at 5°C. The fines were removed by suction and a slurry (made up of the settled gel volume plus an equal volume of buffer) was degassed at 40-50°C using a water-vacuum. Columns were poured from the suspended slurry and allowed to settle under gravity for about 36h, after which the columns were pumped downwards at about 0.2mL/min for 2h. The columns (Wright Scientific, Stonehouse) which these jacketed at 30°C, were one 20 cm x 1.0 cm ID (pumped downwards) in series with two 100cm x 1.0cm ID (pumped upwards). All columns were fitted with adjustable plungers on the "pump side". The system was connected in series and pumped for 72h at 0.2mL/min, after which plungers were adjusted to meet the gel face. The total length of the gel was 122cms.

Samples were applied 'on stream' using a three way tap (Wright Scientific, Stonehouse) on the first column. The column void volume (Vo) was determined using elution times for ferritin (450,000 daltons; BCL, Lewes). All samples were made up in 0.1 mol/L phosphate buffer, pH 6.0 and the column was eluted with the same buffer which was degassed daily.
The buffer was supplied to the column at a flow rate of 0.2mL/min using a Micro Perplex pump, Model 2132 (LKB, Croydon).

4.6.2.2 Application of samples for separation. Biological material was filtered through a 0.22 µm (Nucleopore, Sterilin) bacterial exclusion filter (to prevent microbiological contamination of the column), and aseptic techniques were used to handle and apply these materials. The sample (0.25mL) was placed "on stream" using a 3-way tap (Wright Scientific, Stonehouse).

Between 100 and 250 µL of radioactive urine, urine extract or bile (for subsequent fraction collection and counting) was injected through a septum injector, part 3301 (Omnifit, Cambridge) using a microlitre syringe (0.5mL Hamilton, Whittier).

4.6.2.3 Monitoring Uronic acid. Uronic acid was monitored continuously using the borate-carbazole assay on an autoanalyser system (White & Kennedy, 1979). The system is shown schematically in Fig. 4.6, together with types of tubing and nominal flow values.

The borate reagent contained 25 mmol/L sodium tetraborate (BDH, Poole) in concentrated sulphuric acid and the carbazole reagent was made daily (in advance) by dissolving 0.05g carbazole (Aldrich, Gillingham) in 60mL of absolute ethanol, and diluting to 100mL. The reagents and sample were mixed (Fig. 4.6) and heated at 95°C for 10 min and monitored in a flow cell at 520nm.

The hold-up time in the autoanalyser system was 20 minutes and the response was measured using freshly prepared D-glucurono-6,3-lactone (Calbiochem) at concentrations of 20mg/L in sodium phosphate buffer. The interferences from non-uronic acid material has been reported to be less than 4% (White & Kennedy, 1979).

4.6.2.4 Collecting and monitoring radioactive eluent. Radiolabelled components which had been separated on the column were collected...
Fig. 4.6 Schematic representation of the Autoanalyser system used to monitor column eluent for uronic acid using the borate-carbazole method. The nominal flow rates and types of tubing are:

1) 0.05 mL/min - tygon
2) 0.43 mL/min - acidflex
3) 0.60 mL/min - tygon
4) 0.05 mL/min - solvaflex

After White & Kennedy, 1979.
directly into Beta-vials for 5 min periods (using an Ultrarac Model 7000; LKB, Croydon) and counted in a Rackbeta (4.3) using the scintillation cocktail described (Table 4.3).
2-BROMOETHANAMINE HYDROBROMIDE-INDUCED
RENAL PAPILLARY NECROSIS:
HISTOPATHOLOGICAL AND CYTOCHEMICAL CHANGES
AND FACTORS AFFECTING
THE DEVELOPMENT OF THE LESION

Most of the model systems which can be used to study the pathogenesis of RPN require longterm treatment and they may also be notoriously irreproducible (3.2). Furthermore, in a chronically induced model system it is always difficult to be certain if subtle changes precede, parallel or follow a "primary" lesion. An acutely induced lesion offers the most practical way of assessing what primary pathophysiological changes give rise to a lesion, especially at a molecular level. This, in turn, provides a working hypothesis that can be tested in a chronically induced model system.

Extrapolation of data between systems is an inescapable facet of toxicology, and, although it is fraught with inexactitudes, it remains the fundamental approach through which understanding has been achieved. The investigations described below were undertaken within a framework of such a research philosophy.

2-Bromoethanamine (BEA) hydrobromide was chosen as a suitable model compound to study the molecular pathogenesis underlying the chemical induction of renal papillary necrosis (RPN) because:

A) It induces a papillary specific lesion in all treated animals (Table 3.7).

B) The lesion is of an acute nature (3.2.3.2), occurring within a few days.
C) The acutely induced BEA damage and the chronically induced analgesic "type" lesion both produce similar renal functional and histopathological changes in the rat kidney (3.2.4). The development of the histopathological and the renal functional changes that occur in both the experimental animal models and in human analgesic associated nephropathy (Table 3.8 and 3.9) and strikingly similar.

D) The chemical structure of BEA is simple, thus the synthesis of a radiolabelled molecule for studying absorption, distribution, metabolism and excretion was practicable.

E) BEA is commercially available in a pure (greater than 99%) crystalline, stable form, which is water soluble and has few other hazards associated with its use. Ethylenimine, for example, is not commercially available, it is unstable, explosive and mutagenic.

5.1 THE RESPONSE TO DIFFERENT DOSES OF 2-BROMOETHANAMINE

A variety of BEA dose regimens and routes of administration have been used previously to induce renal papillary necrosis (Table 3.7). These have varied from a single dose of 50mg/kg iv up to 250 mg/kg iv, and multiple daily doses of 100 mg/kg ip for 6 or more days. It was considered essential at the outset of this work to establish a reproducible dose response relationship so that sex related differences and the effects of pretreatment on the lesion could be assessed.

5.1.1 The Dose Response of Male and Female Rats to BEA Administered Intraperitoneally.

a) Animals and treatment. Male and female (200-210g) rats (housed as described 4.1) were given a single dose of freshly prepared BEA (4.1.1) at concentrations of 12, 25, 50, 100, 150 and 200 mg/kg using two rats of each sex for each dose level. Rats were killed after 5 days. In addition two male rats (210g) were dosed daily with 100 mg/kg ip for 4 successive days and killed 5 days after the last dose.
Animals were killed as described and both kidneys, the bladder, spleen and liver were taken for fixing (4.2.1).

b) **Histopathology.** Tissue was fixed for more than 24h, imbedded in wax and sections processed for H & E (4.2.1).

c) **Results.** The renal morphology appeared normal in both sexes given 12 or 25 mg/kg of BEA, but papillary necrosis (of increasing intensity) was seen from a dose of 50mg/kg. The minimal damage (termed intermediate necrosis by Burry, 1968) seen at 50mg/kg included destruction of the interstitial cells, limited necrosis of the loops of Henle and the medullary microvasculature. In addition the epithelia covering the papilla was often hyperplastic over that part of the medulla where necrotic change had taken place (Fig. 5.1.1a). At progressively higher doses of BEA (i.e. 100 and 150 mg/kg) the area of interstitial, nephron loop and capillary necrosis increased. In those kidneys with a normal or hyperplastic covering epithelia the elements of the collecting ducts were always present although they sometimes showed regenerative changes. In more severely affected kidneys (200mg/kg) there was loss of the epithelia covering the papilla, together with necrosis of all of the anatomical parts of the medulla up to (Fig. 5.1.1b), but never beyond the corticomedullary region (termed total necrosis by Burry, 1968). The area of the medulla stump which had "survived" the chemical insult showed signs of regeneration and re-epithelialisation.

The degree of damage showed some biological variability between rats of the same sex, but the general trend in the extent of the medullary necrosis was similar in both male and female rats.

The two animals treated with four daily doses of 100mg/kg showed total medullary necrosis up to the corticomedullary region.

The spleen and liver appeared histopathologically normal in all samples of tissue. The bladder was normal from animals treated with 12 or 25 mg/kg
Fig. 5.1.1a  "Intermediate" renal papillary necrosis showing destruction of most of the medullary elements except the collecting ducts and covering urothelia - after a BEA dose of 50mg/Kg ip. H & E, X 130.

Fig. 5.1.1b. "Total" renal papillary necrosis showing the destruction of all of the elements of the medulla after a BEA dose of 100mg/kg ip H & E, X 80.
Fig. 5.1.1c. Mild hyperplastic changes in the bladder urothelia follow treatment with doses of BEA greater than or equal to 100mg/kg ip. H & E, X 130.

Fig. 5.1.1d Normal bladder urothelia. H & E, X 130.
of BEA, but larger doses caused a flacid bladder, the musculature of which appeared to have lost its contractility. The bladders of animals treated with 100mg/kg BEA or more often filled with a coagulated eosinophilic material. This was often lost during processing or before sections were finally mounted. The transition epithelial lining of bladders from those animals treated with 100 or more mg/kg of BEA also showed mild hyperplastic changes (Fig 5.1.1c), compared to normal controls (Fig.5.1.1d).

**d) Discussion.** The histopathological method for assessing the degree of papillary necrosis is unsophisticated. The locating of this region of the kidney on a histological section is difficult, as is the positioning of the tissue block to ensure that the whole kidney - papillary tip to cortex - is examined. Within the need to execute painstaking histology (to locate the papilla tip) no additional attempt to quantitate the lesion by undertaking serial sectioning through the medulla. Thus it is possible that focal necrotic lesions may have been missed at a dose of 12 or 25mg/kg BEA. As illustrated in Figure 5.1.2a below the only focal lesions detected were limited to the interstitial cells of the papilla apex.

The dose response relation for ip administered BEA was sharp (after what is assumed to be a threshold dose of 50mg/kg) and increased to include areas of necrosis up to, but never beyond the corticomedullary region. This maximal extent of lesion corresponds to that reported previously by Shimamura (1972) who used 6 daily doses of 100mg/kg BEA.

Most of the previously published data have used a single dose or multiple large (> 100mg/kg) doses of BEA (Table 3.8). During the course of these investigations Axelsen (1978b) reported that varying the dose of BEA the degree of necrosis could be varied from minor necrotic foci (80mg/kg sc) to total papillary ablation (250mg/kg sc), although the evidence for this statement was not well documented in his paper. An accompanying communication (Axelsen, 1978a) documented clearly a dose
response relationship between single sc injections of ET and the degree of RPN. This varied over a broad dose range where 0.25 to 2.0mg/kg caused no lesion, 1.25 to 6.25 mg/kg caused intermediate RPN and 4.0 to 8.0 mg/kg caused total papillary necrosis. The lesion was, however, confined to the papilla and did not extend to the corticomedullary junction as described above for BEA. The BEA-induced lesion showed a steeper dose response curve throughout these investigations (although variability did occur) compared to that reported by Axelsen, 1978b. The significance of these differences is discussed further below (see 7.1).

The dose related BEA-induced papillary necrosis was similar in both male and female rats. In contrast, marked sex related differences in the extent of kidney lesions have been reported for certain halogenated solvents (Lock, 1982) and for aminoglycoside antibiotics (Faccini, 1982). Both of these groups of nephrotoxins cause histological changes in the cortex, a site where major sex related morphological and functional differences have been reported (Messow, 1980). Normal male mice have higher glomerular filtration rates and greater relative kidney weights compared to females. Whereas the area occupied by medullary tissue is identical in both sexes, the cortex is larger in the male, but there are fewer glomeruli (although the individual glomeruli are the same size in both sexes). Cuboidal cell transformation in the outer layer of Bowman's capsule occurs in 60% of the glomeruli in male mouse kidney, but only 5% in females. Further, there are differences between the rhythms of excretion of endogenous hippuric acid, when comparing the sexes of both rats and mice (Baunack & Gartner, 1980) and the pattern of urinary protein excretion is characteristic of the sex of a rat (Galaske et al., 1980, and Alt et al., 1980).

The fact that the extent of RPN is independent of sex suggests that the pathogenesis of the BEA-induced lesion may be independant of both cortex...
morphology and function, and relate exclusively to the medulla structure and function.

The significance of the flacid bladders in animals treated with 50mg/kg (or more) BEA is uncertain. PG inhibitors are known to cause loss of tone in bladder smooth muscle (Brown et al., 1980), but other mechanisms (possibly a protracted diuresis or renal debris in the bladder) might produce similar changes.

Medullary and pelvic transitional epithelium hyperlasia are common to papillary necrosis induced using other chemicals, e.g. biphenyls, aspirin and fat-free diet (Hardy 1970a,b; Bokelman, 1971 and Molland 1978a,b). It appears, however, that little attention has been accorded to epithelial changes in the bladder. Recently, long term orally administered aspirin, phenacetin and codeine has been found to cause hyperplastic changes in the bladder (Nanra, R.A., personal communication).

These observations may be directly pertinent to the pathogenesis of transitional epithelium carcinoma that occurs in human analgesic abusers (3.1.3.5), but they have not, apparently, been investigated. Medullary cyclo-oxygenase converts several known urothelial carcinogens (e.g. benzidine) to reactive intermediates (see 2.3.4.5). Other mechanisms, however, such as a changed chemical composition, a decreased osmolality (as a sequel to the necrotic process) or the passage of large amounts of cellular debris out of the kidney could act as an irritant and cause a hyperplastic response from the urothelium. This problem is, however, beyond the scope of these investigations and will not be considered further until Chapter 7, where an attempt will be made to integrate these changes into the overall molecular pathogenesis of analgesic nephropathy.

5.1.2. The Dose Response of Male Rats to BEA Administered Orally and Subcutaneously

a) Animals and treatment. Pairs of male rats (200-220g) were dosed with
BEA at levels of 50, 100, 200, 500 and 1000 mg/kg po (as described 4.1 )
and groups of three male rats were given dose levels of 50, 100 and 200
mg/kg sc. Pairs of animals from these groups were killed after 5 days and
kidneys, bladder, spleen and liver specimens taken for histology (4.2.1).
The two animals treated with 1000mg/kg po were moribund at 24 h and,
therefore, killed immediately. The heart and lungs were taken for
histological examination, in addition to the kidneys, bladder, spleen and
liver.

The single remaining animals from the sc dosed group were kept. The
animal treated with 200mg/kg of BEA sc died after 37 days. Gross autolysis
prevented specimens being taken for histology. The remaining two animals
(treated with 50 or 100 mg/kg BEA) plus an age matched control, were killed
240 days after treatment with BEA.

b) Histopathology. Tissue was fixed for more than 24 h and processed for
H & E (4.2.1).

c) Results. The dose response relationship for animals injected with
BEA via the sc route was similar to that already described for animals
treated intraperitoneally (5.1.1.b) at the same dose level.

BEA caused an apex limited focal papillary lesion in rats given 50mg/kg
(Fig. 5.1.2a and b). The necrosis was confined to the interstitial cells,
and elements of the nephron and the collecting duct were largely
unaffected. Higher doses caused a lesion that occupied progressively
larger areas of the medulla. Rats treated with 200 and 500 mg/kg po showed
loss of non-collecting duct elements of the medulla, a marked hyperlasia of
the covering epithelia and similar changes in the remaining epithelium of
the collecting duct (Fig. 5.1.2c and d).

Dose related damage to the anatomical elements of the medulla followed a
parallel course in rats given BEA orally, i.e. first interstitial cell
loss, transition epithelia hyperplasia, loss of the microvascularity and
Fig. 5.1.2a  The apex limited focal papillary necrosis caused by 50mg/kg BEA po. H & E, X 130.

Fig. 5.1.2b  The apex limited focal papillary necrosis caused by 50mg/kg BEA po. H & E, X 325.
Fig. 5.1.2c  Papillary necrosis caused by 200mg/kg BEA po.
H & E, X 80.

Fig. 5.1.2d  Papillary necrosis caused by 200mg/kg BEA po,
detail of extensive urothelial hyperplastic changes. H & E, X 325
Fig. 5.1.2e  Papillary necrosis caused by 1000mg/kg BEA po.  
H & E, X 100.

Fig. 5.1.2f  Papilla from rat treated with 50mg/kg BEA ip after 240 days.  H & E, X 130.
Fig. 5.1.2g  Mild cortical cystic dilatation at 240 days after 50mg/kg BEA ip. H & E, X 325.

Fig. 5.1.2h.  Re-epithelialised medulla stump and remnants of the papilla ghost 240 days after BEA 100mg/kg ip. H & E, X 80.
Fig. 5.1.2i  Severe cortical scarring and pitting 240 days after BEA 100mg/kg ip. H & E, X 325.
loops of Henle and, finally, at the upper dose levels (1000mg/kg) there was loss of the covering epithelia and the collecting ducts (Fig. 5.1.2e).

In common with rats dosed by the ip route the bladders from rats treated sc with BEA and those given 100mg/kg or more po had lost their normal muscular tone (as assessed macroscopically), some contained a coagulated eosinophilic gel, and microscopically there was some evidence of hyperplasia of the transitional epithelial cells lining the bladder. All other organs, including that from the high dose orally treated animals were histologically normal on H & E sections.

The kidneys from the animal treated with a single 50mg/kg dose of BEA 240 days before (Fig. 5.1.2f) showed a peculiarly re-epithelialised medulla which appeared to contain few, if any, nephron elements. Changes in the cortex (Fig. 5.1.2g) were confined to mild cystic dilatations. At 240 days the entire medulla was absent from the animal treated with a single 100mg/kg dose of BEA (Fig. 5.1.2h). The corticomedullary stump was re-epithelialised and a remnant of the medulla ghost remained in the pelvis. The cortex showed severe scarring and pitting on the surface, and markedly dilated tubules containing eosinophilic material (Fig. 5.1.2i). The aged-matched control kidneys were normal.

d) Discussion. The similarities between the extent of necrosis in rats given varying doses of BEA via the ip and the sc route, suggests that hepatic metabolism plays little, if any, role in the generation of the "proximate" papillotoxin. If hepatic "activation" did occur the ip route might have been expected to produce a more marked effect.

The shift in the dose response relationship when BEA was administered orally might, therefore, be explained by intestinal cell deactivation or delayed absorption and hence less BEA reaching the kidney (see 6.3).

A noteworthy histopathological difference between the parenterally administered BEA and that administered orally was the marked hyperplasia of
the collecting ducts and medulla epithelia when, for example 200mg/kg BEA was given po. Similar changes are described below (5.3.3) for animals treated with anti-oxidants before they were given a BEA insult.

The necrotic changes seen in animals treated with 1000mg/kg of BEA po and killed at 24 h did not have a clearly defined zone between necrosed and viable tissue. This possibly related to the early time point at which animals were sacrificed and reflects a lesion which had not run its full course rather than a qualitatively different pathological change.

The morphological changes 8 months after the BEA-induced RPN present two contrasting pictures depending on the dose of compound administered. The high dose of BEA had caused total ablation of the medulla, followed by its abscission and the re-epithelialisation of the medulla stump. A dose of 50mg/kg BEA, however, left a medulla shell, the surface and collecting tubules of which were re-epithelialised, but between these elements no vestiges of nephron elements or interstitial cells were apparent. This is consistent with the findings of Cuppage & Tate (1974) and Shimamura & Bonk (1976) who reported that epithelia from adjacent undamaged surfaces of the medulla migrated along the intact basement membrane and established a continuity of cuboidal covering, as part of the reparative processes. In common with Shimamura & Bonk (1976) there was, however, no repopulation by medullary interstitial cells. These cells apparently do have the capacity to regenerate. Mitotic figures have been reported in the renal interstitium (Shimamura & Bonk, 1976), and hyperplasia of these renomedullary interstitial cells have been reported to occur in association with Bartters syndrome (Verbeckmoes et al., 1976 and Bartter et al., 1976). On the other hand there is evidence (Dicker & Shirley, 1971, 1973) to show that whereas the cortex undergoes compensation hypertrophy in a uni-nephrectomized animal the medulla does not.

It is difficult to explain the absence of these highly specialised
5.1.3 The Dose Response of Weanling Rats to Intraperitoneally Administered BEA.

The weanling rat may be resistant to a number of nephrotoxic compounds. Thus the effects of BEA were assessed in young animals.

a) Animals and treatment Pairs of male weanling rats (50g) were dosed with BEA (4.1) at levels of 0, 50, 100 and 150 mg/kg ip. The rats were killed 7 days later (4.2.1) and kidneys and bladder taken for histopathology.

b) Histopathology. Tissue was fixed for more than 24 h and processed for H & E (4.2.1).

c) Results. The dose response relationship for BEA in weanling rats differed to that reported above (5.1.1) for adults. Low doses of BEA (50 and 100 mg/kg) caused limited lesions (Fig. 5.1.3a and b), but a dose of 150mg/kg (Fig. 5.1.3a and c) caused total medullary necrosis up to, but not beyond, the corticomedullary junction. The necrosed medulla had undergone abscission and the adjacent viable region had re-epithelialised (Fig. 5.1.3c).

The bladders were normal in rats given up to 100mg/kg, but a dose of 150mg/kg caused a similar loss of contractility to that described above (5.1.1) and minimal changes in the urothelial lining.

d) Discussion. This data suggests that weanling rats are less sensitive to BEA up to doses of 100mg/kg ip, but beyond this the extent of lesion is similar to adult rats. The high dose lesion appears more dramatic, however, because of the early abscission of the necrosed medulla (within 7 days of dosing) and the re-epithelialisation of the stump. Thus the actively growing kidney appears to have a greater reparative capacity than the adult organ.
Fig.5.1.3a. The response of weanling rat kidneys to BEA. Left top, control; right top, 50mg/kg ip; left lower 100mg/kg ip; and right lower 150mg/kb ip. H&E, x3.25

Fig.5.1.3b. The effect of 100mg/kg BEA given ip to a weanling rat, H&E, x100.
Fig. 5.1.3c. The abscission of a necrosed papilla in the kidney of a weanling rat given 150mg/kg BEA ip. H&E, x 80.
It is difficult to explain this reduced dose response. There are a number of morphological features which change with age, such as basement membrane (Ashworth et al., 1960) and the juxtaglomerular apparatus (Vesna & Spomenka, 1980) and several other functional changes have been reported. Organic ion transport is poorly developed and cannot be enhanced when other compounds (e.g. probenecid) are given to very young rats (Stopp et al., 1978) and both the quantity of urinary proteins (in male rats) and its molecular weight distribution varies at different ages (Alt et al., 1980).

The immature kidney is totally refractory to the toxic effects of cephalosporin antibiotics (Wold et al., 1977) and to dichromate and glycerol (Braunlich et al., 1979), but not to mercuric chloride. It has been argued (Wold et al., 1977 and Braunlich et al., 1979) that the absence of a toxic effect relates to the poorly developed organic ion transport mechanism, because increasing sensitivity to nephrotoxins and the development of the transport process are closely paralleled. There are, however, a variety of other functional changes that do also occur in the maturing kidney.

Recently, Kuo & Hook (1980) have reported that the activity of several renal drug metabolising enzymes undergo a complex series of changes as the animal matures. Thus any attempt to predict the age-related sequel to a nephrotoxic insult must depend on some knowledge of the processes involved in generating the proximate toxin (i.e. what enzyme system).

Young rats lack an effective urinary concentrating mechanism which may, in part, relate to the relative absence of interstitial MPS (Abrahams & Pirani, 1966), which has been confirmed chemically by Ber et al. (1969). It has been shown previously that diuresis ameliorates the BEA-induced RPN (Fuwa & Waugh, 1968), that Brattleboro rats are refractory to the papillotoxic effects of BEA until after near-normal urinary concentration has been restored with ADH treatment (Sabatini et al., 1981a) and that El
does not increase the extent of bilirubin related RPN in the Gunn rat (Axelsen, 1980). [The Gunn rat also suffers from a urinary concentrating defect, for reasons that are still not clear (Martinez-Maldonado et al., 1969).]

The possible contribution of urinary concentration mechanism to BEA-induced RPN is considered in more detail below (5.2; 5.3.8; 5.4 and 5.6.2) and in broader terms, as it relates to the analgesic associated lesion, in Chapter 7.

5.2 MORPHOLOGICAL AND HISTOCHEMICAL CHANGES IN THE MEDULLA AND CORTEX DURING THE DEVELOPMENT OF 2-BROMOETHANAMINE-INDUCED RENAL PAPILLARY NECROSIS.

The possible functions of the abundant PoG-GAG matrix surrounding the medullary interstitial cells has already been described (2.3.4.4), as have the changes in MPS staining in the kidneys of human analgesic abusers, (3.1.3.1) and the loss of MPS staining from the papilla of rats with aspirin-, fat-free diet- and BEA-induced RPN. (3.2). The location of this structurally specific macromolecule in the interstitium of medulla (i.e. that part of the papilla which undergoes necrosis) suggested:

A) That the loss of medullary PoG-GAG may, in itself, be an important factor in the molecular pathogenesis of RPN, and

B) The possibility of PoG-GAG as a marker for papillary necrosis, thereby circumvent the need for histopathology and painstaking sectioning.

a) Animals and treatment. The time-course of changes in renal histopathology and histochemistry was followed by administering BEA to 200-220g male rats (number in parenthesis) at levels of 50mg/kg (20), 100mg/kg (10) and 150mg/kg (10) via the ip route as described (4.1.1). Groups of 1 to 4 animals from each dose level, were killed (4.2.1) at 2, 4, 6, 8, 12, 24, 49, 73 and 123 hours after dosing. Single untreated controls were killed at 2, 6, 8, 12, 24, 73 and 123 hours, and fixed for the same
period as the rest of that time group. Only the kidney and bladder were taken from each rat for histology.

b) Histopathology. Tissue was fixed for 22-24 h, imbedded (4.2.1), and serial sections stained by H & E, colloidal iron/Neutral red, Alcian blue, Toluidine blue, Safronin O and Neutral red only (see 4.2.1).

c) Results. The histopathological changes in renal architecture followed the same trend irrespective of dose, but generally the most marked changes were caused by the higher dose levels.

The inner medulla showed a biphasic change. Between 2 and 6 h the collecting ducts were dilated and the tubular epithelium showed marked hydropic degeneration (Fig. 5.2.1a). Between 8 and 12 h cellular architecture was almost normal, but by 24 to 48 h necrosis had occurred. Low doses (50mg/kg, ip) caused destruction of the interstitial cells, the blood vessel walls and the epithelia of the thin limbs of the loop of Henle, but the collecting duct epithelia and the cuboid epithelia covering the medulla were intact (i.e. "intermediate necrosis", Burry, 1967). High doses (100-150 mg/kg) caused "total necrosis" where all of the anatomical elements in the medulla had been destroyed.

Regenerative changes were apparent as early as 24 h after a single dose of BEA administration, and included active cell division and cell migration to re-epithelialise the damaged collecting ducts, papillary surface and the junction between the necrosed and the viable tissue (Fig. 5.2.1b). Regenerative changes were extensive by 123 h.

Eosinophilic casts were present in the collecting ducts from 24 h and the epithelia covering the papilla and lining the pelvis and bladder showed hyperplastic change as early as 24 h after BEA. The papillary hyperplasia was most prominent over the areas of intermediate necrosis (Fig. 5.2.1c) or over the "viable" area adjacent to total necrosis (Fig. 5.2.1d). The hyperplasia of the pelvic epithelium was most marked adjacent to the
Fig. 5.2.1a. Hydropic degeneration in medulla 4h after dosing with 100mg/kg BEA ip. H&E,x 2600.

Fig. 5.2.1b. Regeneration taking place in damaged medulla 24h after 100mg/kg BEA ip. H&E,x 650.
Fig. 5.2.1c. Epithelial hyperplasia over necrosed area 24h after 50mg/kg BEA ip. H&E, x325

Fig. 5.2.1d. Hyperplasia of the papillary urothelia adjacent to viable medullary cells, 24h after 100mg/kg BEA ip. H&E, x325
Fig.5.2.1e. Comparison of MPS staining in the papilla of rat treated with 100mg/kg BEA ip after 4h (left) and control rat(right). Colloidal iron/Neutral red, x80

Fig.5.2.1f. Pronounced granular staining of MPS; left in papilla tip 12h after 50mg/kg BEA ip; right in control papilla. Colloidal iron/Neutral red, x 325
Fig. 5.2.1g. Areas of non-straining occurred around intestinal cells. Left, papilla tip 8h after 100mg/kg BEA ip; right, control papilla. Colloidal iron/Neutral red, x 1300.

Fig. 5.2.1h. Loss of MPS staining from necrosed area 24h after 100mg/kg BEA ip. Left H&E, right colloidal iron/Neutral red, x 130.
Fig. 5.2.11. Loss of MPS staining from necrosed area 24hr after 100mg/kg BEA ip. Left H&E, right colloidal iron/Neutral red, x 325.
totally necrosed papilla. The transitional epithelia of the bladder showed some variable hyperplasia by 73 to 123h.

The cortex was essentially normal up to 73 h but some slight tubular dilatation and increase in the space between the glomeruli and Bowman's capsule were apparent by 123 h.

The earliest histochemical changes in the PoG-GAG matrix were an increase in the intensity of staining (as assessed by colloidal iron, Safronin 0, Alcian blue and Toluidine blue) at 2 and 4 h after BEA, Fig. 5.2.1e compares control MPS staining with BEA treated. Colloidal iron staining provided the most easily visualisable changes; the subtle differences between the red and the orange in Safronin 0 metachromasia were difficult to photograph, as was Alcian blue (because much of the colour was dissolved out on ethanol dehydration) and Toluidine blue (because metachromasia is only pronounced in aqueous phase, but it is largely suppressed in dehydrated and mounted sections). Colloidal iron gave a pronounced granular appearance, particularly at the papillary tip, at 8 to 12 h (Fig. 5.2.1f) and the normal sharp line of demarcation between the PoG-GAG matrix and the epithelial cells was lost and became diffuse at the apex of the medulla. Areas of non-staining surrounded the interstitial cells (Fig. 5.2.1.g). Between 12 and 24 h the areas of intermediate necrosis coincided with areas of diffuse loss of granular colloidal iron staining and both colloidal iron staining and metachromasia were absent from those areas where necrosis was total (Fig. 5.2.1 h & i). The absence of staining from necrosed areas was maintained from 48 to 123 h. The adjacent non-necrosed areas of the medulla showed a narrow zone from which there were diffuse losses of staining, but beyond this the PoG-GAG matrix stained relatively normally.

Some of the cast material within the lumen of the collecting ducts stained intensely with colloidal iron.
d) Discussion. Murray et al., 1972 have previously reported the histopathological changes associated with the development of BEA-induced RPN in female Holtzman rats given 250mg/kg BEA iv (Table 3.7). The earliest changes reported by these authors were necrosis of the limbs of Henle and numerous eosinophilic droplets in the collecting ducts. There were also casts in the limb of Henle. The final lesion was similar to that found in this investigation, where the area of necrosis extended up to, but never beyond the corticomedullary junction.

There were marked differences between both the time course and the dose related effect reported by Murray et al., 1972 (250mg/kg iv within a lesion developing in 4-7 days) compared to that reported above (50mg-150mg/kg ip where the necrotic process was complete in 2 days). It seems unlikely that these differences arise from the different sexes of the rats used in the two studies, because there were no differences in the dose response relationship for male and female Wistar rats. It seems more likely that differences are strain related and that Holtzman rats are less sensitive to BEA than are Wistar rats. Bokelman et al., (1971) have reported that Manor Farm SPF rats were more sensitive to the nephrotoxic effects of a NSAID than were Sprague Dawley CD rats. The initial lesion which occurred spontaneously in the Manor Farm rats was a focal hyperplasia of the papillary epithelia. NSAID caused a dose-dependent increase in the severity of the lesion, multiple polypoid growth and focal necrosis. Similar changes were not seen in the CD rats, either spontaneously, or in response to the NSAID, but treatment did cause a low frequency of focal interstitial necrosis.

In this regard it is perhaps worthy of note that the normal 24-h urine output for the Holtzman rats used by Murray et al., 1972 was higher than that for Wistar rats (of similar weight) used for this investigation (12.5mL versus 8.6mL).
An alternative explanation might be that BEA is an unstable molecule in solution and may not have been prepared freshly and used immediately by Murray et al., (1972) as it was for these investigations.

The accumulation of tubular casts is a feature more prominent in the study of Murray et al., (1972) than observed here, although casts were also present. Indeed cast formation has been reported in several chemically induced models of RPN. These include El (Ham & Tange, 1969), aspirin (Molland, 1976 and 1978a,b), and the biphenyls (Hardy 1970a,b and 1974). Similar changes have also been reported to develop in fat-free diet related RPN (Molland 1978a).

The nature of these casts is uncertain, although Murray et al., 1972 reported strong PAS positive staining of kidney sections indicating the presence of carbohydrate. Recent data, outside of the scope of this dissertation, suggests that glycoprotein material is a major cast constituent (Chapter 7) but, presumably other macro-molecules may also be involved. The significance of cast formation to the pathogenesis of RPN is doubtful, but it may play an important role in the development of secondary cortical changes (see Chapter 7).

The significance of hyperplasia of the papillary urothelia is uncertain. Murray et al., 1972 failed to comment on it, although their photomicrographs show these changes. Similar hyperplastic changes of the papillary or pelvic urothelia (or of the epithelia at the junction between necrosed and non-necrosed medullary tissue) have been reported for El (Ham & Tange, 1969), aspirin (Molland, 1976 and 1978a,b), biphenyls (Hardy, 1970a,b and 1974) and fat-free diets (Molland, 1978a).

The fact that both acute and chronic chemical insult and dietary deficiency cause similar changes suggests that the hyperplastic response is to the developing lesion per se. It might be part of a reparative process which forms a reservoir from which cells can migrate along the
basement membrane to facilitate re-epithelialisation, or it may be due to the changes in osmotic or electrolyte environment. This is unlikely to be related to disruption of the concentrating mechanism alone because the urothelia is normal in the Brattleboro rat (McAuliffe, 1980) where urine osmolality is low. It is possible that the BEA-induced wasting of electrolytes such as $K^+$, $PO_4^{2-}$, $Ca^{2+}$ etc. (Sabatini, 1981b) might, in itself produce the hyperplasia, or failure to reabsorb other constituents of the glomerular filtrate might play a role.

Whatever the processes involved they warrant some research effort in the future in order to throw some additional light on transitional urothelial carcinomas often associated with analgesic abuse (3.1.3.5).

The large quantities of PoG-GAG ground substance in the medulla of control rats has been discussed previously (2.3.4.4), as has its possible role in normal renal function. The possible importance of this group of molecules as a valid marker for monitoring RPN non-invasively was suggested by the absence of MPS staining from the medulla of rats with BEA-induced RPN (Shimamura & Bonk, 1976), and both aspirin- and fat-free diet-induced RPN (Molland, 1978a,b). The data from human analgesic abusers are confusing because both more intense and the absence of staining has been reported (3.1.3.1).

The intense staining for papillary MPS shortly after BEA administration should be interpreted with caution. Although it is possible that the increased staining represents a net increase in the quantities of PoG-GAG ground substance, this is unlikely. The change is probably too marked to represent de novo synthesis as the response is maximal within 1 to 2 h, after which it plateaus. It is possible that BEA inhibits the normal metabolic degradation of these macromolecules, but the turnover of papillary GAG in the rat is typically measured in days rather than hours (Barry & Brownness, 1975).
The staining intensity of the MPS may, therefore, represent the availability of staining sites rather than absolute quantity (see 2.3.4.4). Thus an alternative explanation for the finding is that the medullary PoG-GAG undergoes a conformational change and/or cationic groups are displaced from the GAG polyanions in response to BEA treatment. Both effects would make more binding sites available for interaction with the stain.

There are a number of observations which give circumstantial support to this argument.

1) Initially great difficulty was experienced when attempts were made to cut frozen sections from the kidneys of rats treated with BEA. The tissue showed a marked cryoprotection and was of a rubbery constituency at -70°C (see 6.6.2).

2) BEA caused a marked diuresis shortly after administration (6.1).

3) BEA was found to cause a massive, but transient magnesium ammonium phosphate crystaluria (6.3.6). We have also observed a marked increase in birefringent particles in the nephron and in the tubules; it has not been possible to establish the chemical nature of these crystals although they were acid soluble.

If PoG-GAG conformation did change one might expect a sudden "dumping" of previously bound cationic solutes into solution. This could give rise to a diuretic response and to the precipitation of those products which exceeded their solubility product. This would also explain the cryoprotective effect.

The molecular events associated with the loss of staining are unclear, but the histology suggests a diffusive loss of MPS from the matrix, through the epithelia. The change in PoG-GAG conformation might be an initial stage in the later depolymerisation of these molecules and/or subsequent loss of anionic groups such as carboxyl or sulphates. These changes
closely paralleled the necrotic process with respect to their time course and were confined almost exclusively to those areas where necrosis was found to have occurred. In common with the observations of Shimamura & Bonk, 1976 no "recolonization" of the medulla by interstitial cells from adjacent (apparently undamaged areas) was detected and the necrosed medulla remained free of PoG-GAG, although the "ghost" of the necrosed medulla did strain progressively more strongly with Hale's colloidal iron up to 5 days, but not with the other MPS "selective" stains.

A relationship has been established by other workers between a failure to concentrate urine and the absence of medullary MPS material in Brattleboro rats (Sun, 1980 and McAuliffe, 1980). It seems possible that part of the marked diuresis that developed within the first hours after BEA treatment may be explained by perturbation of the PoG-GAG integrity (during the first hours) and subsequently the loss of these macromolecules. Evidence is presented below to support this hypothesis (6.6).

This broad concept can also be used to explain at least some of the secondary cortical effects that develop after BEA treatment and also the molecular changes associated with the analgesic related lesion in man (Chapter 7). It is obviously not possible to assess if these changes are primary, nor to probe the biochemical mechanism(s) involved without recourse to other techniques (see Chapter 7).

5.3 FACTORS AFFECTING THE DEVELOPMENT OF 2-BROMOETHANAMINE-INDUCED RENAL PAPILLARY NECROSIS

It has often proved possible to derive some insight in the molecular pathogenesis which underlies a toxic lesion by specifically perturbing a biological system before exposing it to the insult.

Only diuresis and anti-diuresis (Fuwa & Waugh, 1968) and reserpine pretreatment (Wyllie et al., 1972) have been reported previously to alter
the course of the BEA-induced lesion. The studies described below were undertaken in order to probe specific areas of the BEA-related pathogenesis.

5.3.1 The Effect of Reserpine Pretreatment on the Development of BEA-induced RPN.

Reserpine pretreatment has previously been reported to ameliorate BEA-induced RPN (Wyllie et al., 1972).

a) Animals and treatment. Reserpine for injection was prepared according to Martindale (1977). Reserpine (Sigma, Poole) 250mg, plus 2mL benzylacohol and 250mg anhydrous citric acid were warmed overnight at 37°C to dissolve; 10mL polysorbate 80 was added, the mixture diluted to 100mL, and passed through a 0.22 μm bacterial filter (Nucleopore, Sterilin) into sterile containers. The solution was stored in the dark at 4 to 6°C. Reserpine-free vehicle was similarly prepared.

Male rats, (220 to 240g) were divided into groups of 4 rats each, treated with a single sc dose of 0.80mg reserpine, followed 18h later by BEA (50 mg/kg ip); a second group of 4 rats were treated with a single 1.60 mg dose of reserpine, followed 18h later by 50 mg/kg BEA given ip; a group given vehicle only was given 50 mg/kg BEA ip, and the fourth group acted as controls and received a 1.6 mg dose of reserpine and no BEA.

Rats were killed after 5 days and the kidneys and bladder taken for histology (4.2.1).

b) Histopathology. Tissue was fixed for more than 24h, and processed for H & E (4.2.1)

c) Results. The rats treated with reserpine only had normal renal and bladder morphology. The BEA-induced lesion was essentially similar in rats pretreated with vehicle and both doses of reserpine and with rats which had received no previous treatment before a 50 mg/kg ip dose of BEA (5.1 and 5.2).
d) Discussion. Wyllie et al., (1972) investigated a group of drugs chosen by these workers to try and prevent or reduce the severity of BEA-induced RPN. The chemicals they used included heparin (to prevent fibrin thrombi), sulphinpyrazone (to prevent platelet aggregation), and several amines with vasopressor activity, such as chlorpromazine, phenoxybenzamine, 6-hydroxydopamine and reserpine. Reserpine was the only compound which ameliorated the BEA-induced lesion. Reserpine prevented the development of RPN in about 20% of the rats studied and markedly reduced the severity of the lesion in up to another 20% of the test animals. In addition reserpine delayed the onset (for 6 to 8 days) of the otherwise immediate diuresis, but once the urinary concentration mechanism was lost these changes continued for at least 20 days (Wyllie et al., 1972). This data was interpreted as suggesting that vasodilatation imparted to the medulla protection against a BEA insult, but it could not be concluded that microvascular occlusion was a predeterminant in the development of RPN, indeed, electron microscopic (Hill et al., 1972) and medullary plasma flow studies (Solez et al., 1974 and Vanholder et al., 1981) discount such conclusions; as does the medullary blood vessel filling investigations described below (5.5).

It is difficult to suggest why reserpine pretreatment in this study failed to confer an ameliorating effect on the BEA-induced lesion while producing a protective effect in the hands of Heptinstall's group. The marked differences between the time-course development of the lesion described by Murray et al., (1972) and these investigations have been discussed (5.2d). The failure to reproduce the papilloprotection described by Wyllie and co-workers may relate to the fact that only 4 animals were studied per group. Alternatively they may arise from strain differences either in the response to, or metabolism of BEA and/or reserpine (5.2d).
5.3.2 The Effect of Pretreatment with Inducers and Inhibitors of Mixed Functional Oxidase Activity and Thiol Protectors on BEA-induced RPN.

a) Animals and treatment. Groups of three rats each (230 to 250g) were treated as described below, at the appropriate time BEA (100 mg/kg ip) was given to two of the rats and the third left as "treatment" control. The rats were killed 4 days later and kidneys and bladder taken for histology.

The groups were treated as follows:-

i) phenobarbitone sodium (PB), 50mg/kg ip, 12-hourly for 48h before and 48h after BEA,

ii) 3-methylcholanthrene (3-MC) 40mg/kg ip in corn oil given 48 and 24h before BEA dosing,

iii) 2-diethylaminoethyl-2,2-diphenylvalerate (SKF-525A)75 mg/kg given ip (SKF-525A was kindly donated by Smith, Kline & French, Welwyn Garden City),

(iv) cobaltous chloride, 60 mg/kg sc 24h before, and simultaneously with BEA dosing,

vi) Acetylcysteine 200 mg/kg po in suspension was administered 24h before BEA and 24-hourly for 72h after dosing.

vii) methionine 200 mg/kg po in suspension was administered 24h before BEA and 24 hourly for 72h after dosing.

vii) controls for BEA response were given saline injections only.

b) Histopathology. Tissue was fixed for more than 24h, and processed for H & E (4.2.1).

c) Results. The control rats given BEA had an RPN of similar magnitude to that already described for a dose of 100 mg/kg BEA alone (5.1 and 5.2). There were no obvious differences between controls and rats pretreated with PB, 3-MC, cobaltous chloride, acetylcysteine or methionine. However, the BEA-induced lesion was more marked in SKF-525A pretreated rats. The area of necrosis in these rats extended towards the corticomedullary
Fig. 5.3.2a. Enhanced papillary necrosis in a rat treating with SKF-525A before BEA-dosing, 100mg/kg, ip. H&E, x 80

Fig. 5.3.2b. Prominent eosinophile casts in the collecting duct in a rat treated with SKF-525A before BEA dosing, 100mg/kg ip, H&E, x 130.
Fig. 5.3.2c. Marked tubular dilatation in the cortex of a rat treated with SKF-525A before BEA dosing, 100mg/kg ip, H&E, × 130.
junction (Fig. 5.3.2a) tubular casts were especially prominent (Fig. 5.3.2b) and the cortical tubules showed extensive tubular dilatation (Fig. 5.3.2c).

Bladder changes were similar to those described above (5.1 and 5.2).

d) Discussion. The failure of mixed function oxidase inducers to alter the BEA-lesion suggests that the liver plays little direct role in either metabolising BEA to its "proximate" nephrotoxin or in converting any papillotoxic compounds to inactive metabolites. This interpretation may, however, be a over-simplification because of the major differences in the response of hepatic and extrahepatic tissue to different inducing agents, e.g. 3-MC affects many organs but PB only induces mixed functional oxidase in the liver and intestinal tract. This problem is complicated further by the different responses to chemicals in the medullary and cortical zones of the kidney and their different response to pretreatment (2.2.4.5). Previously published data on the changes in furan target organ selective toxicity following pretreatment are most complex and must remain for the moment, descriptive rather than mechanistic (Boyd & Dutcher, 1981).

The marked exacerbation of the BEA-induced necrosis following SKF 525A treatment might be interpreted to result from the failure to deactivate a papillotoxic compound. The failure of CoCl₂ to cause a similar effect, together with the failure of two different inducers of xenobiotic metabolism to ameliorate the lesion make such a conclusion equivocal. Whereas SKF-525A may be highly specific in inhibiting xenobiotic metabolism in vitro, this compound is known to cause diverse pharmacological effects (Anders, 1971), most importantly in the kidney. In the kidney SKF-525A causes a loss of blood flow autoregulation, a natriuretic effect (see Ander, 1971) and, perhaps most significantly, an anti-diuretic response (Arima & Kuriaki, 1959). There is also data from Fujimoto & Hakim (1970) which suggests that SKF-525A affected renal
tubular transport mechanisms. This was administered directly to the kidney, using the avian Sperber preparation, in which there was no hepatic metabolism to consider.

Similarly the failure of thiol protectors to alter the BEA-induced lesion could be interpreted as indicating that BEA is not converted to a chemically reactive electrophile which depletes glutathione stores. The uncertainties associated with extrapolating data from hepatic systems to the kidney have already been outlined (2.2.4.5), and above. The lack of protection from thiol agents is of particular interest because BEA can react spontaneously (via the formation of an aziridine ring) with a variety of sulphhydryl containing compounds (Jones, 1973 and Jones & Capps, 1977), under semi-physiological conditions (pH 7.4 phosphate buffer at 37°C).

Thus one might expect that if cyclization of BEA to EI was the rate-limiting step in the formation of the "proximate" papillotoxin in an excess of sulphhydryl containing compound should (assuming it was present in the "correct" metabolic pool), ameliorate the lesion. A number of straight chain brominated compounds (James et al., 1981) such as 1,3-dibromopropane deplete hepatic glutathione and are excreted as mercapturic acid conjugates. The two compounds are not, however, directly comparable because the bifunctionality of the bromine substitution confers totally different physicochemical, metabolic handling and most probably toxicological properties.

5.3.3 The Effects of Pretreatment with Anti-oxidants and Free Radical Scavengers on BEA-induced RPB.

a) Animals and treatment. Groups of 5 rats each (220 to 245g were treated as described below, at the appropriate time BEA was given to 2 rats at 50 mg/kg ip and 2 rats at 100 mg/kg ip. The fifth rat was was left as "treatment" control. The rats were killed 4 days later and the kidney and bladder taken for histopathology. (4.2.1).
i) Vitamin E (α-tocopherol acetate; Sigma, Poole) 100 mg/kg in olive oil (2mL/kg), ip, given 24h before BEA treatment,

ii) Zinc sulphate, (BDH, Poole), 5 mg/kg sc daily starting 24h before BEA dosing. Olive oil (2 mL/kg) was given ip to these animals

iv) Butylated hydroxyanisole (BHA), (Sigma, Poole) 300 mg/kg in olive oil (2 mL/kg) ip given 24h and 1h before BEA dosing.

v) Butylated hydroxytoluene (BHT), (Sigma, Poole) 300mg/kg in olive oil (2mL/kg ip given 24h and 1h before BEA dosing.

vi) Olive oil vehicle only (control group for BEA response) starting 24h before BEA and given daily.

b) Histopathology. Tissue was fixed for more than 48h, and tissue processed for H & E. (4.2.1).

c) Results. Rats treated with 50 mg/kg or 100 mg/kg of BEA alone showed necrotic lesions similar to those already described. There were no obvious differences in response between these rats and those pretreated with zinc sulphate.

Animals pretreated with BHA showed an altered response in one of the two animals treated with 100 mg/kg BEA. The necrosis was not located in the apex of the papilla, but appeared to occur deeper in the medulla. There were no changes in response in other animals.

BHT totally protected one animal treated with 50 mg/kg BEA, but none of the others in this group.

Vitamin E pretreatment altered the response in one of the two rats treated with 50 mg/kg BEA, but had no obvious effect in the other animal or on the rats given 100 mg/kg BEA.

d) Discussion. The generation of free radicals (Demopoulos, 1973) and lipid peroxidation (Di Luzio, 1973) play an essential underlying role in the development of a number of naturally occurring and chemically-induced pathophysiological changes. The high concentrations of polyunsaturated
radical reaction of this sort and might, therefore, account for the site specificity of RPN.

Zinc, among its many roles, is a most important free radical scavenger. The lack of a Zn-related protective effect against BEA-induced papillary necrosis suggests that this lesion may be unrelated to free radical formation. However, the detailed kinetics of zinc have not been well described for the kidney. Thus although the dose of zinc administered does increase renal zinc concentrations (Bonner, 1980), there appears to be no data relating to how this free radical scavenger is distributed along the nephron, nor if it is "free" or metallothionein bound.

It is difficult to decide if the variable, but altered responses which followed BHT, BHA and vitamin E treatment were real or if they should be considered "spurious" and rejected. More recent experience and that described in sections 5.3.4 to 5.3.7 have established that pretreatment can alter the response to BEA. Further we have found that different rats may show a different threshold to BEA, for example in one recent study 2 rats treated with 35 mg/kg BEA developed a focal RPN while a third rat was normal. Thus although the variability in response to anti-oxidant treatment is great, it may be a real effect.

BHA and BHT are both widely used as anti-oxidants in food, and have therefore been extensively evaluated for safety. The full extent of the biological effects of these compounds is complex and not fully documented. Both BHA and BHT prevent lipid peroxidation in vitro, however, both compounds are not equipotent and both perturbate drug metabolic function differently (Vainio, 1974). Further, BHT is metabolised to an intermediate which binds to thiol containing molecules (Nagagawa & Hiraga, 1981). Both BHA and BHT are excreted via the renal route mostly as the glucuronide, less as the sulphate and up to 6% as the free phenol for both
BHA and BHT, although there are marked differences in their individual metabolism (Hathway, 1966). Both compounds may also modify renal function. BHT caused a reduced urine osmolality, but BHA did not when both were given at dose level of 500 mg/kg/day po (Ford et al., 1980a). Both compounds reduced electrolyte excretion, but this effect may have been extra renal due to the anorexic effects produced by treatment. Similarly, BHT depressed organic acid transport more markedly than BHA (Ford et al., 1980b), but these results were equivocal (see Ford et al., 1980a,b for references and discussion) and may be related to other variables, including more subtle effects such as the amount and type of dietary lipids and concomitant intake of other anti-oxidants, e.g. vitamin E (Rowe & Wills, 1976).

Despite the massive body of information that has been published on vitamin E there is very little data on its interaction with the kidney per se. Moore & Sharman (1978) have reported that avitaminosis E caused degenerative changes in the cortex which, in addition, underwent very rapid post mortem autolysis. This suggests an important dietary role for this vitamin in stabilizing renal cellular membranes.

In the present study all of the animals pretreatment with vitamin E were not protected against BEA-induced necrosis of the medullary interstitium, but the covering epithelium was left intact. This may be an important factor for renal recovery and re-epithelialization of the collecting ducts.

On the other hand it may be totally inappropriate to use this data as a basis for developing means of intervening in the course of a chronically induced lesion (7.3.5).

5.3.4 The Effects of Pretreatment with Non-steroidal Anti-inflammatory, and Analgesic- and NSAID-like Compounds on BEA-induced RPN.

Analgesics and NSAID play an important role in the development of RPN.
There was no published data on the interaction between these compounds and BEA. This study was undertaken to establish what type, if any, interaction occurred.

a) Animals and treatment. Groups of 5 rats each (220 to 245g) were treated as described below, at the appropriate time BEA was given to 2 rats at 25 mg/kg ip and 2 rats at 50 mg/kg ip. The fifth rat was left as "treatment" control. The rats were killed 4 days later and the kidney and bladder taken for histopathology (4.2.1).

All the chemicals were individually finely ground in a pestle and mortar and suspended in water containing 0.67% (v/v) Tween 80 (Sigma, Poole) as dispersant. All of the compounds (supplied by Sigma, Poole) were constituted to be given po in 1 mL of vehicle per rat. The compounds were:-

i) Aspirin, 1 mmol/kg, po,
ii) Dexamethasone, 25 mg/kg, po,
iii) Paracetamol, 1 mmol/Kg, po,
iv) Phenacetin, 1 mmol/kg, po,
v) N-Phenylanthranilic acid, 1 mmol/kg, po,
iv) Vehicle only (control group for BEA response only).

The animals were dosed with test compounds 24h and 6h before the BEA was administered. The rats were killed after 4 days (4.2.1).

b) Histopathology. The kidneys and bladder were taken for histology and fixed for more than 24h, and processed for H & E (4.2.1).

c) Results. The rats pretreated with vehicle only showed a response similar to that already described, there were no lesions at 25 mg/kg BEA and 50 mg/kg caused an apex limited lesion, where the covering epithelia was generally left intact.

Dexamethasone pretreatment caused no obvious changes from this.

All the analgesic type compounds caused a markedly increased
sensitivity to BEA, in which there were focal necroses at 25 mg/kg and variable but extensive medullary ablation following 50 mg/kg BEA.

The changes in the bladder were apparent after 25 mg/kg and 50 mg/kg BEA in animals with RPN. Changes were similar to those described (5.1 and 5.2).

d) Discussion The marked synergistic interaction between BEA and analgesic compounds and NSAID analogues (but not a steroidal anti-inflammatory) is an interesting finding. Recently, Wirdnam et al., (1981) repeated this investigation and found that the system is most complex. Pretreatment (24h and 6h before BEA) with low doses of aspirin (0.1 and 0.5 mmol/kg po) greatly exacerbate the RPN caused by a threshold dose of BEA (35 mg/kg ip). High doses of aspirin (2.0 and 5.0 mmol/kg po), however, totally prevented papillary necrosis, although there were changes in the renal interstitium. By contrast, pre-treatment with all dose levels of paracetamol (0.1, 0.5, 1.0, 2.0 and 5.0 mmol/kg po give 24h and 6h before a threshold dose of BEA) exacerbated the RPN.

These results are difficult to interpret without fuller investigations. It is, however, tempting to speculate that the failure to induce RPN using very high doses of aspirin (Rosner, 1976) contrasted with the successful use of low doses (Molland 1976, 1978a,b), may be explained by the high dose protective effect seen by us. However, our findings would not explain the fact that paracetamol appears to be only a weak papillotoxic compound on its own (Molland, 1978a).

The response could be explained by cyclo-oxygenase inhibition before BEA treatment having prevented the synthesis of PG's which normally antagonise the action of ADH (Beck & Dunn, 1981). This might be expected to produce an anti-diuretic state in the kidney which would exacerbate the BEA-induced lesion (see 5.4). The protective effect of high dose aspirin tends, however, to discount this idea. If the lesion is related to the co-
oxygenation of BEA to a reactive metabolite it is possible that low "doses" of irreversible co-oxygenase inhibitors (such as aspirin) might induce enzymic activity and increase the amount of biologically reactive intermediates, but that high doses would totally inhibit the conversion of BEA to its "proximate" papillotoxin. Paracetamol, a relatively poor and reversible inhibitor of this enzyme system might only promote activation and exacerbate the lesion. Whatever the mechanism it demonstrates that complex and subtle interactive effects might alter the development of RPN.

Axelsen (1980) has reported that aspirin treatment greatly increases the frequency and severity of RPN proportionally with dose in the Gunn rat, but paracetamol and phenacetin caused markedly less response. Further, he found no differences between the response of Gunn rats and Sprague-Dawley rats to increasing doses of EI. Axelsen (1980) suggested the potential value of this model for studying the pathogenesis of RPN and for comparing the risk associated with various drugs. He ignored, however, the spontaneous occurrence of the lesion with progressive age as more and more bilirubin-like material is deposited in the papilla. Thus any compounds which displace plasma bound bilirubin (aspirin) would be likely to greatly exacerbate the lesion, while those that are only poorly albumin bound (both paracetamol and phenacetin) might be expected to cause minor changes. Furthermore, the assertion that the Gunn rat is no more sensitive to EI than other species may not be true, because there is a urinary concentration defect in these animals (Martinez-Maldonado et al., 1969) which should afford protection against EI.

Although this approach may have some use as a screening test it must be regarded with circumspection until such time that the effect of other compounds (e.g. sulphonamides and warfarin), which are known to displace bilirubin, have been investigated for their papillotoxic (?) effects.

The interaction between BEA and several likely papillotoxins may
provide a useful means of undertaking short term screening of chemicals which cause, and factors that predispose to, RPN. This is particularly important because previous safety evaluation of the papillotoxic potential of analgesic and NSAID compounds has been complicated by the fact that unknown variables make the induction of RPN most irreproducible (3.2.3), Rosner, 1976. There are differences in the sensitivity of different species (Bokelman et al., 1971), not all animals in a group develop the lesion, even if the likelihood is enhanced by dehydration, long periods of treatment may be required (3.2.3.2) and it is easy to miss focal RPN when kidneys are examined histopathologically.

It is normally only analgesics - NSAID which are scrutinised for this toxicological effect. Thus the true spectrum of chemicals which might cause this lesion remains unchartered and there is no means by which compounds can be assessed and compared.

5.3.5 The Effect of Ethan-1-ol-2-amine (EA Hydrobromide Pretreatment on the BEA-induced RPN Dose Response Curve.

The structural similarities between BEA and EA (a natural component of phospholipids) suggests that BEA might mediate its papillotoxic effects by inhibiting normal interstitial lipid metabolism. Accordingly the papilloprotective effect of EA was assessed.

a) Animals and treatment. The effects of EA on renal morphology was assessed by injecting male Wistar rats (200-225g) with a single ip dose of EA HBr dissolved in saline at concentrations of 25, 50, 100, 200, 500 and 1000 mg/kg. Only one rat was used for each dose level.

Animals were killed after 5 days and the bladder and kidneys of each taken for fixing (4.2.1). Other organs were examined macroscopically.

After the effect of EA, per se, on the kidney had been established (see below) a group of 16 rats (male Wistar, 210-230g) were studied. Groups of 4 rats each were dosed with saline alone, with 10 mg/kg, with 100 mg/kg, or
with 500 mg/kg EA via the ip route. After 35 min the groups were divided equally for BEA treatment; 2 from each group were dosed with 50 mg/kg ip, the other 2 with 150 mg/kg ip.

The animals were killed 5 days later (4.2.1), and both kidneys and bladder fixed from each animal. Other organs were assessed macroscopically.

b) Histopathology The tissues were fixed for 24h and processed for H & E (4.2.1).

c) Results. All organs from the EA treated animals, over the whole dose range, were normal macroscopically. Similarly, the renal morphology was normal over the entire dose range of EA, from 25 to 1000 mg/kg when assessed microscopically.

The animals pretreated with the saline vehicle only showed papillary changes commensurate with the dose of BEA they received, i.e. largely interstitial necrosis at 50 mg/kg and necrosis of most of the medullary elements at 150 mg/kg, as described above.

Treatment with 10 and 100 mg/kg EA produced no obvious changes in medullary response to BEA. Pretreatment with 500 mg/kg EA ameliorated the marked tubular necrosis seen in rats given 50 and 150 mg/kg BEA alone, but there was still interstitial necrosis. The tubule and collecting duct epithelia appeared relatively normal, and the absence of mitotic figures suggesting that this was not a re-epithelialising population.

d) Discussion. The renal medullary interstitial cells are characterised by their high lipid contents, much of which is present as phosphatidylcholine (2.3.4.2). The specificity of BEA for the medulla, particularly the renal interstitial cells suggests that this cell type may be inherently and peculiarly sensitive to certain types of toxic insult.

Phosphatidylethanolamine may be methylated to form phosphatidylcholine in several tissues including liver and brain (Morganstern & Abdel-Latif,
1974 and Tinoco et al., 1979), but there appear to be no data on either the kidney as a whole or the medullary interstitial cells. Choline deficiency causes a renal necrosis which, according to the literature, is confined to proximal convoluted tubule necrosis (Keith & Tryphonas 1978). The cortical location of this lesion suggests that choline although a major constituent of a medullary phospholipid may not be the "direct" precursor of phosphatidylcholine in the interstitial cells.

This study was undertaken to assess if BEA might be a competitive inhibitor of any EA incorporation into medullary renal metabolism. The data from rats pre-treated with 10 or 100 mg/kg EA give an inconclusive picture, although there was an impression that a slight protective effect may have been produced. Rats treated with the highest dose of EA (500 mg/kg) did, however, have a protection against both dose levels of BEA. The large quantity of EA required to produce an amelioration may refute the hypothesis, but equally it may reflect a medullary lipid-phospholipid pool that is only entered via specific intermediates, for example as phosphatidylethanolamine. It is also possible that the highest dose of EA either alters some other aspect of renal function or alters BEA metabolism. Obviously future critical investigations will have to delineate the interstitial phospholipid—fatty acid pools and their metabolic turnover, preferably using an in vivo system where extra-renal metabolic effects are minimised. Alternatively pure cultured renal interstitial cells may offer fundamental data upon which in vivo studies can be based.

The possible importance of papilloprotective substances is described later (7.1.).

5.3.7 The Effect of Experimentally-induced Diabetes Mellitus on the BEA-induced RPN Dose Response Curve.

Diabetic patients may develop RPN as one of the complications that are part of diabetic nephropathy (3.1). The observation that analgesic
and NSAID pretreatment exacerbated the BEA-induced RPN (5.3.4) suggested that the diabetic kidney may be more sensitive to papillotoxic insults. This was studied in animals with experimentally induced diabetes.

a) Animals and treatment. Diabetes was induced in 6 male Wistar rats (120-130g) by injecting 50 mg/kg of streptozotocin (Upjohn, Kalamazoo) iv. The streptozotocin was dissolved immediately before use in 0.1 mmol/L (pH 4.5) citrate buffer. Dr. Peter Evans administered the streptozotocin by tail vein injection.

Rats were left for 72h, then two rats were injected with saline and single rats with 10, 25, 50 and 100 mg/kg BEA ip (4.1). The group was killed 72h later and their kidneys and bladder taken for histology.

b) Histopathology. Tissue was fixed for 24h and processed for H & E (4.2.1).

c) Results. There were no renal abnormalities in the streptozotocin treated rats nor in the diabetic animals treated with BEA and the morphology of all the bladders was also normal.

d) Discussion. Experimentally induced diabetes most often uses streptozotocin because it is target "specific" for pancreatic islet \( \beta \)-cells, and it is less toxic than alloxan. The clinical use of streptozotocin has, however, been limited by its nephrotoxicity, a finding confirmed in experimental animals (Levine et al., 1980) but only using large doses (greater than 100 mg/kg). The dose of streptozotocin used in this study was adequate to cause a diabetic state (blood glucose level 200-300 mg\%, normal value 80 mg \%, Evans 1982), but would not cause nephrotoxicity. It would, however, cause an increased glomerular filtration rate (Carney et al., 1979), and may perturbate both blood pressure and the renin system (Kohler et al., 1980). While some of these changes are a consequence of the diabetic state others are assumed to be caused by a direct nephrogenic effect.
It is difficult to explain why an experimentally induced diabetes should protect against the papillotoxic effect of BEA. This protective effect might relate to the ameliorating effect of the polyuria induced by a diabetic state (see 5.4). Recently, rats with streptozotocin-induced diabetes have been reported to be protected from the cortex directed nephrotoxic effects of a number of chemicals (Vaamonde et al., 1981). Although there are circumstances where renal cells remain refractory to a second challenge after a primary insult (Magos, 1982), it is difficult to accept this as an explanation for these findings because streptozotocin causes damage to the proximal tubules, but not the medulla (Levine et al., 1980).

5.3.8 The Effects of Partial Renal Papillectomy on the Development of BEA-induced Renal Papillary Necrosis.

Previous work by Hardy (1970a,b, 1974) showed that surgical abscission of the apex of the papillae prevented the development of secondary cortical cystic dilatation in rats treated with biphenyls such as N-phenylanthanilic acid and diphenylamine. The biphenyl-induced lesions are apparently confined to the papillary tip irrespective of dose, whereas the BEA-induced lesion, as reported earlier in this thesis, shows a dose response relationship from a focal apex limited lesion to total medullary ablation depending on dose (5.1), route of administration (5.1) and pretreatment (5.3).

5.3.8.1 The effect of partial renal papillectomy on renal morphology. The changes in renal morphology following partial papillectomy were assessed at various time intervals after surgical manipulation.

a) Animals and treatment. A group of 14 male Wistar rats (55-65g) were subjected to unilateral partial papillectomy (4.1.3.3). Two rats failed to recover from anaesthetic and the kidneys were used as "zero day" papillectomy. Pairs of animals were randomly selected for killing at 2, 8, 15, 23, 26 and 45 days.
Post mortem tissue was taken within 5 min of death. Live animals were killed as described (4.2.1) and the kidneys and bladder taken for histology. One pole was cut off the partially papillectomized kidney to identify it. Tissue was fixed for 26 h and processed for H & E and MPS staining (4.2.1 & 4.2.2).

c) Results. The kidneys taken from rats at day zero showed a clean abscission line where the papilla tip had been removed. Within 2 days there were signs of re-epithelialisation, (Fig. 5.3.8a) which was complete by 8 days (Fig. 5.3.8b) and remained stable thereafter. Most of the animals suffered from a minor, but persisting haematuria and there was evidence of bleeding from the truncated papilla in all but one of the rats studied at 45 days. Erythrocytes formed small casts in the ureter (Fig. 5.3.8c) or in the medulla stump (Fig. 5.3.8b).

The rest of the papillectomized kidney and the contralateral kidney were normal. There was no MPS staining immediately behind the line of abscission and there was a general impression that MPS staining was also reduced in the partially papillectomized medulla (Fig. 5.3.8d) compared to the contralateral kidney. (Fig. 5.3.8e).

d) Discussion The major difference between these results and those reported by Hardy (1970a,b) is the protracted haematuria that followed the surgical abscission of the papilla in most of the animals. All the blood appeared to be from the papilla tip, and the pelvis contained epithelia-covered blood clots. Similar protracted haematuria was reported by Molland, 1978a. Recently, (Thurston, H., Personal communication) has suggested that this relates to the low levels of vitamin K in most commercial rat diets and that haematuria can be prevented by administration of adequate doses of phytomenadione pre- and post-surgically.
Fig. 5.3.8a. Partially papillectomized medulla stump 2 days after resection, H&E, x 130.

Fig. 5.3.8b. Partially papillectomized medulla stump 8 days after resection, H&E, x 160.
Fig. 5.3.8c. Formation of blood casts in the ureter adjacent to resected medulla stump after 26 days, H&E, x 130.

Fig. 5.3.8d. Distribution of MPS in medulla stump 8 days after partial papillectomy. Colloidal iron/Neutral red, x 130.
5.3.8.2. The effect of partial renal papillectomy on BEA-induced papillary necrosis and secondary cortical changes.

a) Animals and treatment. A group of 8 male Wistar rats (50-60g) were subjected to unilateral partial papillectomy (4.1.3.3) and left to recover for 21 days. Rats were randomly divided into two equal groups, one of which was treated with 50mg/kg and the other group given 150mg/kg. Two rats from each group were killed after 7 days and the remaining two from each group at 30 days.

b) Histology. Rats were killed as described (4.2.1) the pole was cut off the papillectomized kidney (for identification purposes) and the tissue fixed for 25h and processed for H & E (4.2.1).

c) Results. The results were variable within dose and time groups, but the partially papillectomized kidney was always less affected by BEA treatment than its contralateral kidney. The most striking example of the protective effects of partial papillectomy was seen in rats treated with 50mg/kg BEA after 7 days (Fig. 5.3.8f) and 150 mg/kg BEA after 30 days (Fig. 5.3.8 g). Here small fragments of ghost papilla were all that remained in the pelvis of the cystic contralateral kidney, whereas there was some necrosis at the medulla stump (in the partially papillectomized kidney) and the cortex appeared normal.

Bladder calculi were found in one of the rats 30 days after treatment with 150mg/kg of BEA. Figure 5.3.8h shows the gross changes that had taken place in the bladder. The inorganic chemical composition of these calculi are discussed below (see 6.3.6) together with their possible significance.

d) Discussion. Findings from the partial papillectomy model indicate that there may be a fine distinction between the biphenyls (which only necrosed the surgically ablated region), BEA where it afforded protection and aspirin (Molland, 1978a) and EI (Hardy, T.L., personal communication) where the surgical resection had no effect on
Fig. 5.3.8e. Distribution of MPS in normal kidney papilla 8 days after resection of the contralateral papilla shown in Fig. 5.3.8d. Colloidal iron/Neutral red, x 130.

Fig. 5.3.8f. Comparison of the protective effects of partial papillectomy in response to BEA 50mg/kg ip after 7 days. Left partially papillectomized kidney, right contralateral control. H&E, x 3.25.
Fig.5.3.8g. Comparison of the protective effects of partial papillectomy in response to BEA 150mg/kg ip after 30 days. Left partially papillectomized kidney, right contralateral control. H&E, x 3.25

Fig.5.3.8h. Bladder of a partially papillectomized rat which contained calculi 30 days after treatment with 150mg/kg BEA ip. H&E, x 65.
related to strain or sex differences, or the persistence of haemorrhaging in both this study and that of Mollands, or to the fact that Hardy used a dose of El that would cause total ablation of the medulla.

The fact that partial papillectomy can confer protection against RPN caused by BEA raises some interesting possibilities, the understanding of which may help explain the molecular pathogenesis. Hardy (1970b) showed that uninephric partially papillectomized rats had the same urine concentration capacity as uninephric controls. This suggests that the papilla tip may not be an essential part of the concentrating apparatus. Indeed rats with N-phenylanthranilic acid-induced RPN (a papilla, tip limited lesion) regain most of their concentrating function (Hardy, 1970a).

This supports the idea that partial papillectomy does not, per se, cause a concentrating defect which would, in itself, ameliorate the BEA-induced lesion. The reduction of MPS staining in the partially papillectomized kidney may, however, relate to an altered concentrating capacity within the medulla, that is compensated for elsewhere in the kidney (see 7.1).

Alternatively, the interstitial cell at the papilla tip may have a specialised function that makes them more susceptible to insult; from which a progressing pathobiological change is generated to adjacent interstitial cells. The interstitial cells in the papilla apex are morphologically different (Bohman, 1980), in that they are round and lack the many stellate processes found on other medullary cells.

5.4 THE EFFECTS OF DEHYDRATION ON THE RESPONSE OF THE RENAL PAPILLA TO 2-BROMOETHANAMINE.

Dehydration has often been cited as an exacerbating factor in the
development of renal papillary necrosis in both man (3.1.2) and in the experimentally induced lesion (Table 3.5).

The studies described below were undertaken in the Department of Biological Sciences, University of Natal, Durban, South Africa. Facilities were kindly made available by Professor Pat Berjak.

a) Animals and treatment. Two groups of 9 male Wistar rats (University of Natal stock) were housed in open bottomed cages (over soft wood shavings) in a natural light cycle for four days. The rats were maintained on Epol rat cubes (Epol Oil and Cake Mill, Durban) and tap water was available ad libitum.

The water was withdrawn from one group (the dehydrated group) for 24h before BEA dosing. BEA was administered at the following levels (number of rats in parenthesis), 0 (1), 12.5 (1), 25 (2), 50 (2), 100 (2) and 150 (1) mg/kg ip to both groups and water was returned to the 24h dehydrated group.

The dehydrated rat treated with 150mg/kg BEA died within the first 20h (no tissue was taken for histology because of autolysis) and one of the two dehydrated rats (given 100 mg/kg BEA) died about 50h after dosing. Both kidneys and the bladder were removed within 2h of death for fixing.

The remaining rats were killed 72h after BEA dosing (4.2.1) and the kidneys and bladder from each fixed in formal-saline for 24h.

b) Histopathology. Tissue was wax imbedded and sections processed for H & E (4.2.1).

c) Results. The kidneys from controls and 12.5mg/kg BEA treated rats were microscopically normal. BEA doses of 25mg/kg and more caused a dose response RPN similar to that described above (5.1). There were, however, differences between the two groups. The response was far greater in dehydrated rats, where necrosis affected more of the medulla and secondary cortical cystic tubular dilatations were far more marked.
d) **Discussion.** The lower threshold dose at which BEA caused a necrotic lesion in these Natal rats, compared to those of University of Surrey stock, could be explained on the basis of genetic, dietary and/or environmental differences.

Fuwa & Waugh (1968) showed that diuresis prevented the development of the BEA-induced lesion and anti-diuretic hormone exacerbated it. Recently, Sabatini et al., (1981a) reported that after the administration of ADH to Brattleboro rats they became sensitive to the papillotoxic effects of BEA, but not until the urine concentrating ability had been restored to near normal.

The enhanced sensitivity to BEA in an anti-diuretic state (as opposed to the protective effect of a diuresis) has always been explained on the basis of augmented counter current concentration increasing the medullary concentrations of the papillotoxin. Perhaps, however, a distinction should be made between the increased medullary concentrations of BEA (because of enhanced counter current concentration) and an increased sensitivity of medullary cells to BEA when they are biochemically programmed for anti-diuresis. The medullary concentrations of BEA will normally be reduced because BEA causes a diuresis (see 6.1.1). Shortly after its administration and well before the earliest light microscopic changes are apparent. Only electron microscopy will provide an unequivocal answer, but it suggests that BEA may alter a cellular function before it disrupts the architecture (i.e. loops of Henle etc.) of counter current concentration. This suggests a "biphasic" diuresis which is promoted in the early stages by changes in cellular function and later by loss of the loops of Henle and their associated structures. The disruption of these cellular processes by BEA may be a key factor in the pathogenesis of the lesion. It also seems unlikely that maximal counter current concentration is a prime important
factor. Because we have recently found that the threshold dose of BEA in the Gerbil is 200mg/kg. This is surprising in a species which concentrate urine to a maximal 5500 mOsm/l (Black, 1965), a value twice that of the rat. Thus if urine concentrating capacity was the only determinant of sensitivity to BEA the Gerbil should be more sensitive than the rat. The morphology of the Gerbil kidney does, however, differ from that of the rat, in that the medulla is more (and the cortex less) prominent, and a greater proportion of long loops of Henle in the Gerbil contribute to a more efficient kidney for counter current concentration. Thus the greater stress to the cellular processes of anti-diuresis in the rat may increase the sensitivity of the medulla to BEA. Alternatively there may be other species related factors which account for these differences.

5.5. THE EFFECT OF 2-BROMOETHANAMINE ON THE FILLING OF THE PAPILLARY MICROVASCULAR SYSTEM.

Several workers have suggested that compromised medullary vascular filling may cause anoxia and ischaemic injury, which subsequently precipitates renal papillary necrosis (3.2.2.2). The evidence in favour of such a mechanism is conflicting (3.2.5.2). The degenerative vascular changes associated with aspirin-induced papillary necrosis (Molland, 1976, 1978a), and with the lesion reported in human analgesic abusers (Burry et al., 1977; Burry 1978 and Mihatsch et al., 1980 a, b, c) is thought by many, to substantiate a cause-and-effect relationship. It is, however, impossible to be certain if these microvascular changes precede, parallel or follow a chronically induced necrosis in the medulla.

Published data from an acutely induced renal papillary necrosis is conflicting. Consensus favours the view that the process of necrosis follows or at least parallels microvascular changes (Ham & Tange, 1969;
Murray et al., 1972; Hill et al., 1972; Wyllie et al., 1972; Solez et al., 1974 and Vanholder et al., 1981), but several workers (Davies, 1970; Cuttino et al., 1981) disagree. These studies each suffer from one or more experimental design flaws. Firstly, Indian ink has commonly been used as a convenient source of colloidal carbon in order to assess medullary vascular filling (Ham & Tange, 1969, Davies, 1970 and Molland, 1978). Drawing ink contains a number of additives (e.g. phenol and glue), however, which might cause unpredictable vaso-responses in a kidney that has already been exposed to a chemical insult. Secondly, the colloidal nature of the carbon particles impart a substantial oncotic pressure, which could cause haemodynamic changes within the kidney in response to a hypertonic lead. Thirdly, both ethylenimine (Axelson, 1978) and 2-bromoethanamine (5.1) cause a dose related lesion, the effect of which plateaus at high concentrations. Previous studies (Murray et al., 1972; Hill et al., 1972, Wyllie et al., 1972; Solez et al., 1974 and Cuttino et al., 1981) used doses of 2-bromoethanamine (250 mg/kg) which in our experience (see previous sections) caused extensive ablation of the medulla. Finally, work from Galli et al., 1979 has recently highlighted the marked haemodynamic changes that occur in the kidneys of anaesthetised animals.

The aim of this study was to follow the time course changes in the renal microvascularity and morphology after a dose of 2-bromoethanamine adequate to cause papillary necrosis, but insufficient to ablate the entire medulla. Vascular filling was assessed using pure iso-osmotic colloidal carbon (at 37°C) administered to conscious, unrestrained rats that had been chronically cannulated via the right external jugular vein.

a) Animals and treatment. Chronic venous cannulation (4.1.3.5) was performed on 15 male rats (200-240g) husbanded as described previously
All animals were prepared surgically on the same day, after which they were housed individually for 48h before being randomly assigned for treatment. 2-Bromoethanamine (50mg/kg ip) was administered to 12 rats and 3 animals served as saline treated controls. Single control rats were killed at 2, 8 and 26h after saline treatment, and 2-bromoethanamine treated rats (numbers shown in parenthesis) were killed at 2 (3), 4 (2), 8 (3), 26 (2) and 48h (2).

Iso-osmotic colloidal carbon solution (2mL at 37°C, prepared as described (4.2.4) was injected into conscious unrestrained rats (over a 1 minute period) via the exteriorised cannula. The rat was then killed immediately (by cervical dislocation) and the kidneys dissected free, and fixed (4.2.1) for 24h.

b) Histopathology. One kidney was dehydrated, imbedded in wax (4.2.1) and several sections, including the papilla tip, were processed for H & E, colloidal iron, and Safronin O metachromasis (4.2.2).

The contralateral kidneys were dehydrated and embedded in celloidin (4.2.5). Thick sections (100 µm), to include the papilla, were cut on a sledge microtome and mounted as described (4.2.5).

c) Results

i) Histopathology. H & E sections from BEA treated rats showed a biphasic change with an early dilatation of the collecting tubules and hydropic degeneration (2h) followed by a period when the medullary architecture was apparently normal (4h). After 8 h early necrotic changes were apparent by 26 h there was "intermediate" papillary necrosis and by 48 h necrosis was "total", with active regenerative changes in the areas adjacent to tissue destruction.

ii) Histochemistry. Medullary MPS matrix stained more intensely between 2 to 4 h, but by 8 h those areas undergoing early necrotic changes showed both areas of non-staining and diffuse loss of staining.
Fig. 5.5a. Filling of renal microvasculature in control rat injected with colloidal carbon. Celloidin section, x 80.

Fig. 5.5b. Pronounced microvasculature filling of the outer medulla with colloidal carbon 4h after BEA 50mg/kg ip. Celloidin section, x 80.
Fig. 5.5c. Advanced signs of papillary necrosis 26h after BEA 50mg/kg ip. H&E, x 130.

Fig. 5.5d. Continued microvascular filling of papilla tip in the contralateral kidney to that shown in Fig. 5.5c. Celloidin, x 130.
There was an absence of matrix staining from necrosed areas from 26 h onwards, but the medulla "ghost" gave a background staining of increased intensity.

iii) Thick section microvascular filling. The distribution of colloidal carbon in control animals showed a uniform capillary network in the glomeruli of the cortex and the sub-cortical zone was relatively avascular except for a few large vessels and some capillary plexuses (Fig. 5.5a). The outer medulla was characterised by a series of compact vascular bundles (containing the vasa recta) associated with a network of dense capillary plexuses (Fig. 5.5a). The papillae were filled to the tip by a fine capillary network.

At 2 and 4 h after 2-bromoethanamine treatment the vascularity of the cortex was decreased in favour of a pronounced filling of the outer medulla, a pattern that was maintained up to 26 h (Fig. 5.5b). There were some slightly irregular large vessels in the sub-cortical zone, but there were no obvious changes in the filling of the inner medulla. Wax imbedded sections for the contralateral kidney showed early necrotic changes at 8 h, but thick sections showed that the capillaries of the inner medulla were wider than controls and that there was marked filling of the plexuses at the papillary tip. At 26 h (when necrosis was advanced in the contralateral kidney, Fig. 5.5c) the microvasculature of the papillary tip still showed marked, but irregular filling (Fig. 5.5d). Only when the necrotic process was complete at 48 h was the damaged papilla avascular.

d) Discussion. Papillary microvascular changes have been assumed to be the most significant morphological factor underlying this necrotic lesion (see 3.2.5.2). It is, however, impossible to establish a cause and effect relationship between microvascular degeneration, ischaemic injury and necrosis of the papilla in those circumstances where the
lesion develops over weeks, months or years. 2-bromoethanamine offers a more decisive answer to the question - is vasospasm or microvascular degeneration in the papilla an essential prerequisite to necrosis?

Both the histopathological and the cytochemical changes that occurred during the development of the lesion in chronically cannulated rats were similar to those already described in animals that had had no surgical pretreatment (5.2). The lesion developed slightly earlier in chronically cannulated animals, but the "degree" of necrosis was unaltered. The reasons for this contracted time scale in the development of necrosis are unknown, but may be a consequence of the anaesthetic, surgical "trauma" (although the procedure was in itself subject to little, if any, body fluid losses), individual housing or, perhaps, intravenous loading with small volumes (0.05mL) of 0.9% (m/v) NaCl when the cannulae were flushed.

The distribution of colloidal carbon represents microvascular filling and not haemodynamics, although it is tempting to interchange the two. The control sections showed the classical intra-renal blood distribution (2.1). Early 2-bromoethanamine-induced changes are consistent with the pronounced and sustained diuresis where blood would be drawn away from the cortex in favour of the medulla. (Early & Friedler, 1964, 1965 and Chuang et al., 1978).

An increased flow through the vasa recta would serve to "wash-out" the high solute concentration associated with counter current concentration. It is still not certain if diuresis initiates the vascular response or vice versa.

The histochemical changes in the staining of mucopolysaccharide material followed an identical course to that already described (5.2).

There was no evidence to suggest that either medullary microvascular spasm or vascular degenerative occlusion developed in the early stages
of necrosis. In fact extensive medullary colloidal carbon filling was
apparent at 8 h when the contralateral kidneys showed considerable early
necrotic changes. Even at 26 h, when necrotic changes had run their full
course, the papillae showed marked, but irregular filling.

It was only at 48 h that the necrosed papillary tip failed to show any
colloidal carbon filling. It is most improbable that the administration
of the colloidal carbon could, in itself, "open", degenerated vessels
because the iso-thermal, iso-osmotic solution was administered via the
right ventricle, and would therefore reach the kidneys at normal blood
pressure.

The contribution of subtle changes in renal haemodynamics to the
pathogenesis of chemically induced papillary necrosis are difficult to
assess because of the complexities of renal vascularity, the factors
controlling blood flow and the technical difficulties associated with
defining renal haemodynamics. The data presented above clearly suggests
that 2-bromoethanamine-induced renal papillary necrosis is unrelated to
ischaemic injury caused by microvascular occlusion or vasospasm. The
possibility that medullary anoxia develops as a consequence of
haemostasis associated with dilatation of the vasa recta cannot,
however, be excluded. The data from Solez et al., 1974 and Vanholder et
al., 1981 showed that plasma flow through the papilla was normal or
elevated (6 and 24 h, respectively) after giving BEA, but the doses of
BEA used by these workers was much higher than that used in the present
studies.

5.6 CHAPTER SUMMARY

A single dose of BEA administered ip, sc or po - caused a specific
necrosis of the renal papilla in all treated animals. For a given set of
conditions (i.e. dose and route of administration) the BEA-induced
papillary lesion was reproducible and there were no obvious differences in renal sensitivity between the sexes. The extent of the lesion was dose and route dependent, varying from an apex limited focal lesion confined to the renal medullary interstitial cells (50mg/kg po), to a necrotic change affecting all anatomical elements within the papilla, except the collecting ducts and the covering epithelia (50mg/kg ip or sc, or 200mg/kg ip po). Higher doses still (equal to or greater than 100mg/kg sc or ip, or 500 mg/kg po) caused the necrosis of all the elements of the papilla up to, but never beyond, the corticomедullary junction.

Hyperplasia only occurred in the papillary epithelium when there were necrotic changes in the tissue underlying it. When the papilla was totally necrosed the epithelia which covered the medullary stump was similarly hyperplastic. There were also hyperplastic changes in the epithelia of the bladders of rats given high doses of BEA.

Weanling rats were less sensitive to doses of BEA up to 100mg/kg ip, but 150mg/kg caused total ablation of the medulla up to the corticomедullary junction.

The time-course changes in renal morphology and histochemistry were assessed at 2, 4, 6, 8, 12, 24, 49, 73 and 123h after doses of 50, 100 and 150mg/kg BEA given ip. Morphologically the changes included hydropic degeneration and tubular dilatation between 2 and 6h, but the appearance was normal between 8 and 12h. The earliest signs of necrosis were apparent at 24h after BEA dosing, extensive by 48h and repairative processes were well advanced by 73h. The changes occurred at similar times irrespective of dose, but they were more extensive for higher doses of BEA.

The changes in the staining of medullary MPS showed most dramatic changes. Within 2h of BEA treatment the MPS stained most intensely, a
feature that was maintained for 8h. During this time, however, the appearance became vesicular and there appeared to be diffusive losses of staining at the junction between the ground substance and the urothelium in the papilla. The loss of staining was extensive by 12 to 24h and both paralleled and was confined to those areas in which necrotic changes were occurring or had taken place. By 48h most MPS staining had been lost from the necrosed areas although there was staining in adjacent regions in which necrotic change had not occurred. From 73h after the BEA insult this staining pattern was maintained, i.e. positive (although not normal) staining in the non-necroosed region and the absence of MPS from the necrosed papilla.

Pretreatment of rats with reserpine, drug metabolism inducers (phenobarbitone and 3-methylcholanthrene) or inhibitors (cobaltous chloride), sulhydryl protectors (acetylcysteine and methionine) or a free radical scavenger (zinc sulphate) failed to perturbate the dose response relationship to BEA.

Pretreatment with the anti-oxidants BHT, BHA and vitamin E protected some of the animals from BEA-induced RPN, while high doses of ethanolamine (500 mg/kg) caused a consistent, marked amelioration where only the interstitial cells were necrosed and the urothelium appeared normal after either 50 or 150 mg/kg BEA ip. Streptozotocin treatment caused diabetes and also totally protected against the subsequent papillotoxicity of BEA.

Pretreatment with SKF-525A exacerbated the lesion, and aspirin, paracetamol, phenacetin, or N-phenylanthranilic acid administration before BEA also caused a markedly increased response. This was not the case when dexamethasone was given before BEA.

Partial renal papillectomy did not, in itself, prevent a BEA-induced necrosis of the remaining medulla stump, but the necrosis was always far
less severe than the comparable area on the contralateral kidney. The protective effect was more obvious after 30 days, at which time the partially papillectomized kidney appeared essentially normal except for some necrotic change at the medulla stump). The contralateral kidney, however, showed gross secondary cystic dilatation and only remnants of the necrosed medulla were left in the renal pelvis.

Dehydration for 24h before BEA dosing served to lower the threshold at which necrosis was induced from 50 to 25mg/kg. These hydropenic animals did not survive high doses of BEA (100 to 150mg/kg ip), whereas normally hydrated rats did.

The effect of BEA on the filling of medullary microvasculature was assessed at various time intervals after dosing by injecting iso-osmotic, iso-thermal colloidal carbon intravenously into conscious unrestrained animals (that had been chronically cannulated) shortly before they were killed. BEA caused an increased filling of the outer medullary blood vessels between 2 and 6h after dosing. The microvascular integrity was still functional when early necrotic changes were apparent in the contralateral kidney at 8h and even at 26h when necrosis was total there were still vascular tracts containing colloidal carbon up to the papilla tip. Only 48h after BEA was the vascularity to the papillary tip obliterated.
In order to describe the complete molecular pathogenesis underlying this acutely induced RPN both the bioconversions of BEA requires elucidation and the kinetics of these metabolites need full delineation. Although BEA has a simple molecule structure, it may undergo a variety of "spontaneous" chemical changes (Dermer & Ham, 1969) and there are also a substantial number of routes through which it might undergo bioconversion, (Fig. 6) at least some of which could be analogues of endogenous substances. The most appropriate practical approach for investigating biotransformation is the use of isotopically labelled molecules. 2-Bromo-[\textsuperscript{14}C]ethanamine has recently been synthesised (4.4).

In order to understand the histochemical changes in MPS staining followed by BEA treatment (5.2) their relation to biochemical changes in the medullary PoG-GAG matrix, as reflected by tissue and urinary turnovers, also required examination.

6.1 URINARY CHANGES FOLLOWING BEA ADMINISTRATION.

BEA has been reported by others to cause a urine concentrating defect and increased 24-hour urine volumes (see Table 3.7). In the light of a different histopathological time-course, compared to published data (5.2), changes in urinary volume, osmolality and pH were reassessed.
Fig. 6. Schematic pathway for the possible chemical and biological transformation of 2-bromoethanamine.
Table 6.1 The effect of BEA on urinary volume, pH and osmolality.

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† Animal treatments were as follows:--
Rat A, saline; B & C, 50mg/kg BEA ip and D & E, 100mg/kg BEA ip

§ No urine (NU) was voided spontaneously over this period.
a) Animals and treatment. Urine was collected from 5 male Wistar rats (240-250g) housed in metabolic cages (4.1.1.2) where food and water were available ad libitum. Control 24-hour urines were collected and the volume, pH (using a micro pH electrode) and osmolality (using a Knauer semi-micro-osmometer, Department of Clinical Chemistry at St. Lukes Hospital, which was calibrated before and during each set of measurements at 1000 mOsm/kg) were measured. One rat was injected with saline, two rats with BEA 50mg/kg ip and the remaining two with BEA 100 mg/kg ip.

Spontaneously voided urine was collected two hourly for 8 hours, then after 24h and 48h, and the volume, pH and osmolality of each sample measured. Water intake was not measured.

b) Histopathology. The rats were killed (4.2.1) at 48h, the bladder and kidneys taken from each animal, fixed for 26h, and processed for H & E stained sections (4.2.1).

c) Results. The histopathology was constant with the dose of BEA administered, as described above (5.1 to 5.5).

The changes in urinary volume, osmolality and pH are shown for the individual rats in Table 6.1.

d) Discussion. The marked loss of urine concentrating function caused by BEA became apparent within the first hours after administration. Because of the uncertainties associated with the period of time which precedes each spontaneous bladder voiding it is impossible to relate these urine volumes to renal functional change for any one time interval. The decrease in urine osmolality from 800-1000 mOsm/kg to less than the normal plasma value (290 mOsm/kg) in 3 of the 4 treated animals by 24h suggests total disruption of the normal concentrating processes. The data shows no clear time course trends in volume or osmolality changes that might be used for assessing the degree of necrosis.
The loss of urine concentrating ability reported above is consistent with published data (Wyllie et al., 1972). These authors only measured 24-hour urinary changes, however, and assumed that the marked urine concentration defect reflected the necrotic loss of the thin loops of Henle. Our data shows the substantial loss of concentrating function shortly after BEA. It is impossible (without further investigations) to define the cause of this rapid "diuretic" response. It may reflect an early perturbation of those renal elements which concentrate urine, as a prelude to their ultrastructual disruption, or it may represent a non-specific "flushing response" to a renal irritant.

The maximum drop of urinary pH by 1.6 units (although more modest over short time periods) is also difficult to explain, without further investigations. It is, however, tempting to speculate that both the BEA-induced volume and pH changes relate to alterations in the medullary PoG-GAG matrix. Changes in MPS staining have been associated with changes in the urinary concentration process (2.3.4.4), and the absence of MPS staining is synonymous with a concentrating defect in the Brattleboro rat (Sun, 1980 and McAuliffe, 1980). A depolymerisation of PoG-GAG or loss of acidic groups (see 6.6.2) from the medullary matrix could cause a drop in urinary pH. These changes are discussed further below (7.1).

6.2 THE DISTRIBUTION OF RADIOLABELLED 2-BROMOETHANAMINE

Whole-body autoradiography provides essential information on the absorption, distribution and routes of excretion of labelled compounds and provides a valuable foundation upon which more detailed kinetic studies can be based. It does not, however, distinguish between parent compound and metabolites. The distribution of compounds within organs is best assessed using autoradiography at the light microscopic level.
6.2.1 The Whole-body Distribution of $^{14}$C-BEA.

a) Animals and treatment. Two male Wistar rats (180g) were dosed with 5 μCi $^{14}$C-BEA (in 50mg/kg freshly prepared BEA (4.1.1), given ip). One rat was killed, by cervical dislocation, after 1h and the other rat at 2h after dosing. Rats were immediately frozen (4.2.6.1) and stored at -70°C until processed as described (4.2.6.1). Whole-body sections were exposed to X-ray film for 21 days and developed as described (4.2.6.1).

b) Results. Figure 6.2.1a and b shows the autoradiographic distribution of BEA derived material at 1 and 2h after dosing. Each time point is represented by sections taken at two different levels through the same animal. At 1h after dosing BEA derived radiolabelled material was visualised (Fig. 6.2.1a) most strongly in the bladder, the peritoneal cavity, the kidney, the caecum, stomach and liver. After 2h most radioactivity was located in the bladder, stomach caecum, kidney and liver (Fig. 6.2.1b).

c) Discussion. Intraperitoneally administered BEA is rapidly absorbed (it was absent from the peritoneal cavity by 2h) and distributed to the kidney, bladder and liver.

The high intra-gut concentration of an intraperitoneally administered dose suggests that stomach wall secretion may play an important role in BEA absorption. The flux from the peritoneal cavity into the stomach might contribute to the prominent distribution of labelled BEA to the liver, via the portal system, presumably before it entered the systemic circulation. The intra-gastric flux is not unique to BEA for significant amounts of EI derived radiolabel in stomach, caecum and both the large and small intestine after this compound had been given intraperitoneally (Wright & Rowe, 1967).

One of the autoradiographs at both 1h and 2h (arrow) includes a section through the kidney where part of what appears to be a papilla
Fig. 6.2.1. Whole-body autoradiographs of rats treated with radiolabelled BEA. Sections at two levels are shown each with its autoradiograph.

B  Bladder       L  Liver
Br Brain        Lu Lung
Gi Gastrointestinal tract  St Stomach
H  Heart
K  Kidney

White arrow highlights the pelvic cavity or a section through the medulla.

a) 1 hour after dosing, page 211

b) 2 hours after dosing, page 212
tip shows more labelling than the cortex. These sections are, however, oblique and should be interpreted with care, because the more intense labelling may represent the contents of the renal pelvis, rather than an accurate representation of distribution of radioactivity in tissue. Further, BEA-derived material is being assessed autoradiographically.

6.2.2 The Distribution of $^{14}$C-BEA in the Kidney Studied at a Light Microscopic Level.

Attempts to assess the distribution of BEA (using the methods described below) had to be abandoned when it was discovered that kidneys could not be frozen at -70°C, because of a marked cryoprotective effect. Failure to achieve rapid freezing produced extensive ice crystal artefacts which destroyed most of the morphological features of the kidney. In contrast, the renal mass from a control rats (not treated with BEA) froze normally and was used to produce sections which showed excellent morphological features.

During the course of the work described below (6.3.1) large amounts of inorganic material was found to precipitate in urine and birefringent crystals were observed in tissue sections. This suggested that a rapid deposition of solute into solution (within the kidney) most likely explained the cryoprotective response to BEA. It seemed reasonable to assume that reducing the renal solute load, by giving rats distilled water, ad libitum, in place of tap water might reduce this effect. Recent preliminary experiments, not described here, showed that the dose response curve to BEA was unaltered in rats given distilled drinking water for 6 days and that kidneys taken from rats both 1 and 2h after BEA dosing froze without cryoprotection and produced artefact-free cryostat sections.

a) Animals and treatment. Six male Wistar rats (245 to 250) were housed, as described (4.1), but given distilled water ad libitum, after
10 days one rat was dosed with 50mg/kg ip of freshly prepared unlabelled BEA (4.1.1) and killed (4.2.1) after 1h, the kidney excised and snap frozen (4.2.3). Cryostat sections were cut (without any signs of cryoprotection), flash evaporated on slides, stained with 1% Toluidine blue (4.2.2.1) and found to be morphologically normal. Within an hour of cutting these sections (i.e. 3-4h after dosing the "test" rat an additional 4 rats were dosed with 50mg/kg ip; this contained 50 μCi 14C BEA for two of the rats, (4.1.1). Rats were killed after 1h, as described above, and kidneys snap frozen. These samples of tissue also froze without apparent signs of cryoprotection, but cryostat sections showed significant ice crystal artefacts when examined microscopically.

The autoradiographic procedures were undertaken at Ciba-Research as detailed above (4.2.6). The kidneys from rats not given radiolabelled BEA were used for assessing positive and negative chemography. Tissue was exposed against Ilford Type K2 in individual boxes for 7, 14 and 21 days, before fixing and processing as described (4.2.6.2). Slides were stained with H & E or Toluidine blue (4.2.2.1).

b) Results. The autoradiographs developed after 14 days gave the most satisfactory BEA exposure to background ratio. The sections which contained no radiolabelled material showed neither positive nor negative chemography. The disruption of the renal morphology made it impossible to define any cellular distribution of silver granules in animals given radiolabelled BEA.

The zonal distribution of labelled material was, however, assessed by counting silver grains in a high power field (X 400). Ten fields in each of the inner medulla, the outer medulla, the juxtamedullary zone and the superficial cortex were counted on two sections from each animal. The distribution showed a modest "concentration" gradient from the cortex (1), the juxtamedulla (1.5 to 2), to whole medulla (2 to 3).
There was, however, no obvious gradient across the medulla between the papilla, tip and the outer zone.

c) Discussion. It is difficult to explain the occurrence of the ice artefacts in the second batch of rats. In view of the fact that this experiment represented the use of the last batch of available radiolabelled material meticulous detail was paid to preparing, storing and transporting the tissue.

The data on the distribution of BEA cannot be interpreted. The formation of ice crystals is synonymous with slow freezing, which will, in turn allow water soluble molecules to diffuse. This, together with the disruption of morphological features, means that it is impossible to assess if the modest medulla-cortex gradient represents a uniform distribution or if radiolabelled material was, for example concentrated:-

i) within specific cell types and/or

ii) within the tubules and collecting ducts.

Furthermore, the capillary volume fraction of the medulla is twice that of the cortex, (Beewkes, 1980). Thus the apparent but modest concentration gradient between these two areas in the kidney may reflect, in part, radiolabelled material which is located "in" the vascular system.

This question still remains important to the understanding of BEA-induced RPN and future effort will need to be expended in developing an approach to produce artefact-free freezing of kidneys from BEA-treated animals. Longer periods of distilled water administration may be useful or freezing mixtures in equilibrium with liquid air might overcome the problem.

6.3 THE EXCRETION OF RADIOLABELLED 2-BROMOETHANAMINE.

The autoradiographic distribution of BEA suggested that preliminary
quantitative assessment of the excretion of radiolabelled material could be usefull undertaken in anaesthetised rats, where the bladder and bile duct had been cannulated.

Crystalline material was found to be deposited in bladder cannula within 1 to 2h of BEA dosing. This was shown to be inorganic material by flame charring and the full identification of this substance, together with the bladder calculi taken from one of the BEA-treated partially papillectomized rats (5.3.8.2) is described below (6.3.6).

The total amount of radiolabel administered to each rat was estimated by liquid scintillation counting of an accurately measured aliquot of the carbon-14 containing BEA dose (in triplicate).

The data for recovery of BEA-derived radiolabelled material has a coefficient of variance between 30 and 60% and is, therefore, not amenable to statistical analysis for differences between groups. Results are presented, therefore, as the range of data and its means. Differences in trends are, however, discussed.

6.3.1 The Excretion of Radiolabelled BEA in the Urine and Bile of Anaesthetised Rats.

The persistant labelling of the rat liver as detected autoradiographically (6.2.1) suggested that the bile might be a route for the excretion of BEA derived material.

a) Animals and treatment. Rats (5 male Wistar weighing 180-200g) were anaesthetised (4.1.3.1) and the bladder and bile duct of each cannulated (4.1.3.2). The animals were left for 20 min and the biliary and urinary flow rates measured at 5 min intervals. Flow rates were estimated from the period of time to fill a 5 µL capillary tube.

One rat with grossly impeded urine flow rates was excluded from the study. The right external jugular vein was exposed (4.1.3.5) and 2 rats were dosed with 50mg/kg BEA iv, and the other 2 rats with 100mg/kg BEA
b) Collection of urine and bile and quantitation of radiolabel recovery. Urine and bile were collected into pre-weighed containers which were changed at intervals shown in Table 6.3.1. The vials were re-weighed, and duplicate aliquots (20 µL of urine or 100 µL of bile) were removed for liquid scintillation counting (4.3), after which vials were weighed again. The total recovered radioactivity, for any time period, was based on the weight of liquid removed for quantitation, related to the total weight collected. In addition, an estimate of bile and urine flow rates was made from weight differences after removing aliquots of known volume compared to the total weight of liquid collected.

The counting efficiency was assessed using ¹⁴C-labelled internal standards and recounting those vials containing bile (4.3).

c) Results. Table 6.3.1 shows details of the dose of BEA, the amount of label, and the biliary and urine recoveries. The figures in parenthesis represent the estimated mean bile and urine flow rates respectively.

d) Discussion. The bile flow rates were about 800 to 1000 µL/h during the 20 min period which preceded the administration of BEA. This is just below the range (1000 to 1200 µL/h) for Wistar rats reported by Brauer et al. (1951). Similarly the urine flow rates were constant at 90 to 120 µL/h (a value somewhat less than 150 to 480 µL/h reported by Ross, 1972).

The administration of BEA caused no changes in the urinary flow rate, in these cannulated rats, in contrast to the marked diuretic response observed in in vivo studies (6.1) within the first few hours of dosing. The biliary flow rate fell markedly over the 5h collection period. It
Table 6.3.1 Recovery of radiolabelled material excreted in bile and urine from anaesthetised rats given \([1-^{14}C]\)BEA by the intravenous route.

<table>
<thead>
<tr>
<th>Number of Rats</th>
<th>Dose of BEA (mg/kg)</th>
<th>Activity µCi/Rat (kBq/Rat)</th>
<th>Time in Hours</th>
<th>Mean Bile Range</th>
<th>Σ</th>
<th>Mean Urine Range</th>
<th>Σ</th>
<th>Σ Bile+Urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 (iv)</td>
<td>50</td>
<td>1.55 (57.4)</td>
<td>0 - 1</td>
<td>4.70 (784) a 4.60-4.80</td>
<td>4.70</td>
<td>12.16 (122) b 6.67-17.64</td>
<td>12.16</td>
<td>16.86</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 - 2</td>
<td>2.95 (776) 2.00-3.10</td>
<td>7.65</td>
<td>6.49 (89) 5.23-7.75</td>
<td>18.65</td>
<td>26.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 - 3</td>
<td>1.53 (640) 1.05-2.00</td>
<td>9.18</td>
<td>3.9 (86) 3.36-4.44</td>
<td>22.55</td>
<td>31.73</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3 - 4</td>
<td>0.83 (515) 0.67-0.98</td>
<td>10.01</td>
<td>3.31 (99) 3.14-3.48</td>
<td>25.86</td>
<td>35.87</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 - 5</td>
<td>0.6 (445) 0.60 b 10.61</td>
<td>1.85</td>
<td>1.26-2.44 27.71</td>
<td>38.32</td>
<td></td>
</tr>
<tr>
<td>2 (iv)</td>
<td>100</td>
<td>1.64 (60.7)</td>
<td>0 - 0.5</td>
<td>0.86 (443) 0.82-0.87</td>
<td>0.86</td>
<td>5.08 (74) 2.68-7.49</td>
<td>5.08</td>
<td>5.93</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.5 - 1</td>
<td>0.98 (467) 0.91-1.04</td>
<td>1.82</td>
<td>6.26 (79) 3.78-8.74</td>
<td>11.34</td>
<td>13.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 - 2</td>
<td>1.26 (386) 1.23-1.30</td>
<td>3.08</td>
<td>8.88 (103) 8.78-9.89</td>
<td>20.22</td>
<td>23.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 - 3</td>
<td>0.68 (299) 0.63-0.73</td>
<td>3.76</td>
<td>4.43 (95) 3.96-4.90</td>
<td>24.65</td>
<td>28.41</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3 - 4</td>
<td>0.45 (232) 0.39-0.50</td>
<td>4.21</td>
<td>3.09 (90) 2.94-3.25</td>
<td>27.74</td>
<td>31.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 - 5</td>
<td>0.44 (276) 0.40-0.49</td>
<td>4.65</td>
<td>1.97 (95) 1.74-2.20</td>
<td>29.71</td>
<td>34.36</td>
</tr>
</tbody>
</table>

a mean flow rate µl/hour  
b both recoveries 0.50%
is noteworthy that the 100mg/kg dose of BEA caused a more marked decrease in the biliary flow rate, which was reduced to about half of its pretreatment value within 30 min. The reasons for this dramatic change obviously warrant future research. They may explain the lower recovery of radiolabelled material in bile compared to the animal treated with 50mg/kg BEA.

Surprisingly, up to 11% of recovered BEA derived material was in the bile. Dissociated BEA has a molecular weight of 125 (the debrominated compound would have a molecular weight of 45) and consequently even if this molecule was conjugated with glucuronic acid it might not be expected to exceed the molecular weight threshold of about 200 to 300 for the rat (Smith, 1973). This suggests that BEA may either be an exception to the biliary molecular weight threshold limit, or it is conjugated through more than one functional group, or with glutathione (see 6.4 for further discussion).

This kinetic data showed that radiolabelled material was largely excreted in the urine, and to a lesser extent in the bile after an iv dose supports the autoradiographic distribution (6.2.1).

6.3.2 The Excretion of Radiolabelled BEA in Urine, Breath and Faeces.

The full assessment of BEA metabolism was carried out in metabolic cages.

a) Animals and treatment. Male Wistar rats (6 animals weighing between 180 and 190g) were housed individually in glass Metabowls as described previously (4.1.2.1) for 24h with the pumps running. BEA 50mg/kg was given ip to 3 rats and 100mg/kg (po) to 3 rats (see Table 6.3.2 for amount of radioactivity given per rate).

b) Collection of urine, faeces and exhaled material and quantitation of radiolabel recovery. The urine collection containers were changed every 4h for the first 12h then at 24, 48 and 72h. The urine
was made up to a convenient volume, mixed and a measured aliquot removed for liquid scintillation counting. Faeces and both acid and alkaline traps were changed every 24h. Radioactivity was measured in each as described 4.3.

c) Results. Table 6.3.2 shows the mean recoveries in urine and faeces and from the two types of breath traps. The figures in parenthesis are the range for the three animals in each group.

d) Discussion. The urinary recoveries over the first 4 to 8h period are similar to those already reported (6.3.1) for the cannulated anaesthetised rat. In these non-anaesthetised animals the measurement of BEA excreted by the kidney has been delayed because it was measured in spontaneously voided urine. Were it not for the marked diuretic effect caused by BEA the urinary time-course recoveries would obviously give less kinetic information. Approximately 90% of the recovered BEA was excreted in the urine, 5-7% in the breath (mostly as CO₂) and 2-3% was recovered in faeces.

The biliary recoveries were up to 11% (6.3.1) of the excreted radiolabelled product, an amount 3 to 5 fold higher than the faecal recoveries. This suggests an active enterohepatic metabolic cycling of some BEA-derived product, a factor which may contribute to the different recovery profile for a dose of BEA given via the ip route compared to that given po. The data showed too much variance and has too few points for statistical analysis, but the trends in urinary recovery suggests that there may be a delay in gastric emptying or a substantial entero-hepatic circulation which initially prevents BEA-derived material reaching the systemic circulation, from which it would be rapidly cleared by the kidney. An enhanced entero-hepatic cycling, or reduced oral absorption, may also be supported by higher faecal recoveries and higher CO₂ recoveries (possibly representing more intestinal or hepatic
<table>
<thead>
<tr>
<th>Number of Rats</th>
<th>Dose of BEA (mg/kg)</th>
<th>Route</th>
<th>Activity uCi/Rat (kBq/Rat)</th>
<th>Time in Hours</th>
<th>Urinary</th>
<th>HCl trap</th>
<th>CO₂</th>
<th>Mean Faecal</th>
<th>Total</th>
<th>Σ</th>
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<td>3</td>
<td>50</td>
<td>ip</td>
<td>1.97</td>
<td>0-4</td>
<td>28.25</td>
<td>28.3</td>
<td>-</td>
<td>-</td>
<td>28.3</td>
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<td></td>
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<td>(17.87-41.67)</td>
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<tr>
<td></td>
<td></td>
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<td></td>
<td>4-8</td>
<td>12.48</td>
<td>40.7</td>
<td>-</td>
<td>-</td>
<td>40.7</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>(6.57-19.47)</td>
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<td></td>
<td>8-12</td>
<td>11.43</td>
<td>52.2</td>
<td>-</td>
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<td>(5.88-17.40)</td>
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<td>3.7</td>
<td>1.8</td>
<td>70.50</td>
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<td>24-48</td>
<td>3.43</td>
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<td></td>
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<td>48-72</td>
<td>0.75</td>
<td>68.0</td>
<td>ND</td>
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<td>0.2</td>
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<td>(0.57-1.05)</td>
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<tr>
<td></td>
<td>100</td>
<td>po</td>
<td>1.48 (54.8)</td>
<td>0-4</td>
<td>16.09</td>
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<td>(14.39-17.67)</td>
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<td>9.70</td>
<td>25.79</td>
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<td>25.79</td>
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<td>8.79</td>
<td>34.58</td>
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<td>34.58</td>
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<td>(5.99-12.52)</td>
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<td></td>
<td></td>
<td>12-24</td>
<td>19.64</td>
<td>54.22</td>
<td>1.7</td>
<td>4.2</td>
<td>2.6</td>
<td>62.72</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(18.43-20.51)</td>
<td></td>
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<td></td>
<td></td>
<td>24-48</td>
<td>10.96</td>
<td>65.18</td>
<td>0.2</td>
<td>0.8</td>
<td>0.5</td>
<td>75.18</td>
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<td>(7.75-15.42)</td>
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<td></td>
<td>48-72</td>
<td>1.1</td>
<td>66.28</td>
<td>ND</td>
<td>ND</td>
<td>0.1</td>
<td>76.38</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td>(1.00-1.20)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

* Value determined every 24h  
ND = No Data available
degradation) after oral dosing. The total recovery was, however, similar when BEA was given by either route which precludes malabsorption.

From the recovery of BEA-derived material at 24, 48 and 72h the total amount of product excreted appears to be asymptotic. The failure to recover more than 70-76% of the administered dose suggests that 24-30% of BEA metabolites are either incorporated into metabolic pools where they have a slow turnover or these products covalently bind to structural components.

The chemical conversion of BEA to El, a powerful alkylating agent, (3.2.3.2), together with the fact that both compounds cause RPN, has led several workers to suggest that EI is the "proximate" toxin in BEA-induced RPN. The similarities and differences between BEA and EI excretion and metabolism will be considered below (7.1).

6.3.3. The Effect of Reserpine Pretreatment on the Excretion of Radiolabelled BEA in the Bile and Urine of Anaesthetised Rats.

Data published by Wyllie et al., (1972) suggested that reserpin pretreatment ameliorated the BEA-induced RPN. Accordingly, at the same time as the histological assessment of the effect of reserpin on BEA-induced RPN (5.3.1) was in progress the urinary and biliary excretion of radiolabelled BEA was also studied.

a) Animals and treatment. Three male Wistar rats weighing from 200-210g were given a single 0.80mg reserpin (5.3.1) 18h before 50mg/kg in BEA. They had been anaesthetised and cannulated as described (6.3.1a). Resting bile and urinary flow rates were measured before 50mg/kg BEA containing 2.08 μCi (77kBq)/rat was given iv (see 6.3.1a).

b) Collection of urine and bile and quantitation of radiolabel recovery. Urine and bile were collected into pre-weighed containers and aliquots taken for liquid scintillation counting as described...
Table 6.3.3  The effects of reserpine pre-treatment (0.80 mg/rat sc given 18h before) on the recovery of BEA-derived radiolabel excretion in the bile and urine of cannulated anaesthetised rats.

<table>
<thead>
<tr>
<th>Time in hours</th>
<th>Mean Bile</th>
<th>Range</th>
<th>Σ</th>
<th>Mean Urine</th>
<th>Range</th>
<th>Σ</th>
<th>Σ Bile + Urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 0.5</td>
<td>1.35</td>
<td>0.82-1.92</td>
<td>1.35</td>
<td>5.65</td>
<td>0.55 - 8.27</td>
<td>5.65</td>
<td>7.00</td>
</tr>
<tr>
<td></td>
<td>(670)*</td>
<td></td>
<td></td>
<td>(120)*</td>
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</tr>
<tr>
<td>0.5 - 1</td>
<td>1.60</td>
<td>1.08-1.98</td>
<td>2.95</td>
<td>3.94</td>
<td>2.60 - 4.60</td>
<td>9.59</td>
<td>12.54</td>
</tr>
<tr>
<td></td>
<td>(680)</td>
<td></td>
<td></td>
<td>(111)</td>
<td></td>
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</tr>
<tr>
<td>1 - 2</td>
<td>2.32</td>
<td>1.81-2.60</td>
<td>5.27</td>
<td>6.12</td>
<td>5.49 - 6.62</td>
<td>15.71</td>
<td>20.98</td>
</tr>
<tr>
<td></td>
<td>(620)</td>
<td></td>
<td></td>
<td>(134)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>2 - 3</td>
<td>1.39</td>
<td>1.24-1.48</td>
<td>6.66</td>
<td>4.54</td>
<td>3.92 - 4.99</td>
<td>20.25</td>
<td>26.91</td>
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<tr>
<td></td>
<td>(516)</td>
<td></td>
<td></td>
<td>(142)</td>
<td></td>
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</tr>
<tr>
<td>3 - 4</td>
<td>0.85</td>
<td>0.77-0.89</td>
<td>7.51</td>
<td>2.80</td>
<td>2.26 - 3.44</td>
<td>23.05</td>
<td>30.56</td>
</tr>
<tr>
<td></td>
<td>(411)</td>
<td></td>
<td></td>
<td>(130)</td>
<td></td>
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</tr>
<tr>
<td>4 - 5</td>
<td>0.56</td>
<td>0.48-0.60</td>
<td>8.07</td>
<td>2.04</td>
<td>1.62 - 2.45</td>
<td>25.09</td>
<td>33.16</td>
</tr>
<tr>
<td></td>
<td>(330)</td>
<td></td>
<td></td>
<td>(143)</td>
<td></td>
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</tr>
</tbody>
</table>

† Each rat given 2.08 μCi (77 kBq) of BEA (50mg/kg iv)

§ Mean for 3 rats

* Flow rate μL/h.
6.3.1b.
c) **Results.** Table 6.3.3 shows the mean urinary and biliary recoveries, together with the range, sum and estimated flow rates. The total sum recovery is shown in the righthand column.

d) **Discussion.** The bile flow rates which started in the range of 800-1000μL/h fell rather rapidly over the course of the experiment, while urinary flow rates were relatively constant. Whereas the bile flow rates were less than animals given the same dose of BEA only, urinary flow rates averaged at 130 μL/h a value greater than that previously observed ( 100 μL/h, see Table 6.3.1). The total recoveries of radiolabelled material was about 2% less in both bile and urine. These differences are unlikely to be significant in the light of the inter-animal variability.

Further discussion is considered below (6.3.4) where the results of the in vivo BEA-derived radiolabel excretion has been presented.

6.3.4 The Effects of Reserpine Pretreatment on the Excretion of Radiolabelled BEA in Urine, Breath and Faeces.

The effect of reserpine pretreatment on BEA excretion was also assessed in conscious unrestrained rats.

a) **Animals and treatment.** Rats (male Wistar weight 180-190g) were housed individually in Metabowls (4.1.2.1) for 24h before radiolabelled BEA was given. Six hours after the start of this acclimitisation period each of the 3 rats was given 80mg reserpine sc (5.3.1). Each rat was dosed with 50mg/kg BEA (given ip) which contained 1.87 μCi(69kBq).

b) **Collection of urine, faeces and exhaled material and quantitation of radiolabel recovery.** Urine, faeces, and exhaled material were collected and the recovery of radiolabelled material quantitated as described above (6.3.1b).

c) **Results.** Table 6.3.4 presents the mean recoveries for urine
d) Discussion. Animals pretreated with reserpine excreted a mean sum urinary value more than rats treated with BEA alone (Table 6.3.2). Both the alkaline component in breath and the labelled CO₂ and the faecal recoveries were less than rats that had been treated with BEA alone.

The 0 to 72h faecal recoveries are lower than the amount of biliary radiolabel collected in 5h, confirming the likelihood of an enterohepatic circulation (6.3.2d).

Reserpine pretreatment failed to provide any papilloprotective effect (5.3.1) and the possible reasons underlying the failure to reproduce the results of Wyllie et al. (1972) are discussed above (5.3.1d).

6.3.5 The Excretion of BEA-derived Radiolabelled Material after Subcutaneous Dosing, During a Diuresis and After Pretreatment with Non-labelled BEA.

Having previously defined aspects of the excretion of BEA derived radiolabel when the material was given ip, po and iv, one rat was similarly studied after the parent compound was given sc. The effect of an induced diuresis on BEA excretion was also assessed because of the ameliorating effect attributed to this state (Fuwa & Waugh, 1968).

a) Animals and treatment. Male Wistar rats (weighing 180 to 190g) were housed individually in glass Metabowls (4.1.2.1) for a 24h acclimatisation period before radiolabelled material was administered. One rat was given 50mg/kg BEA (non-labelled) ip at the start of the acclimatisation period. Twenty-four hours later another dose of BEA (50mg/kg ip) containing radiolabelled material was administered. A state of diuresis was induced in a second rat which was placed on 5% glucose drinking water for the 24h before BEA (50mg/kg ip) and for the
Table 6.3.4 The effect of reserpine pretreatment (0.8 mg/rat sc given 18h before) on the recovery of BEA-derived radiolabel excreted in the urine, faeces and breath of rats.

<table>
<thead>
<tr>
<th>Time in Hours</th>
<th>Mean$\text{(Range)}^*$</th>
<th>% Urinary $\Sigma$</th>
<th>Mean Breath CO$_2$ (Range)</th>
<th>Mean Faecal $\Sigma$</th>
<th>Total $\Sigma$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 4</td>
<td>31.96 (29.67-33.58)</td>
<td>31.96</td>
<td>-</td>
<td>-</td>
<td>31.96</td>
</tr>
<tr>
<td>4 - 8</td>
<td>23.21 (15.54-27.47)</td>
<td>55.17</td>
<td>-</td>
<td>-</td>
<td>55.17</td>
</tr>
<tr>
<td>8 - 12</td>
<td>5.80 (4.96-6.41)</td>
<td>60.97</td>
<td>-</td>
<td>-</td>
<td>60.97</td>
</tr>
<tr>
<td>12- 24</td>
<td>6.89 (6.80-7.01)</td>
<td>67.84</td>
<td>1.00 (0.69-1.04)</td>
<td>1.87 (1.57-2.13)</td>
<td>0.73 (0.46-0.93)</td>
</tr>
<tr>
<td>24- 48</td>
<td>1.89 (1.53-2.21)</td>
<td>69.73</td>
<td>0.38 (0.12-0.95)</td>
<td>0.93 (0.43-1.33)</td>
<td>0.20 (0.16-0.24)</td>
</tr>
<tr>
<td>48- 72</td>
<td>0.79 (0.68-0.75)</td>
<td>70.52</td>
<td>0.10</td>
<td>0.07</td>
<td>0.07 (0.05-0.12)</td>
</tr>
</tbody>
</table>

$^+$ Each rat given 1.87 μCi (69 k Bq) of BEA (50mg/kg ip)

$^*$ Figures in parenthesis give range for the group.

$^\S$ Mean for 3 rats
duration of urinary collection. The third rat was injected with BEA (100mg/kg) but administered subcutaneously. The BEA dose contained 1.68 μCi (62kBq) radioactivity per rat.

b) **Collection of urine, faeces and exhaled material and quantitation of radiolabel recovery.** These were undertaken as described above (6.3.1b).

c) **Results.** Table 6.3.5 presents the urine and faeces and air trap recoveries for the individual rats.

d) **Discussion.** The recoveries after the sc administration of BEA are essentially similar to those reported for ip administration, suggesting that the molecule is rapidly absorbed to the systemic circulation. The amount of BEA-derived material recovered from a rat in a mild state of diuresis was similar to that reported for animals treated with BEA alone. Less radiolabelled material was, however, excreted as CO₂ and in faeces.

The most significant result is the 10% increase in urinary recovered radiolabelled material when a second dose of BEA was administered. The faecal and breath trap recoveries were also reduced, the greatest reduction being the basic exhaled component trapped in HCl. It is difficult to explain this increased urinary recovery, although several possibilities exist. It is uncertain what role diuresis per se plays in this effect. Diuresis induced by glucose drinking water (Table 6.3.5) failed to increase C-14 BEA clearance, although the 24-h urine volume (21.3mL) from BEA and glucose was less than that caused by BEA treatment alone (27.2mL). Fundamental differences in the mechanism responsible for the diuretic state might, however, cause markedly different handling of molecules within the kidney. Thus, for example, BEA might be reabsorbed by the intact kidney, even in a state of diuresis, but not reabsorbed when structure and functional integrity is damaged.
Table 6.3.5 The excretion of BEA-derived radiolabelled material when given subcutaneously, 24h after a non-labelled dose of BEA and to a rat with an existing diuresis.

<table>
<thead>
<tr>
<th></th>
<th>Collection Period h</th>
<th>Sub-cutaneous Recovery %</th>
<th>BEA-treated Recovery %</th>
<th>Glucose Diuresis Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Urine</td>
<td>0 - 4</td>
<td>32.28</td>
<td>32.28</td>
<td>35.96</td>
</tr>
<tr>
<td></td>
<td>4 - 8</td>
<td>13.44</td>
<td>45.72</td>
<td>25.05</td>
</tr>
<tr>
<td></td>
<td>8 -12</td>
<td>4.10</td>
<td>49.83</td>
<td>7.88</td>
</tr>
<tr>
<td></td>
<td>12 - 24</td>
<td>14.71</td>
<td>64.54</td>
<td>9.36</td>
</tr>
<tr>
<td></td>
<td>24 - 48</td>
<td>4.29</td>
<td>68.83</td>
<td>2.05</td>
</tr>
<tr>
<td></td>
<td>48 - 72</td>
<td>1.19</td>
<td>70.02</td>
<td>1.07</td>
</tr>
<tr>
<td>HCl trap</td>
<td>0 - 24</td>
<td>0.71</td>
<td>0.71</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>24 - 48</td>
<td>0.33</td>
<td>1.03</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>48 - 72</td>
<td>0.31</td>
<td>1.35</td>
<td>0</td>
</tr>
<tr>
<td>Alkaline (CO₂) trap</td>
<td>0 - 24</td>
<td>2.07</td>
<td>2.07</td>
<td>1.02</td>
</tr>
<tr>
<td></td>
<td>24 - 48</td>
<td>0.03</td>
<td>2.10</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>48 - 72</td>
<td>0</td>
<td>2.10</td>
<td>0</td>
</tr>
<tr>
<td>Faeces</td>
<td>0 - 24</td>
<td>2.22</td>
<td>2.22</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>24 - 48</td>
<td>0.44</td>
<td>2.66</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>48 - 72</td>
<td>0.02</td>
<td>2.68</td>
<td>0.10</td>
</tr>
<tr>
<td>Total Recovery</td>
<td>0 - 72</td>
<td>76.15</td>
<td>84.06</td>
<td>71.45</td>
</tr>
</tbody>
</table>

a 50mg/kg given 24h before a labelled dose of BEA.

b 5% Glucose drinking water given 24h before a labelled dose of BEA.
Arruda's group have reported extensive electrolyte wastage in BEA treated animals. Similarly, those BEA-derived metabolites which were cationic (see 6.5.2) should be cleared preferentially from the kidney of a BEA pretreated animal because of the fall in urinary pH (Table 6.1) provided their pKa was high enough.

Approximately 25% of an administered dose of BEA was not recovered after 72h (Table 6.3.2) in rats given a single dose of BEA. This suggests that some BEA-derived material is irreversibly bound into a metabolic (e.g. phospholipid) or a non-metabolic pool (e.g. a macromolecule) where it has a prolonged half-life. Thus greater urinary recoveries after a dose of BEA may arise because of the blocking of these binding sites.

The phenomena of increased recovery of BEA (given as a second dose) may illuminate aspects of the metabolism of this compound. Alternative techniques such as perfused kidney or renal slices may give a better insight into the mechanism, but this type of investigation may not be helpful until more is known about the structure of BEA metabolites.

6.3.6 Identification of urinary crystals and bladder calculi.

a) Identification of urinary crystals. The crystalline material in the urine of anaesthetised bladder-cannulated rats was identified by shape and solubility (4.5.1) infra-red spectrum (4.5.2) and X-ray microprobe analysis (4.5.3).

b) Identification of the bladder calculi. The inorganic constituents in the calculi were identified by their infra-red spectrum (4.5.2) and by X-ray microprobe analysis (4.5.3).

c) Results. The shape of the urinary crystalline material was that of the classical "coffin lid" which together with the solubility of the material in dilute mineral and acetic acid, but not alkaline, alcohol, acetone, ether or on heating in aqueous solution suggested magnesium
Fig. 6.3.6a-c Infra-red spectra of:-
a: urinary crystalline material passed from the bladder of anaesthetised rats treated with BEA,
b: reference spectra of magnesium ammonium orthophosphate, and
c: bladder calculi found in a partially papillectomized rat after BEA treatment.

A polystyrene film was used to provide an accurate calibration of the spectra at 1583 and 1601 cm$^{-1}$. 
Fig. 6.3.6d-f  X-ray energy dispersion spectra of:–

d: urinary crystalline material passed from the bladder of anaesthetised rats treated with BEA,
e: reference spectra of magnesium ammonium orthophosphate, and
f: bladder calculi found in a partially papillectomized rat after BEA treatment.
ammonium phosphate hexahydrate (MAP). This was confirmed by infra-red analysis (Fig. 6.3.6a) which differed only slightly from a chemically pure standard (Fig. 6.3.6b). (The urological nomenclature will be applied to material of urinary origin, i.e. magnesium ammonium phosphate, where the chemical standard is correctly called ammonium magnesium orthophosphate). X-ray micro-probe analysis did, however, show striking differences between the MAP (Fig. 6.3.6d), which contained substantial amounts of potassium, and the chemical standard (Fig. 6.3.6e) which was potassium free, but contained a trace of chlorine ions.

The bladder calculi produced an infra-red trace (Fig. 6.3.6c) that differed substantially from that of the chemical standard, but still showed prominent phosphate absorption at 950 to 1200 cm$^{-1}$ and a broad area at 2900 to 3250 cm$^{-1}$ which indicates MAP. The sharp absorption at 1390 cm$^{-1}$ and the shoulder at 300 cm$^{-1}$ suggest that traces of oxalate may also be present. The x-ray micro-probe analysis (Fig. 6.3.6f) confirms the presence of magnesium and phosphate, but also shows a small amount of calcium.

d) Discussion. The identification of any urolithiases is most difficult and depends, for its validity, on the use of several complementary methods. Thus crystal shape and solubility, together with the IR spectrum, confirmed that this material was predominantly MAP. X-ray microprobe analysis showed the presence of a substantial amount of potassium in addition to magnesium and phosphorous. The consistent ratio of these three elements in a large number of crystals which had been washed free of all urinary material suggests that the crystalline material is either a mixture of MAP and magnesium potassium phosphate or more likely a magnesium ammonium potassium phosphate complex, neither of which appears to have been described before.
The analysis of calculi is made more difficult by the presence of organic matrix material (this will impart its own characteristic to a IR spectrum) which binds the inorganic crystalline mass together and because calculi are heterogeneous (Oliver & Sweet, 1976 and Nordin et al., 1979). Although the calculi were largely MAP they did contain both oxalate and calcium.

The formation of urolithiases is a complex process (Nordin et al., 1979) dietary inorganic intake plays an important loading role in which saturation and super-saturation favour the formation of crystal nuclei products, but this may be prevented by inhibitors of crystallisation. One of these, perhaps the most important, are the glycosaminoglycans. If microcrystals are formed they may re-dissolve or be excreted as such, or may act as nuclei for the growth of larger crystals and eventually calculi. (Nordin et al., 1979 and Cheng, 1980).

There appears to be little published data on the formation of urinary MAP crystals per se and most inferences must be drawn from the massive body of literature on urinary tract calculi. MAP is encountered relatively infrequently as the major component of urinary stones, although it is often associated with calcium oxalate and phosphate stones and calculi. MAP is, however, the major component of "staghorn" calculi which form in, and may block, the renal pelvis and cause progressive damage. The aetiology of urinary MAP deposits in man are explained by the concomitant pre-disposing factors such as dietary inorganic loading, dehydration and organic matrix being excreted etc. and, most importantly, the presence of a urinary tract infection with an organism that splits urea to ammonium ions. This provides not only the cation for the complex, but also increases urinary pH above 7, a factor which because of the acid solubility of MAP, encourages the crystals to form (Nordin et al., 1979 and Cheng, 1980).
Obviously, this scenario cannot explain the acutely induced and short lived crystalluria following BEA. Furthermore, the formation of MAP-like crystals when the urinary pH was acidic (6.1) is difficult to explain. The presence of potassium ions suggests that from a sudden increase in magnesium or phosphate concentrations within the kidney. It is thus tempting to suggest that the BEA-induced increase in MPS staining (5.2) represents a conformational change in PoG-GAG, as a result of which the polyanionic role of the medullary interstitium would be altered. Thus a large amount of previously bound solute might be released, exceed the solubility product and precipitate. The acidity of the urine would dissolve these crystals and produce a solute loaded kidney and an osmotic flushing. This view is supported by the osmotic response (although other mechanisms may be envolved), the marked swelling of the kidneys and the dramatic cryoprotective effect after BEA treatment. Substantial electrolyte wastage has been shown to occur 24h after BEA treatment by Sabatini et al. (1981b).

An increase in acid soluble bi-refringent material has been found in kidney sections from BEA treated animals since these studies were completed, but it has not been possible to identify crystals in spontaneously voided urine. Failure to find crystals may relate to the decreased urinary pH which would favour dissolution.

The presence of "MAP" based bladder calculi in one BEA treated rat is best regarded with circumspection. Calculi do occur spontaneously in laboratory animals (Cheng, 1980) although it is difficult to induce them at will because of the many variables involved. Furthermore most rats were killed within 7 days of treatment a period which is generally regarded as too short for calculi to develop. The problem, as it relates to human analgesic abusers is, however, discussed further below (see 7.2).
6.4 THE EFFECT OF PARTIAL PAPILLECTOMY ON THE EXCRETION OF RADIOLABELLED 2-BROMOETHANAMINE BY THE UNINEPHRIC RAT

Data presented above (5.3.8.2) established that partial papillectomy ameliorated the BEA-induced RPN compared to the contra-lateral kidney. This raised the possibility that partial papillectomy might reduce the clearance of BEA through the surgically manipulated kidney in favour of other routes of excretion, such as the contra-lateral kidney or the bile.

6.4.1 The Effect of Partial Papillectomy on the Excretion of Radiolabelled BEA From Anaesthetised Rats.

a) Animals and treatment. Three male weanling Wistar rats (50g) were subjected to partial renal papillectomy as described (4.1.3.3) and left to recover for 21 days, after which the partially papillectomised kidney was removed from one rat (the uninephric normal) and the normal kidney removed from the other two rats (the uninephric papillectomised) using the technique described above (4.1.3.4). After a recovery period of 9 days the animals (weight 240-250g) were anaesthetised and the bladder and bile ducts cannulated (4.1.3.2). After a stabilisation period radio-labelled BEA 50mg/kg (see Table 6.4.1 for amount of radio-activity per rat) was given via the right external jugular vein and bile and urine collected over timed periods for 5h.

b) Collection of urine and bile and quantitation of radiolabel recovery. Urine and bile were collected into pre-weighed containers and quantitated as described above 6.3.1b.

c) Results. Table 6.4.1 shows the recoveries of radiolabelled BEA in urine and bile, over the 5h collection period, together with their estimated flow rates.

d) Discussion. The bile flow rates were similar, but slightly higher than those previously reported in these investigations (see 6.3.1) and
Table 6.4.1  A comparison of biliary and urinary recoveries of BEA-derived material administered to cannulated anaesthetised uninephric rats with normal or partially papillectomized kidneys.

<table>
<thead>
<tr>
<th>Surgical Manipulation</th>
<th>Time Period of Collection (h)</th>
<th>Time Period of Collection (h)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-0.5</td>
<td>0.5-1</td>
<td>1-2</td>
</tr>
<tr>
<td>Normal † Recovery %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninephric Σ recovery %</td>
<td>2.40</td>
<td>3.74</td>
<td>7.12</td>
</tr>
<tr>
<td>Flow uL/h</td>
<td>1040</td>
<td>590</td>
<td>770</td>
</tr>
<tr>
<td>Papillectomised † Recovery %</td>
<td>1.66</td>
<td>1.00</td>
<td>2.49</td>
</tr>
<tr>
<td>Flow uL/h</td>
<td>800</td>
<td>436</td>
<td>556</td>
</tr>
<tr>
<td>Papillectomised § Recovery %</td>
<td>2.06</td>
<td>2.36</td>
<td>2.68</td>
</tr>
<tr>
<td>Uninephric Σ recovery %</td>
<td>2.06</td>
<td>4.42</td>
<td>7.10</td>
</tr>
<tr>
<td>Flow uL/h</td>
<td>1004</td>
<td>1072</td>
<td>996</td>
</tr>
</tbody>
</table>

† Radioactivity 1.29μCi/Rat (48kBq/rat)
§ Radioactivity 1.90μCi/Rat (70kBq/rat)

ND No Data for this time point
* Σ recovery does not include urine sample from 4 to 5h.
declined over the 5h collection period as described above (6.3.1). The recovery of radiolabelled material in bile was similar in the normal and both partially papillectomised uninephric animals, and essentially the same as for normal (two kidney) rats.

The urinary flow rates were higher than those reported in previous experiments using this model, and possibly related to the additional workload undertaken by the uninephric kidney in which there is compensatory hypertrophy in the cortex but not in the medulla (see below) Two of the three rats showed a diminution in urinary flow for a few hours after BEA, but there was an easily recognisable increase in urinary flow rates thereafter. The recoveries of radiolabelled material were essentially similar from animals with a normal kidney compared to those with the partially resected papilla, but there was a reduction in the total mount of radiolabelled product cleared by the uninephric kidney (irrespective of whether it had been surgically manipulated previously or not). This reduced urinary clearance of radiolabelled BEA is opposite to the response which would have been predicted. Molland (1978b) showed that uninephrectomised rats were more sensitive to aspirin. This was explained in terms of the observation (Dicker & Shelley, 1971, 1973) that compensatory hypertrophy affected only the cortical mass and left the medullary mass unaltered. Thus the workload on the cortex would be reduced by hypertrophy, but increased in the medulla, which would have to process the total filtered volume. The net outcome appears to be a greatly increased sensitivity of the medulla to insult, a finding confirmed for other analgesics (Tange, J.D., personal communication), although there is no data for BEA.

Our data does, however, suggest that both the normal and the partially papillectomised kidneys are exposed similarly to BEA and excretion is
not shunted to the biliary route.

6.4.2. The Effect of Partial Papillectomy on the Excretion of Radiolabelled BEA From Conscious Rats.

a) Animals and treatment. Two male weanling Wistar rats (60g) were subject to partial papillectomy as described (4.1.3.3) and a third was sham operated where the papilla was exposed, manipulated, but not resected. After a recovery period of 21 days the rats were uninephrectomised (4.1.3.4) to give one rat with a sham operated kidney, one with a normal kidney and the third with a partially papillectomised kidney.

After 7 days the rats (250-260g) were housed individually in glass metabowls for a 24h acclimatisation period (4.1.2.1) with the pumps running. Each rat was dosed with 50mg/kg BEA (4.1.1) ip containing 2.71 μCi labelled BEA per rat.

b) Collection and quantitation of excreted radiolabelled material. Urine, exhaled air, and faeces was collected for 72h at intervals already described (6.3.2b) and quantitated (4.3).

c) Results. Table 6.4.2 shows the urinary, expired air trap and faecal recoveries of BEA-derived radiolabelled material for each collection period, cumulatively and total excreted products.

d) Discussion. The total urinary and breath trap recoveries were essentially the same in the normal and in the papillectomised rats, although less was recovered from the papillectomised rat in the earlier collection period (0 to 12h). The sham operated rat excreted more radiolabelled material via its urine and breath, but slightly less in faeces, than the other two.

The excretion via the different routes is, however, within a few percent for each of the three types of surgically manipulated animal, which (as a group) fit well within the range of biological variability
Table 6.4.2  Recovery of radiolabelled material excreted in urine, breath and faeces from uninephric rats that were partially papillectomized, sham operated or normal after administering [1-14C]BEA intraperitoneally.

<table>
<thead>
<tr>
<th></th>
<th>Collection Period h</th>
<th>Sham Uninephric Recovery %</th>
<th>Normal Uninephric Recovery %</th>
<th>Papillectomized Uninephric Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Σ%</td>
<td>Σ%</td>
<td>Σ%</td>
</tr>
<tr>
<td>Urinary</td>
<td>0 - 4</td>
<td>31.85</td>
<td>31.85</td>
<td>23.88</td>
</tr>
<tr>
<td></td>
<td>4 - 8</td>
<td>5.71</td>
<td>37.56</td>
<td>9b</td>
</tr>
<tr>
<td></td>
<td>8 - 12</td>
<td>12.92</td>
<td>50.48</td>
<td>21.60</td>
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<td></td>
<td>12 - 24</td>
<td>17.65</td>
<td>68.13</td>
<td>19.86</td>
</tr>
<tr>
<td></td>
<td>24 - 48</td>
<td>3.46</td>
<td>71.59</td>
<td>3.58</td>
</tr>
<tr>
<td></td>
<td>48 - 72</td>
<td>1.25</td>
<td>72.84</td>
<td>0.90</td>
</tr>
<tr>
<td>HCl trap</td>
<td>0 - 24</td>
<td>1.63</td>
<td>1.16</td>
<td>1.42</td>
</tr>
<tr>
<td></td>
<td>24 - 48</td>
<td>0.14</td>
<td>1.77</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>48 - 72</td>
<td>0.16</td>
<td>1.93</td>
<td>0</td>
</tr>
<tr>
<td>CO₂</td>
<td>0 - 24</td>
<td>2.09</td>
<td>2.09</td>
<td>1.37</td>
</tr>
<tr>
<td></td>
<td>24 - 48</td>
<td>1.18</td>
<td>3.27</td>
<td>1.24</td>
</tr>
<tr>
<td></td>
<td>48 - 72</td>
<td>0</td>
<td>3.27</td>
<td>0.13</td>
</tr>
<tr>
<td>Faeces</td>
<td>0 - 24</td>
<td>0C</td>
<td>0</td>
<td>1.56</td>
</tr>
<tr>
<td></td>
<td>24 - 48</td>
<td>1.21</td>
<td>1.21</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>48 - 72</td>
<td>0.19</td>
<td>1.40</td>
<td>0.09</td>
</tr>
<tr>
<td>Total</td>
<td>0 - 72</td>
<td>79.44</td>
<td>76.06</td>
<td>73.41</td>
</tr>
</tbody>
</table>

a  Dose of radioactivity 2.71 µCi (100 KBq) per rat
b  No urine passed from 4 - 8h
c  No Faeces passed from 0 - 24h
reported elsewhere (see 6.3.2).

The data suggests that partial papillectomy does very little to shunt the excretion of BEA away from the kidney to either the biliary system, to the liver for metabolism to \( \text{CO}_2 \) or to the lungs for exhalation as the volatile basic product(s). This favours the concept (5.3.8.2d) that the papillary tip is particularly sensitive to insult, either because of its morphology (i.e. its position in the kidney in relation to concentration gradients) or because the apex of the kidney has some specific or well defined biochemical function(s) which make them a target for certain types of toxins.

On the other hand, the kinetic data from spontaneously voided rat urine is subject to timing uncertainties where more subtle changes in urinary excretion would be masked by the variance. More importantly, the lack of information on the metabolites of BEA dictate that it is impossible to fully define any altered response because only total radioactivity is measured. Attempts to delineate some of the characteristics of the excreted BEA-derived materials are described below (see 6.5).

6.5 URINE AND BILIARY METABOLITES OF BEA-DERIVED RADIOLABELLED MATERIAL

The synthesis of radiolabelled BEA provided the essential "tool" for establishing the chemical nature of the excreted BEA-derived material.

Samples of urine and bile from studies described above (6.2-6.4) were stored frozen and pooled at the completion of each investigation, to be used for developing the separation techniques that would precede a full structural identification (a task beyond the scope of this dissertation).
6.5.1 Extraction of BEA-derived Radiolabelled Material.

6.5.1.1 Organic phase extraction from different pH buffered systems.

a) Buffer and solvent systems. Aliquots of BEA-derived material in urine (0.5mL) was mixed with an equal volume of 1.0M buffer in screw topped tubes with teflon liners. The buffers were glycine-NaOH, pH 10.0, Na-phosphate, pH 7.0, and acetate-acetic acid, pH 4.0. Organic phase extraction was undertaken using 1mL of hexane, benzene ethyl ether, chloroform and ethyl acetate; from each of the three pH systems in duplicate. Tubes were shaken for 20min, centrifuged at 3000 rev./min to separate the solvents. Both the organic solvent (0.5mL) and the aqueous phase (0.5mL) were transferred to glass scintillation vials, the organic solvent and aqueous phase were evaporated under a gentle stream of nitrogen (so as to remove quenching solvent material), 0.5mL water was added and the system counted as described (4.3).

b) Results. Less than 1% of the total radioactivity per tube was transferred to the organic phase. This was lowest for the apolar solvents (hexane 0.01%) and highest for ethyl acetate (0.73%). There was no obvious pH related effect in improving the transfer of radiolabelled material.

c) Discussion. The data suggests that all of the BEA-derived radiolabelled material is of a highly polar nature, where no functional cationic or anionic group could be converted to a unionised form.

The recovery data may, however, be misleading and represent an increasing solubility of aqueous phase in the less apolar solvents rather than extraction, because solvents were not dried before aliquots were taken for counting. Obviously, solvent-solvent extraction was inadequate for isolating BEA-derived material.

6.5.1.2. Extraction of BEA-derived, radiolabelled products from freeze-dried material. In the preliminary phase of this study it was found that at least 95% of radiolabelled material could be extracted
from rotary evaporated urine or bile using the methanol and sonification procedure described below. The likely thermolability of the BEA metabolites and the difficulties associated with rotary evaporation (frothing and bumping) indicated the necessity of a using freeze-drying method.

a) Rotary evaporation of urine and bile. Samples of urine and bile were rotary evaporated at 37 to 39°C and the viscous residue was dried (at about 1mm Hg) in a vacuum desiccator (over silica gel) for 48h. The clear "distillate" did not contain any radioactivity.

b) Freeze-drying of urine and bile. Distilled water diluted urine or bile (this was necessary because of their high solute contents which depresses the freezing point and caused thawing during the process of freeze-drying) was shell-coated and freeze-dried for 24 to 48h and stored at -20°C until it was used.

c) Solvent extraction. Aliquots of methanol were added to the container in which urine or bile had been dried and the container sonicated for 15 to 20 min periods at RT, after which the solid material was left to settle and the solvent aspirated off. This was repeated 4 or 5 times, the extracts pooled and evaporated down under pure dry N₂. The resulting viscous extract was stored at -20°C.

d) Recovered radiolabelled material. The large amount of solute left in dried urine and bile made it difficult to assess exactly how much radiolabelled material was not extracted. The recovery of radiolabelled material was estimated to be about 97% and 98% from methanol extract of rotary evaporated and freeze-dried products respectively.

d) Discussion. The absence of radioactivity in the distillate of the rotary evaporated products suggested by the lack of solvent extraction, that neither ethylenimmine nor the free bases of BEA or EA
were present in significant amounts in the urine. These compounds are volatile and would have distilled over.

6.5.2 Separation of BEA-derived Radiolabelled Material By T.l.c.

6.5.2.1 T.l.c. of urinary and biliary BEA-derived products. A methanol extract of the rotary evaporated urine and bile was used in the early part of these investigations for t.l.c. separation of BEA-derived material.

a) Thin layer chromatographic separation. Aliquots of the methanol extracts of dried urine and bile (20 to 50 μL portions) were spotted onto either cellulose or silica gel t.l.c. plates under a stream of dry N₂ (4.3.5) interspersed with BEA and EA radiolabelled standards which were run simultaneously. The solvent systems are described above (4.3.5.1).

Aliquots of aqueous material from the methanol extracted urine and bile were also spotted on the t.l.c. plates.

b) Autoradiographic visualisation of BEA derived material and detection of functional -NH₂ groups. The t.l.c. plates were dried and then placed against X-ray film as described (4.3.4.2) for 60 days, and developed. The t.l.c. plates were sprayed with fluorescamine to detect primary amine groups (4.3.5.4).

c) Results. Fig. 6.5.2.1a and b and Fig. 6.5.2.1a and b show chromatographic separation of BEA-derived material. Those areas with functional primary amine groups are marked "+".

The chromatograms which failed to give a possibly useful separation are not shown.

d) Discussion. The separation on cellulose (Fig. 6.5.2.1a and b) was well defined for BEA and EA standards, but BEA-derived material showed a number of component with concave and convex shapes. Similar, but less pronounced separation artefacts were apparent on silica gel
Fig. 6.5.2.1a. Autoradiograph of BEA-derived material separated on cellulose using solvent system iv.

1) BEA
2) EA
3) Residue left in extracted bile
4) Methanol extract of bile
5) Residue left in extracted urine
6) Methanol extract of urine

Broken line represent solvent front.
Fig. 6.5.2.1b. Autoradiograph of BEA-derived material separated on cellulose using solvent system ii. 1 to 6 are the same as Fig. 6.5.2.1a.
plates (Fig. 6.5.2.2a and b).

The EA showed several minor impurities and the BEA showed several minor radiolabelled components most probably because of chemical decomposition from prolonged storage.

The BEA-derived biliary and urinary material was free of both BEA and EA as assessed by both $R_f$ and primary amine reacting groups. Further, the dissimilarities between the pattern of $R_f$ and primary amine groups in biliary compared to urinary material suggests that few if any of the 6 to 8 metabolites in each were chromatographically the same. There was, however, a substantial amount of labelled material left at the origin which represent compounds which were not assessed.

N.B. It is not certain if differences between separated components in the methanol extract of bile and urine compared to the aqueous dissolved residue represents chemically different components or different interfering factors).

In view of the potential chemical reactivity of BEA the chromatographic profile of labelled components formed in vitro between BEA and urinary and biliary constituents was also investigated and described below.

6.5.2.2 Assessment of interaction between BEA and endogenous urinary or biliary substances. It was obviously necessary to establish if BEA reacted chemically with endogenous substances in urine and bile before a concerted effort was made to identify BEA metabolites.

a) Preparation of "spiked" biliary and urinary samples. A single Wistar rat (250g) was housed in a metabolic cage (4.1.1.2) for 24h and urine collected on ice. The rat was then anaesthetised (4.1.3.1), the bile duct cannulated (4.1.3.2) and bile collected for 4h. The urine (8mL) and bile (2.4mL) and saline were each "spiked" with 105 d.p.m. [1-$^{14}$C]BEA containing 8.2 and 8mg of nonlabelled material,
respectively in methanol. The material was left overnight at 4°C, rotary evaporated (6.5.1.2a) and methanol extracted (6.5.1.2c).

b) T.l.c. separation, autoradiography and visualisation. T.l.c. plates were spotted with "spiked" material as well as BEA and EA standards and both urinary and biliary BEA-derived material obtained as in (6.5.2.1). The plates were run, dried, autoradiographed for 90 days and visualised (6.5.2.1).

c) Results. Fig. 6.5.2.2a and b show the t.l.c. separation on silica gel. Separation on cellulose supports or in different solvent systems was inferior. NH$_2$ positive groups are marked "+".

d) Discussion. This investigation was qualitative, rather than quantitative, so no attempt was made to measure radiolabel recoveries etc. The large number of components in the BEA and EA were chemical impurities that had been formed over many months, fresh BEA had been used for spiking the samples.

The BEA-spiked saline produced a component which chromatographed the same as BEA, but both bile and urine contained several minor components which were chromatographically different to BEA. There were, however, no concurrence between these components (as far as R$_f$ and primary amine groups) and those extracted from urine and bile after BEA had been administered.

From this data it is obvious that BEA may react chemically with endogenous components. This approach cannot exclude BEA reacting with different endogenous substances in the in vitro and the in vivo situation. Similarly, it cannot exclude such chemical reactions between BEA congeners and "in vitro" or "in vivo" produced endogenous material.

This observation has two important consequences. Firstly, it highlights the need to collect specimens (for future structural
Fig. 6.5.2.2a. Autoradiograph of BEA-derived material separated on silica gel using solvent system i.

Fig. 6.5.2.2b. Autoradiograph of BEA-derived material separated on silica gel using solvent system ii.
elucidation) under conditions where labile products will be most stable. Secondly, it warns of the need to carefully evaluate all tentative structural data generated from excreted BEA to avoid chemical artefacts. Finally, it was uncertain if these artefacts were generated partially or entirely by rotary evaporating, a factor which prompted the study described below (6.5.3.2) in which spiked material was freeze dried.

6.5.3. Separation of BEA-derived Radiolabelled Material By Liquid Chromatography on a Molecular Exclusion Column.

BEA-derived radiolabelled material was originally applied to the Bio-Gel P-2 column to establish if:-

A) Any radioactivity eluted at the void volume. This would suggest that BEA was covalently bound to a urinary excreted macromolecular constituent, a factor which might be important in defining the molecular pathogenesis of the lesion (3.2.5.4).

B) There were any similarities in the distribution of radiolabel and uronic acid containing material to suggest that BEA affected the medullary PoG-GAG directly.

C) BEA could indeed polymerise to polycations as was suggested (see Fig. 6).

The radiolabelled material separated into 5 major and several minor components and provided a superior chromatographic system to answer several questions which could not be assessed on the t.l.c. systems already used.

6.5.3.1. Comparison of the BEA-derived radiolabelled components in a methanol extract of freeze-dried urine and in freeze dried urine.

a) Preparation of solutions. The unused urine from a rat treated with radiolabelled BEA from another study was divided into two approximately equal (5mL) aliquots, and freeze-dried (6.5.1.2b). One
was extracted with 1mL portions of methanol 5 times and the extracts pooled and made up to 5mL (6.5.1.2c). The other was reconstituted with 5.0mL 0.1 mol/L pH 6.0 Na phosphate buffer 4h before use to allow dissolution. About 4h before use 1mL of methanol extract was evaporated using dry N₂ and reconstituted with 1mL 0.1mol/L, pH 6.0 Na phosphate buffer.

b) Chromatographic separation and quantitation. Aliquots of 0.25mL were injected from a 0.5mL gastight syringe (Hamilton), through the septum port directly onto the column (4.6.2) and fractions collected at 5 min intervals for 12 h. Each fraction was counted individually with a known counting efficiency (Table 4.3).

In addition, triplicate 10 μL aliquots of the injected solution were counted (Table 4.3.1) so that the total amount of activity injected could be calculated and the sum of the radioactivities in each fraction used to ascertain the recoveries from the chromatographic separation.

c) Results. The estimated recovery of radiolabelled material exceeded 96%. Fig. 6.5.3.1 shows the radio-chromatographic profile for the methanol extract and for the unextracted urine.

d) Discussion. The high recoveries of injected material suggests that very little adsorption takes place. The profiles for the BEA-derived material from an extract of urine and from urine are most similar. There are, however, subtle differences between the two radiochromatographic profiles, but these may relate to slight changes in the compression of the gel. The peaks are sharp and relatively slight changes in retention time might result in a lot of counts going into the adjacent vial. Data presented below (see 6.5.4) suggests that at least some of these changes are due either:

i) to failure to extract the missing peaks using methanol,

ii) due to chemical decomposition when the dried samples were
Fig. 6.5.3.1. Comparison of the radiochromatographic elution profiles of BEA-derived material in urine and the methanol extract of freeze-dried urine.
extracted with methanol, or

iii) solubility problems/chemical decomposition when the methanol extract was taken to phosphate buffer.

Perhaps the most significant finding is that very little radiolabelled material (less than 0.1%) was of high enough molecular weight (greater than 2000 daltons) to be excreted at the void volume. Further this was present in both the urine and its methanol extract. It therefore seems unlikely that BEA-derived material which is covalently bound to kidney structural macromolecules is excreted in the urine unless the macromolecules are largely degraded prior to excretion.

6.5.3.2. Comparison of the chromatographic behaviour of bile and urine BEA-derived radiolabelled material.

The small but significant biliary excretion of BEA-derived radiolabelled material has already been discussed (6.3) in the light of the low molecular weight of the parent compound. The separation of labelled urinary material into several different molecular weight components prompted a similar study on biliary excreted BEA-derived material.

a) Preparation of bile and urine. A small amount of pooled bile from previous studies (that had been stored at -20⁰C) was thawed, left at RT for 4h with mixing, centrifuged (3000 rev./min for 20 min) and used. Freeze-dried urine was prepared as described above (6.5.3.1a).

b) Chromatographic separation and quantitation. Aliquots of bile or urine were injected, fractions collected and quantitated as described above (6.5.3.1b). Separate aliquots were also counted for estimation of recoveries.

c) Results. The recovery from urine was 98% but from bile only 92%, Fig. 6.5.3.2 shows the radiochromatographic profile for bile and urine.

d) Discussion. More of the biliary BEA-derived components eluted
Fig. 6.5.3.2. Comparison of the radiochromatographic elution profiles of a methanol extract of freeze-dried biliary and urinary BEA-derived material.
from the column between 2 and 5h and less from 5 to 7h than was the case for urine. This confirms the t.l.c. data presented below that there are few, if any similarities between biliary and urinary BEA-derived material. It was not possible to establish which of these components also formed between bile and BEA in vitro.

The shorter elution times (i.e. higher molecular weight) of these components in bile probably explains why they had been excreted by this route. The future study of BEA bioconversion will thus have to establish the full structural identity of these biliary components before the metabolism of urinary BEA-derived material is fully described.

6.5.3.3. **An assessment of in vitro BEA stability using liquid chromatography.** BEA is an unstable chemical in solution and, therefore, some of the BEA-derived urinary material might represent either polymers or products that had been formed chemically from endogenous urinary substances and either BEA or one of its unstable congeners. The stability of BEA was studied in both urine and in buffer.

   a) **Spiking of urine and buffer.** Aliquots of approximately $10^5$ d.p.m. of $[1-^{14}C]$BEA in 2mg of non-labelled material (dissolved in methanol) was transferred to 25mL sterile plastic containers (Sterilin, London) and evaporated with $N_2$.

   A male Wistar rat (200g) was housed in a metabolic cage (4.1.1.2) and urine collected directly onto the labelled BEA, at $0^\circ$C for 24h. Part of the urine was used, the rest freeze-dried and reconstituted with 2.5mL of 0.1 mol/L, pH 6.0 Na phosphate buffer.

   The stability of BEA was also assessed in 0.1 mol/L, pH 6.0 Na phosphate buffer 24h after reconstitution, and after freeze drying and reconstituting again.

   b) **Chromatographic separation and quantitation.** Aliquots of 0.15 mL were injected, separated, fractionated, quantitated and recoveries
estimated as described (6.5.3.1b) for the following:-

i) Spiked pH 6.0 buffer - 24h at 0°C

ii) Spiked pH 6.0 buffer freeze-dried and reconstituted.

iii) Spiked urine collected 24h at 0°C.

iv) Spiked 24 h urine, freeze-dried and reconstituted.

c) Results. Fig. 6.5.3.3a-d shows the radiochromatographic profile for BEA in the 4 different systems studied.

The total number of d.p.m. differs depending on the volume from which the sample was taken (e.g. the 24h urine volume was about 8mL, whereas the other volumes were 2.5 mL). The recoveries of eluted material were in excess of 95% of the injected material.

The freshly prepared BEA in buffer showed two minor "leading" shoulders (Fig. 6.5.3.3a) but these were increased after freeze-drying and reconstitution (Fig. 6.5.3.3b). The spiked urine showed both a leading and a tailing component (Fig. 6.5.3.3c). After freeze-drying, however, there was a most substantial loss of the original component (which eluted at 6h) in favour of a series of higher molecular weight substances (Fig. 6.5.3.3d).

d) Discussion. The 0.1 mol/L, pH 6.0, Na phosphate buffer was used as the liquid phase in which to assess BEA stability because it was the liquid phase used for the column chromatographic system, and, fortuitously it also provides a pH similar to that observed in rat urine after BEA dosing (see section 6.1).

BEA appears to be relatively stable in both pH 6.0 buffer and in rat urine (pH 7.3) at 0°C for 24h. Freeze-drying and reconstituting, altered the molecular weight distribution only slightly in buffer but caused most dramatic changes in the urine profile.

It is probably not valid to extrapolate these findings on BEA
Fig. 6.5.3.3. Comparison of the stability of radiolabelled BEA in buffer (pH 6.0, 0.1 mol/L phosphate) and in urine.

a) BEA spiked into buffer 0°C 24h
b) BEA spiked into buffer 0°C 24h, freeze-dried and reconstituted
c) BEA spiked into urine 0°C 24h
d) BEA spiked into urine 0°C 24h, freeze-dried and reconstituted
stability to the urine of a BEA treated rat, because the urinary constituents differ from a non-BEA treated rat, the pH is lower and BEA metabolites may be more or less stable than the parent compound. The major finding of significance is that freeze-drying may drastically alter the nature of BEA-derived material and might have contributed artefacts (the exact nature of which are not well understood) to the studies described so far and those reported below.

6.5.4 The Effects of Selective Enzymic Digestion on the Chromatographic Separation Profile of BEA-derived Radiolabelled Material.

A preliminary attempt to assess the effect of sulphatase and beta-glucuronidase digestion on BEA-derived material had proved inconclusive because of the artefacts associated with the t.l.c. separation. The successful separation of several components by column chromatography (6.5.3) offered an improved approach.

a) Enzymic digestion of BEA-derived material. Aliquots of reconstituted freeze-dried urine (0.20mL) were placed in 1mL Reactive-vials (Pierce & Warriner, Cheshire) with PTFE lined screw top closures. An equal volume (0.20mL) of enzyme or buffer only was added. The enzymes were:

i) Sulfatase (Sigma, Poole) 200 Units plus 5mg saccharolactone (Sigma, Poole) in 0.2 mol/1 Na acetate buffer, pH 5.0.

ii) Ketodase (W. Warner, Hampshire) 1250 Units in 0.2 mol/1 Na acetate buffer, pH 5.0.

iii) Trypsin (Sigma, Poole) 7000 BAEE Units in 0.1 mol/L Na phosphate buffer, pH 7.4.

iv) Collagenase (Sigma, Poole) 1250 Units in 0.1 mol/L Na phosphate buffer, pH 7.4.

v) Hyaluronidase (Sigma, Poole) 1000 NF Units in 0.1 mol/L Na phosphate buffer, pH 7.4.
vi) Chondroitinase ABC (Sigma, Poole) 5 Units, in 0.1 mol/L Na phosphate buffer, pH 7.4.

vii) Chondroitinase AC (Sigma, Poole) 5 Units, in 0.1 mol/L Na phosphate buffer, pH 7.4.

These were digested at 37°C for 24h and then frozen and stored at -20°C until they were required. In addition two vials of urine only were mixed with an equal volume of pH 5.0 acetate buffer and another two with pH 7.4 phosphate buffer only. One at each of these pH values was incubated for 24h and the other frozen at -20°C until used.

b) Chromatographic separation and quantitation. The frozen control vials or enzymic digests were thawed before use, left for 1h at RT to allow dissolution and centrifuged (3000 rev./min. for 20 min) to remove all the particulate material. Aliquots of 0.15mL (only 0.10mL in the case of sulfatase which is a most insoluble enzyme) of the supernatant was taken into a gastight syringe, injected, separated etc. as described above (6.5.3.1b).

c) Results. There were essentially no differences in the radiochromatographic profile of the separation using either the pH 5.0 or the pH 7.4 buffered material both before and after incubation at 37°C for 24h. A representative separation is shown in Fig. 6.5.4a. Sulphatase caused no change (Fig. 6.5.4b), but all the other enzymic digestion caused the loss of one or more components eluting at about 6 or 7h in favour of an increased amount of material (of higher molecular weight) eluting between 3 and 5.5h (Fig 6.5.4c-h).

d) Discussion. These results are difficult to interpret. Selective enzymic digestion would have been expected to release a radiolabelled molecule from its sulphate or glucuronide conjugate, or to cleave off carbohydrate and/or peptide groups. The nett effect would be a reduced molecular weight and a longer elution time. All the enzymic related
Fig. 6.5.4. Comparison of the effect of enzymic digestion (24h at 37°C) on the radiochromatographic elution profile of BEA-derived material in rat urine. Shown are:-

a) Control urine
b) Sulphatase digested
c) Ketodase digested
d) Trypsin digested
e) Collagenase digested
f) Hyaluronidase digested
g) Chondroitinase ABC digested
h) Chondroitinase AC digested.
changes, however, were a shift from low to higher molecular weight. This suggests that molecules may, in fact, be binding to urinary or enzymic components such as polypeptides or oligosaccharides, (this seems unlikely because the proteolytic or carbohydrate degrading enzymes should digest these species). Alternatively enzymic digestion may release a BEA-derived molecule which can then react with another carrier component in urine covalently or, perhaps, hydrophobically or the chromatographic separation of these components may not have been based on purely molecular weight characteristics. In any event these changes are minor and account for the loss of only a few percent of the undigested BEA-derived material.

6.6 THE EFFECT OF 2-BROMOETHANAMINE ON THE URINARY AND MEDULLARY PROTEOGLYCAN-DERIVED MATERIAL.

BEA caused a marked increase in the histochemical staining of the medullary PoG-GAG matrix (5.2) followed by losses which were confined to that area of the papilla which underwent necrosis. The studies described in this section were undertaken to confirm these changes biochemically.

6.6.1 The Effect of BEA on the Polydispersion of Urinary Uronic Acid Containing Material.

The molecular weight distribution of urinary substances containing uronic acid (as a specific PoG-GAG marker) were followed in response to BEA insult.

a) Animals and treatment. Three male Wistar rats (220-230g) were housed for a 24h acclimatisation period in metabolic cages (4.1.2.2) where food and water were freely available. Urine was collected at 0°C from each rat for 24h before ip dosing with 100mg/kg BEA (4.1.1) and thereafter for 4 periods each of 24h. Immediately after collection the urine was frozen and stored at -20°C until the experiment had been concluded, when it was freeze-dried.
b) Analysis of the polydispersion of uronic acid containing material. Each lyophilised urine sample was reconstituted on the day of use with 10mL 0.1mol/L phosphate buffer, pH 6.0 as described (4.6.4) left to dissolve at RT for 4h and both sterilised and cleared of much inorganic particulate material (which was insoluble) by passage through a 0.22 μm filter. Aliquots (0.25mL) were applied to the Bio-gel P2 column via a 3-way tap and the eluant was monitored continuously for uronic acid at 520 nm using the borate-carbazole assay (see 4.6).

c) Results. Fig. 6.6.1 a-d shows a typical series of traces for different molecular weight components containing uronic acid for the 24h collection period before and for four consecutive 24-h collection periods after BEA.

d) Discussion. The BEA caused a sustained marked increase in the high molecular weight component that eluted at 2h. The loss of intermediate and low molecular weight material over the first 48h after BEA treatment may relate to the loss of papillary MPS staining. Data presented below (6.6.2) confirms that BEA caused loss of $^{35}$S from the medulla but this could represent desulphation of the linear carbohydrate chain. The increase in intermediate and low molecular weight components between 48 and 96h may represent a typical compensatory response by the kidney, where the remaining viable interstitial cells were synthesising more PoG-GAG.

The paucity of data on medullary interstitial PoG-GAG dynamics does, however, make it difficult to give meaning to these data without recourse to other techniques (see 7.3).

6.6.2 The Effect of BEA on Medullary Proteoglycan Derived Material.

Experiments which will not be reported here showed that both $^{35}$SO$_4^{2-}$ and $^{14}$C-glucosamine (given ip or sc in single or multiple doses) formed part of a complex series of metabolic pools before they were excreted in
Fig. 6.6.1. Comparison of the elution profiles of urinary material containing uronic acid (measured by the borate-carbazole assay, and separated on the basis of molecular weight) from a rat treated with 50mg/kg BEA ip. The traces represent normalised responses for:

- a) 24-h control period
- b) 0 to 24h,
- c) 24 to 48h,
- d) 48 to 72h, and
- e) 72 to 96h after BEA.
urine, faeces and (for glucosamine, breath). The administration of BEA to animals with radiolabelled PoG-GAG failed to show any differences in metabolic profiles of urinary, faecal or breath excreted label probably because of:-

A) The small amount of labelled material incorporated into the papilla, compared to that incorporated into the rest of the body (less than 0.01%) even when given sc.

B) The rapid turnover of labelled material between different molecular weight groups in the urine.

The complexities of, and lack of data on, medullary PoG-GAG biodynamics dictated that only very limited interpretation could be made of changes in its radiolabelled material at this stage. Possible directions to be followed in future, to better define medullary PoG-GAG dynamics and methods for "specifically" radiolabelling of this tissue are discussed below (see 7.3).

6.6.2.1 Time-course changes in tissue sulphate turnover was assessed as a gross measure of PoG-GAG changes.

a) Animals and treatment. A group of 9 male Wistar rats (150g) were each injected sc with 300 μCi of carrier-free $^{35}$SO$_4$ (Amersham International, Amersham).

b) Tissue radiolabelled sulphate and its quantitation. Rats were killed intermittently up to 5 days (see below) at this time 2 of the remaining 4 rats were treated with 50mg/kg BEA ip. The radiolabelled sulphate treated animals (number in parenthesis) were killed at 1(1), 3(2)5,(2) and 8(4) days (2 of which had been BEA treated) by cervical dislocation, opened by a mid-line incision and the following organs removed kidney, bladder, liver, heart, lung, spleen and thigh muscle. All the non-renal tissue was snap frozen (4.2.3). The kidneys were held on both dorsal and anterior faces and a slice of tissue (about 2mm wide)
was cut sagittally through the centre of the kidney, from the pelvis to the outer circumference using two blades held parallel in a device similar to that described by Simonnet et al. (1980). This slice of tissue (which contained the papilla) was also "snap frozen". It took about 20 to 30s from the time of killing to have both slices of kidney frozen.

The tissue was kept at -30°C and duplicate samples of up to 100mg were transferred to weighed glass vials, reweighed, digested (4.3.3) and counted; each vial was recounted with an internal standard (4.3). The slice of kidney was separated into cortex, corticomedulla and medulla and each counted separately.

c) Results Table 6.6.2 shows the amount of $^{35}$S label in each tissue at 1, 3, 5 and 8 days without BEA treatment and when BEA was given on day 5 and animals killed on day 8.

d) Discussion Radiolabelled sulphate provides a more specific marker of PoG-GAG (Kennedy, 1979) than does a $^{14}$C-carbohydrate precursor (e.g. glucosamine), which, in the medulla, may also be incorporated into glycogen (Darnton, 1969a) or particularly into glycoprotein such as Tamm-Horsfall glycoprotein (Dawnay, 1981).

The high cortex and especially the high medullary sulphate levels in the first few days, probably represents filtered and urinary $^{35}$SO$_4^{2-}$ this would explain the marked decreases by the 5th day.

The residual radiolabel in the papilla of BEA treated animals was about half of that in non-treated controls. In contrast the radiolabel in other tissues was similar, irrespective of treatment, and any differences were of the same order as that seen for duplicates within the same group (i.e. no more than 10 to 15% of their means).

These results confirm biochemically that after BEA treatment loss of MPS staining relates to loss of the $^{35}$S, which could only be presented as
Table 6.6.2

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Non-BEA treated d.p.m. per 100mg tissue on day</th>
<th>BEA treated d.p.m. per 100mg tissue on day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1*</td>
<td>8*</td>
</tr>
<tr>
<td>Papilla</td>
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<td>19558</td>
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<tr>
<td>Corticomedulla</td>
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<td>9432</td>
</tr>
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</tr>
<tr>
<td>Spleen</td>
<td>52254</td>
<td>2871</td>
</tr>
</tbody>
</table>

a  BEA (50mg/kg) given on day 5 after $^{35}$SO$_4^{2-}$ was given (300μCi/rat)

*  Single value

†  Mean of two different animals.
35\textsuperscript{S}O\textsubscript{3}\textsuperscript{−} on GAG. These would probably serve to bind colloidal iron or interact with one of the other MPS-specific stains. Because of the complexity of the PoG-GAG molecule, however, this relatively unsophisticated approach can give little additional information. Autoradiographic techniques, would have to be used to establish that the loss of 35\textsuperscript{S} is, indeed, from necrosed regions of the papilla. It is possible that BEA removes the -SO\textsubscript{3}\textsuperscript{−} groups off the carbohydrate backbone. At first consideration similar studies using a labelled carbohydrate precursor could better define these changes but carbohydrates are not confined to the PoG-GAG pool only (see above) and the low incorporation of less than 0.01% makes the use of \textsuperscript{14}C material unpractical from a cost point of view especially if the morphological significance of these changes were to be assessed autoradiographically.

Possible alternative ways to get more radiolabelled precursor into the papilla are discussed below (7.3.3).

6.7 \textbf{CHAPTER SUMMARY}

BEA causes a marked sustained diuresis within the first hours of administration during which time the urinary pH fell to about 6.0 and urine osmolality decreased to 300-400 mOsm/kg from about 1000 mOsm/kg.

The macroscopic distribution of BEA-derived radiolabelled showed that intraperitoneally administered BEA-derived radiolabel was rapidly absorbed, there was no \textsuperscript{14}C material left in the peritoneal cavity after 2h. The BEA-derived material was distributed most prominently in the bladder, the stomach and upper gastro-intestinal tract and the kidney. Significant amounts were also present in the liver. The light microscopic distribution of radiolabelled material showed a modest two to three fold concentration in the medulla, but freezing artefacts destroyed the morphology, most likely caused some translocation and prevented more definitive localisation.
Intravenously administered [1-\(^{14}\)C]BEA was excreted in both the urine (21 to 30%) and in the bile (4.65% after 100mg/kg and ±10% after 50mg/kg) during the first 5h in anaesthetised rats. In conscious rats between 71 and 76% of the administered dose was recovered in 72h; 66 to 81% in urine, 3 to 5% as CO\(_2\), 0.5 to 2% trapped in HCl as an alkaline breath component and 1 to 3% in faeces. Between 71 and 95% of total urinary excreted radiolabelled material was excreted in 24h. The excretion profile was similar for different routes of administration and largely unaffected by pretreatment, except where non-labelled BEA (50mg/kg) had been given 24h before, in which case the total urinary excretion increased to 84% of the dose administered.

The partially papillectomized kidney handled the BEA load indistinguishably from the normal kidney.

BEA-derived radiolabelled products could not be extracted into organic solvent, irrespective of the pH. Most of the radiolabel was, however, extracted into methanol from a freeze-dried or rotary evaporated residue. Bile and urinary material were each resolved into at least 6 to 8 components on t.l.c., but there were no chromatographic concurrence between materials from the two excretory routes. Liquid chromatography on a molecular exclusion column resolved urinary BEA-derived substances into 3 major and several minor components, generally of higher molecular weight than the parent compound. The biliary material separated into a similar number of components, but there was a predominance of higher molecular weight material. The digestion of urinary BEA-derived material with deconjugating enzymes (β-glucuronidase and sulphatase) and with proteolytic and GAG degrading enzymes caused only minor changes in the chromatographic profile of labelled material. BEA is relatively stable in acidic buffer, but reacts chemically with endogenous urinary substances, a change which was more
prominent after freeze-drying.

BEA-caused a crystaluria which consisted of a magnesium ammonium potassium phosphate complex within a few hours of administration to anaesthetised rats. The polydispersion of uronic acid containing urinary material varied substantially in the period that followed BEA treatment. The high molecular weight material increased, intermediate weight material decreased and then rebounded, but the final post-treatment pattern of polydispersion differed from control. BEA caused a substantial loss of $^{35}$S from the papilla but not from other tissue.
CHAPTER 7

THE MOLECULAR PATHOGENESIS OF
2-BROMOETHANAMINE—INDUCED RENAL PAPILLARY
NECROSIS, ITS SIGNIFICANCE TO OTHER TYPES OF
CHEMICALLY INDUCED PAPILLARY NECROSIS IN ANIMALS AND MAN
AND POSSIBLE FUTURE RESEARCH DIRECTIONS

Toxicology is a developing science where all too frequently an observed biological response has been interpreted as the major event underlying the molecular changes which lead to the development of a lesion. It is often too easy to provide a cogent explanation (via, both misguided inductive or misconstrued deductive reasoning) to link such a change with the final effect. It is much more difficult to prove cause, especially when (as has been the case) too much of toxicological research has used a uni-disciplinary "shot-gun" approach to probe multidimensional and many faceted problems. Too often concepts derived from such research efforts are so deeply ingrained in scientific literature that generations of "scientific" imitators establish them as dogma.

The discussion that follows will try to avoid these pitfalls and will attempt to develop a working hypothesis to explain the molecular pathogenesis of RPN (and its sequelae), in both the acutely and the chronically induced lesions, in experimental animals and in man.

7.1 THE MOLECULAR PATHOGENESIS OF 2-BROMOETHANAMINE-INDUCED RENAL PAPILLARY NECROSIS

This project was undertaken to define specific aspects of the histopathological and biochemical changes associated with the use of
BEA, a model compound which caused RPN within 24 to 48h.

7.1.1 The Absorption, Distribution and Excretion of BEA.

BEA was shown (6.2) to be rapidly absorbed from the intra-peritoneal site of administration and the urinary excretion profile of BEA-derived material was similar when it was given either ip or sc which suggests an equally rapid uptake from the sc sites. The urinary excretion profile from a rat given BEA po showed some delay in reaching a peak urinary excretion. This might be explained by a delayed absorption, or by an enterohepatic circulation, where more of the orally administered BEA would undergo a "first pass effect" and, therefore, delay its entry into the systemic circulation (see also 7.1.2 below).

The autoradiography showed that intra-peritoneally administered BEA-derived radiolabelled material was present in largest amounts in the stomach and upper gastro-intestinal tract, the kidney and most particularly the bladder. Significantly less labelled material was present in the liver.

This might be explained by a movement of BEA-derived material from the circulating blood into the stomach or by the upper gastro-intestinal tract wall secreting BEA-derived material from the peritoneal cavity and (perhaps) from the liver. This would establish an important vascular and extra-vascular flux which would be expected to contribute to both the hepatic uptake and excretion (see also 7.1.2).

The BEA-derived material was very largely excreted via the kidney. Up to 89% of the excreted dose (70% of the administered dose) was excreted in the urine, 96% in the first 24h (6.3). This fulfills the toxicokinetic requirement for producing a direct nephrogenic effect by presenting the kidneys with substantial quantities of BEA-derived material. Assuming that 30 to 40% of a 50mg/kg dose was excreted in the first 4h, during which the urine volumes were typically 6mL in the
In conscious rat the concentration of BEA derived material would be of the order of 15-30 mmol/L (see 6.3 to 6.4 for these figures). BEA does, however, give rise to a number of chromatographically separable species. Thus assuming that any of these 3 to 6 major components is pure, no single BEA-derived congener would exceed a concentration of about 2 to 10 mmol/L.

The defining of these high urinary concentrations of BEA-derived material contribute very little to the understanding of the molecular pathogenesis, per se, because neither the chemical nature of these components is known nor is the precise localisation of total radioactivity at a cellular level established.

Murray et al. (1972) first suggested that the cyclization of BEA to EI was a mandatory step in the process of BEA-induced RPN. The hypothesis has not, however, been proven. Wright & Rowe (1967) are the only workers to have published data on the distribution and metabolism of $^{14}$C-EI. There are certain similarities between the absorption, distribution and excretion of EI and BEA (metabolism is considered below 7.1.2), but there are also marked differences.

Wright & Rowe studied the organ distribution of EI at 24 and 96h. Particularly large amounts of activity were found in the liver, and also the pancreas, kidney, spleen, red cells and bone marrow at 24h. Very large residues of radiolabel were also found in the upper gastrointestinal tract, following an intra-peritoneally administered dose. The persistent labelling of liver, pancreas, kidney, spleen and red cells at 96h shows that EI-derived radiolabel was part of a "metabolic pool" with a slow turnover. No attempt was made to define how EI was distributed within the kidney.

The excretion of EI, in common with BEA, included an exhaled basic component (this was not identified) about 1 to 2%, as well as CO$_2$ (3 to
5%), and a low faecal recovery (1 to 5%). The urinary recoveries were, however, lower (50%) although most of the C-14 had, in common with BEA, been excreted in the first 24h. The total recovery was of the order of 55 to 60%, a value much lower than BEA. This discrepancy would most likely be a consequence of macromolecular alkylation by EI. The protracted labelling of the liver, spleen, pancreas, kidney and erythrocytes probably reflect this alkylation.

While the stomach pH may have a marked stabilising effect, the potential for BEA to cyclize could be realised either before it reached the stomach or once it left it. Thus although there are some marked differences between the absorption, distribution and excretion of EI it is impossible to exclude the possibility that at least part of the administered BEA was converted to this aziridine ring, without recourse to metabolism.

7.1.2 The Metabolism of BEA

A substantial amount of the total dose (about 80%) of labelled BEA was recovered, mostly in the urine, but none as BEA or EA. The site and type of BEA metabolism is uncertain but some of this compound may undergo intestinal or hepatic metabolism to form part of an enterohepatic circulation. Up to 10% of administered BEA was recovered in the bile within 5h of administration, but no more than 4% was recovered in faeces in 72h. It is likely that more BEA-derived material passes through the biliary system than was, in fact, observed in the anaesthetised rat, where the bile flow rates decreased substantially over the duration of the procedure, a change which may have limited biliary excretion.

The enigmatic biliary excretion of a low molecular weight compound such as BEA (6.3.1) is only partially explained by the higher molecular weight BEA-derived material in bile (6.5.3.2). The presence of this
chromatographically different material in bile substantiates the extra-
renal metabolism of some BEA. These intestinal or hepatic metabolites
appear to be unrelated to the *in vitro* papillotoxic effects of BEA,
however, because neither inducers nor inhibitors of mixed functional
oxidase activity (particularly hepatic) perturbated the necrotic
lesion.

The different chromatographic profile of BEA-derived radiolabelled
material in bile and in urine, together with the low faecal recoveries,
suggests that the biliary products undergo other metabolic processes
before urinary excretion. The failure to identify either sulphate or
glucuronide metabolites in urine suggests that other types of
metabolism occur (see Fig. 6). Few of the metabolites separated by
t.l.c. gave a positive primary amine reaction with fluorescamine
(6.5.1) which suggests that if a glutathione conjugate was formed
initially it may be present as the metabolised mercapturic acid or that
the primary amine group of the BEA-derived product is altered (e.g.
acetylated).

Only a relatively small amount of BEA-derived CO₂ was excreted. This
suggests that the C-1 position is protected from metabolic degradation.
No attempt was made to study the volatile basic component that was
exhaled.

These metabolic studies shed little light on the likely conversion
of BEA to EI *in vivo*. It is tempting to compare the amount of CO₂
derived from EI, but it cannot be ascertained from the data of Wright &
Rowe (1967) if the molecule was labelled on both or only one carbon
atom. In common with our findings for BEA-derived material urinary EI-
derived material could not be extracted into organic solvents at
acidic, neutral or basic pH. EI-derived material was resolved into 2
components on an anion exchange column, one of which was shown to form
at least 4 components on paper chromatography using a solvent system which gave no separation of BEA-derived material in our hands. The other was said to separate into "a large number" of components, but no further data was given (Wright & Rowe, 1967). A comparison of the BEA and EI metabolism data, therefore, gives no equivocal data on the conversion of BEA to EI.

It is thus impossible to be certain if BEA or its cyclized congener EI is the proximate or the ultimate papillotoxin. We have been unable to find any volatile BEA-derived radiolabelled component in urine, which suggests that no EI is excreted per se. Similarly there was no BEA excreted in urine (as assessed by t.l.c.) per se. The data presented above (6.5.3.3) does, however, show that BEA reacts with endogenous urinary compounds. Thus if BEA was excreted it might undergo chemical change either in the urine or during isolation. The same is likely for EI.

The elucidation of the structure of the major BEA chemical reaction products and biological metabolites will give an important insight into the molecular pathogenesis of RPN. This will confirm some of the possible routes suggested in Fig. 6. More importantly it will give some idea of the formation of covalent bonds with endogenous substances, which might disrupt function or structure. This is also a necessary pre-requisite to identifying "proximate" papillotoxic BEA derived molecules for subsequent assessment. Finally, it is only once the chemical structures of these compounds has been elucidated that the distribution of radiolabelled carbon atoms derived from the specifically tagged 2-bromo[1-14C]ethanamine can be devised. This will answer the question on the role of EI formation as an intermediate in the production of the dehalogenated products. If the carbon atom adjacent to the amine is the only one to carry the label, then EI could
not have been an intermediate. If the radioactivity is distributed between C-1 and C-2 it would suggest that EI was an intermediate, and if the label is equally distributed EI would be an obligatory intermediate.

7.1.3 BEA in Relation to the Commonly Investigated Mechanism Underlying Papillotoxicity.

The various theories that have been suggested to explain the biochemical mechanisms underlying the pathogenesis of RPN have been reviewed above (3.2.5). It has not been possible to assess each of them critically with respect to the BEA-induced RPN, but some general conclusions can be drawn.

Autoradiography at the light microscopic level suggested that BEA-derived material was concentrated only modestly across the kidney (6.2.2). The freezing artefacts (associated with the tissue changes which preceded the development of the lesion) prevented precise information on the distribution of the BEA-derived material in relation to medullary morphology. It is only when this information is available that the question of the significance of the counter current concentration effect on BEA-derived material can be answered equivocally.

The micro-vasculature of the medulla showed no early vascular occlusion after a BEA insult, even when the necrotic changes in the contralateral kidney were quite substantial. Thus vasospasm does not appear to be a contributing factor to the development of anoxia or ischaemic injury that might cause RPN. The colloidal carbon filling method used in this study (5.5) could not, however, differentiate anoxia that developed as a consequence of haemostasis caused by the vaso-dilatation which was observed. Published plasma flow data after a BEA insult does not, however, appear to support haemostasis (Solez et
al., 1974 and Vanholder et al., 1981). These studies cannot, however, exclude the possibility that BEA insult might cause an markedly increased oxygen requirement.

The possible role of an enzymic conversion of BEA to a biologically reactive intermediate cannot yet be completely resolved. This study failed to identify BEA-derived radiolabelled material as part of a urinary macromolecule (greater than 2000 daltons). This suggests that if a nucleophile was generated, it either bound to a structural or functional component which was not lost to the urine per se (a possibility which seems unlikely because of the substantial destruction of the medulla and the marked diuretic response) or it bound to micromolecules. This seems more likely and may explain the increased molecular weight of BEA-derived products (7.1.2) especially that excreted in the bile. These may not be fragments of macromolecules, because proteolytic and glycolytic enzymic digestion failed to alter the chromatographic profile of BEA-derived urinary products.

There is no direct data to support or deny the interaction between BEA and the prostaglandins. The exacerbation of the BEA-induced RPN by analgesic pretreatment (5.3.4) may be totally unrelated to the inhibition of PGE$_2$ synthesis (3.2.5.3) and relate rather to one of the other many metabolic effects caused by these compounds (see below, 7.1.4).

No attempt was made to study the effect of BEA on the intermediate metabolism of the medulla, because of the problem of cellular heterogeniety. Possible future approaches to resolving this problem are discussed briefly below (7.3.4).

Very recent investigations (once again outside of the scope of this dissertation) have shown that there is an immunological response to BEA insult. The immunoperoxidase method has shown that substantial
Deposits of IgM (but not IgG or IgA) occur in the necrosed medulla and some glomeruli within 3 days of administering BEA. Surprisingly, this pattern of immunodeposits (only IgM) is maintained, almost unchanged, for at least 54 days. The earliest deposits appear to be a consequence of the BEA insult and do not seem to contribute to the lesion, but may play some role in the sequelae.

At this stage it is not possible to explain why other immunodeposits do not follow IgM. It seems reasonable to speculate that the IgM deposits form in response to the lesion, but the rapid destruction of the medullary microvasculature prevents further immunological responses from occurring.

7.1.4 The Effect of BEA on Proteoglycan, Glycosaminoglycans and Tamm-Horsfall Glycoprotein.

The histopathological data from this study (5.1) and from other studies on BEA (Wyllie et al., 1972) EI (Ham & Tange, 1969) involving fat-free diet and aspirin (Molland 1978a,b), the biphenyl (Hardy 1970a,b, 1973) and human analgesic abusers (Burry et al., 1977) have highlighted the resistance of the collecting duct urothelia and that covering the papilla to necrosis. The earliest medullary elements to have been necrosed were the loops of Henle, the capillaries and the interstitial cells. It is, however, difficult to be certain if the intact urothelia represents the original cells or a population which had re-epithelialised the basement membrane (Cuppage & Tate, 1975).

The increase in MPS staining intensity, followed by loss of staining, has not previously been described for BEA-induced RPN. The exact nature of this biphasic change still has to be fully studied, but several preliminary comments can be made. BEA causes a marked change in urinary PoG-GAG-derived material. There is a sustained increase in a high molecular weight material, and an early marked loss of substances
of intermediate molecular weight, followed by a rebound in which one of
the components was absent (6.7.1). BEA also caused a loss of
radiolabelled sulphate from the papilla tip, but not from other renal
and non-renal tissue.

Because of the paucity of data on the dynamics medullary PoG-GAG, it
is necessary to interpret these observations speculatively. Other
techniques will be required to probe the exact molecular changes.

Previously published data, for example, the electronmicroscopic
study of Hill et al., (1972) give no insight to these changes because no
effort was made to fix PoG-GAG in situ, which we now know to be
essential (Furusato, 1977).

It seems likely that the early increase in staining intensity
represents conformational changes where more staining sites are
available rather than increased synthesis or decreased degradation
because of the rapid nature of the change. This is consistent with the
formation of urinary crystals reminiscent of magnesium ammonium
phosphate, but actually being atypical and containing large amounts of
potassium. This could arise from a disruption of the polyanion ion
exchange nature of the ground substance, a concept supported by the
marked cryoprotective effect of BEA (6.2.2) and the sudden diuresis it
causes (6.1) when large amounts of solute were released to solution.
The exact cause and nature of these changes awaits future research. They
could arise from direct effects on the interstitial or other cells, or on the
matrix itself.

Similarly, the loss of staining needs to be fully defined. It may,
for example, represent only the loss of sulphate anionic groups (6.7.2)
as shown by the experiments described above. It was not possible to
define the changes in terms of loss of labelling from the polysaccharide
back-bone of the GAG, because it was not practicable to get enough $^{14}C$
precursor incorporated. The marked changes in urinary uronic acid (6.7.1) do, however, suggest that this system is markedly perturbated by BEA, but the exact change is still to be established. The loss of staining may relate to failure of the renal interstitial cells to synthesise "charged" PoG-GAG, from the leakage of proteoglycan degrading enzymes from medullary or cortical elements of the kidney or, alternatively due to the activation of a poorly understood oxidative-reductive depolymerisation which can occur in many types of macromolecules, especially the glycosaminoglycans (Pigman et al., 1961).

Whatever the cause, the consequences might be expected to be far reaching. They would explain the diuresis, loss of concentrating ability, slight aciduria (described above) and the electrolyte wastage reported by Sabatini et al., 1981.

Assuming that the primary effect is directly and specifically on the ground substance one might expect subsequent damage and loss of the "delicate" morphological structures in the medulla (i.e. the loops of Henle and microvasculature) when their supporting matrix was lost. This would explain why the final collapse of medullary microvascularity only occurred after MPS staining had been lost. One of the many unresolved questions which still perplex nephrophiles is how the medullary interstitial cells can function in an osmotic environment which varies from a few hundred to several thousand mOsm/kg. It seems possible that, the PoG/GAG matrix modifies markedly the osmotic effect on these cells, loss of which would leave them exposed to altered osmolality with subsequent damage. The consequences of the loss of medullary interstitial cells are discussed below (see 7.1.5).

Our interest has very recently been focussed on Tamm-Horsfall glycoprotein (THGP) a macromolecule (7 x 10^6 daltons) which appears to
be synthesised specifically by and associated only with the distal nephron. Its exact physiological role is uncertain, but it is thought to "waterproof" the distal nephron (and thus contribute to urinary concentration) and prevent bacterial attachment to the urothelia. In disease it forms the major component of hyaline casts, it may be an essential organic constituent of calculi and there is some evidence that it is potently immunogenic (Hoyer & Seiler, 1979 and Dawnay, 1981).

We have recently studied the time-course changes in renal THGP distribution after a BEA insult, using an immunoperoxidase antiperoxidase method. THGP was lost from the distal nephron about 12h after BEA, subsequently strongly positively staining material deposited in the collecting ducts of those areas where necrosis had or was taking place, and from where loss of MPS staining had occurred. There was a consistent impression that those ducts of Bellini which contained the largest amounts of THGP positive material drained that part of the kidney in which secondary cortical cystic dilatation of the nephron was by far the most marked (Wirdnam et al., 1981). Rats pretreated with low doses of aspirin before BEA (5.3.4d) showed massive cystic dilatation and had, in addition, heavy deposits of THGP positive material in the Bowman's capsule around the glomerula tuft. A full discussion on the possible significance of these changes is beyond the scope of this dissertation, but will be considered briefly below (see 7.1.6).  

7.1.5 BEA, Medullary Interstitial Cells and The Possible Consequences of Interstitial Cell Damage or Loss.

The failure of medullary interstitial cells to repopulate a necrosed papilla, even when the basement membrane is re-epithelialised, may have several far reaching sequelae.

7.1.5.1. The loss of medullary ground substance synthesis. The absence
of these cells precludes the synthesis of medullary PoG-GAG ground substance. Thus the medulla would be without:-

A) Its immense water holding capacity.

B) The elastic matrix which supports the "delicate" elements of the region such as the thin loops of Henle and capillaries.

C) The polyanionic ion exchanger on which cations could be stored in their transition from filtration to reabsorption.

D) The highly negatively charged barrier which (also possibly provides the source of at least some of the urinary mucoid material) prevents bacterial attachment, infiltration and colonisation.

E) Urinary PoG-GAG is thought to have an important role in preventing urinary supersaturation and therefore the formation of inorganic crystal which would act as nuclei for the growth of calculi.

The absence of PoG-GAG may be an important underlying cause of the electrolyte wastage (Sabatini, 1981b) and result in calcification within the injured medulla (Shimamura et al., 1974). Experimentally induced renal infections have been reported to be established readily after, but not before, BEA treatment (Thiele, 1974). The failure of BEA treated rats to develop infection may relate to the lack of challenging organism from their "protective" environment.

7.1.5.2 The perturbation of medullary PG synthesis. Interstitial cells are a major site of PGE₂ synthesis (2.3.4.2), but the role it plays in renal function remain uncertain. The BEA-induced destruction of the interstitital cells may offer an important means by which to probe the intra- and extra-renal functions of PGE₂ synthesis in this region of the kidney.

7.1.5.3 The antihypertensive contribution of medullary interstitial cells. The role of both medullary PG and lipids in preventing hypertension have been reported above (2.3.3.2).
Shimamura (1976) was only able to induce hypertension in male Fischer 344 rats previously treated with BEA if they were salt loaded. Heptinstall et al. (1975) however, induced a frank hypertension (mean increase in blood pressure of 15 mm Hg) in female Holtzman rats (given a similar dose of BEA, 250mg/kg iv) within a few weeks. Those rats treated with BEA in which one kidney was also clipped showed a far greater rise in blood pressure than was caused by clipping only. There was an identical response in the increase in the normalised heart weight (i.e. BEA and clip greater than clip alone, which was greater than BEA). These findings have recently been confirmed (Thurston, H.B., personal communication) where salt-loading was found to be a necessary prerequisite to increase blood pressure. The clipping of one renal artery in the BEA treated (salt free) rats caused a more pronounced hypertension than did clipping alone. In this respect it is interesting that whereas removing the clip from normal rats is followed by a normalisation of blood pressure , but this was not the case in BEA pretreated rats.

7.1.6. BEA-induced RPN, Primary Lesion and Secondary Effects.

These studies suggest that one of the earliest effects of BEA is on the medullary ground substance, which for reasons that are still not clear, undergoes conformational changes as a prelude to the loss of staining and necrosis of the "sensitive" elements of the medulla, such as the loops of Henle, the capillaries and the interstitial cells.

The techniques used in this study have not enabled the fine differentiation between a primary effect on the interstitial matrix as opposed to the interstitial cells to be made. The early changes in the ground substance were, however, present at the same time as a diuresis, well before light microscopic changes were apparent in the morphological elements which are responsible for countercurrent
This loss of urine concentrating capacity continued when the morphological apparatus was lost.

During the course of medullary MPS changes, particularly when staining was lost and necrosis apparent THGP from the distal nephron formed heavy deposits in the ducts of Bellini. There is inadequate data, at this time, to explain the THGP casts but it seems reasonable to suggest that some, or all, of the following factors could play a role. THGP forms insoluble aggregates from solutions of acidic pH and of high ionic strength (Hoyer & Seiler, 1979). These conditions of increased hydrogen ion (6.1) and electrolyte concentration are met shortly after BEA dosing. It is possible that the normal turnover and excretion of medullary PoG-GAG would, by like-change repulsion, prevent THGP aggregates depositing on the collecting duct urothelia. When the process of necrosis had destroyed these substances THGP deposits might attach to the collecting duct walls.

It is tempting to speculate that the cystic (dilated) nephron and the glomeruli ensheathed in THGP arise because of the "back-pressure" that develops in a blocked collecting duct system. All of the current theories on the pathophysiology of renal haemodynamics (Maher, 1981 and Oken, 1981, 1982) discount this, however. The answer may be as simple as an osmotic effect caused by THGP or other macromolecules confined to the nephron, or it may be more complex. Whatever the cause it seems to be the secondary cortical changes which lead to degenerative responses, loss of viable functioning nephron failure. The route(s) by which the THGP reaches the glomeruli and the consequences of its presence are, at this stage uncertain.

Some commentary, both factual and speculative, must now relate these changes to the molecular pathogenesis of the chronically-induced types of RPN.
7.2 POSSIBLE MOLECULAR CHANGES THAT UNDERLY CHRONICALLY INDUCED PAPILLARY NECROSIS IN BOTH ANIMALS AND IN MAN

Rather than trying to extrapolate data from an acutely induced RPN (in a unipapillary species) to a chronically induced lesion an attempt will be made to highlight parallel and possibly related events in both the experimental models and in the human clinical situations.

7.2.1 Histopathological Similarities.

The pathology of analgesic-induced RPN in man and in animals shows several specific and common features akin to those described for the BEA-induced model. These include loss or altered MPS staining (most prominently at the papilla tip) for aspirin (Molland, 1978a,b) and in man (Burry, 1968 and Gloor, 1978), calcification in aspirin treated rats (Molland, 1978a,b) and in humans (Burry et al., 1977). Calcification may, however, only be a sign of tissue injury. There are heavy hyaline casts in the collecting ducts of rats (Molland, 1978a) and man (Burry et al., 1977) and hyalinised glomeruli have also been reported (Burry, 1968).

7.2.2 Toxicokinetics of Chemicals Which Cause RPN Chronically and the Earliest Morphological and Functional Changes.

There is extensive data to show that most analgesics are excreted predominantly via the kidney (see data of Ransford et al., 1981 and review by Prescott, 1979) although many of the NSAID (e.g. indomethacin) have a major biliary excreted component (see Smith, 1973).

Toxicokinetics appears to play a more important role than has hitherto been appreciated. It has, for example, recently been shown that intravenous administered aspirin (Tange, J., personal communication) and indomethacin (Burnett, R., personal communication) can be used to cause RPN more rapidly and reproducibly than has so far
been reported. Obviously, when given by other routes lower concentrations of the parent compound reach the kidney.

The earliest morphological changes in aspirin-induced RPN in the rat occur in the interstitial cell (Molland, 1976, 1978a,b), a finding which could not be confirmed in man because of the late stage at which the lesion is invariably identified.

The most obvious functional change in response to biphenyl insult (Hardy, 1970a) by the rat is the loss of urinary concentrating ability. Nanra (1980) has highlighted this functional change early in the course of analgesic abuse, although it is often overlooked. In this respect parallels could be drawn between:-

i) the lack of MPS staining in the Brattleboro rat and their urine concentrating defect,

ii) the perturbation of medullary MPS staining and the loss of concentrating ability in the BEA treated rat, and

iii) the similar response in analgesic abusers.

There are other areas where similar parallels can be drawn.

7.2.3 The Effects of Analgesics and NSAID on Medullary Proteoglycan, Glycosaminoglycan and Glycoprotein.

There is now a substantial body of information which shows that analgesics and NSAID inhibit the incorporation of $^{35}$SO$_2^-$ into PoG-GAG synthesised by cartilage in vitro (McKenzie et al., 1976; Palmoski & Brandt, 1980; Palmoski et al., 1980 and Dekel et al., 1980). There were, however, differences between the absolute and the comparative efficacy of any one of the compounds tested. Corticosteroids also inhibited PoG-GAG synthesis, but only when present at concentrations 2 to 3 orders of magnitude greater than the analgesics and NSAID (Dekel et al., 1980).

Recently, Comper et al. (1981) have reported that the anti-
inflammatory compounds also prevent the release and the degradation of PoG, in vitro an observation which may relate, at least in part, to the inhibitory effects these compounds have on the synthesis, release or action of proteoglycan degrading enzymes (Ackerman et al., 1981). The topic is still, however, equivocal because Videman et al., 1981 were unable to show any of these effects on rabbit cartilage in vivo.

There is a lack of direct experimental information concerning the effect of analgesics or NSAID (or indeed any chemicals) on medullary PoG-GAG metabolism. The work of Nanto et al., (1964) showed that phenacetin feeding caused marked changes in urinary GAG. Their data showed a slight increase in the amount of uronic acid compared to hexosamine and a change in the electrophoretic pattern of GAGs to include more negatively changed material. This was, however, a short-term study (14 days) using low doses (10mg per adult Wistar rat) and no histopathological data was available. Weldrake (1975) reported that aspirin (given orally to male Wistar rats, 900 mg/kg day for 21 days) decreased labelled glucosamine turnover in renal mitochondria and microsomes, but had no effect on sulphate. No attempt was made to differentiate between medullary and cortex effects, furthermore the half-life was assessed by total residual activity in the intra-cellular organelles.

Molland (1976, 1978a,b) found an extensive loss of medullary MPS within 8 weeks of giving 250mg/kg/day aspirin to rats. It is, however, uncertain if this was a consequence of earlier damage to the interstitial cells or not.

7.2.4 The Sequelae to Altered Medullary PoG-GAG, THGP and Interstitial Cell Function in the Development of RPN.

The administration of analgesics to experimental animals may produce a diverse range of other effects. There are invariably heavy deposits
of inorganic material in the medulla after aspirin (Molland, 1976, 1978a,b). Aspirin treatment has also been shown to predispose rats most markedly to the challenge of renal infection (Vivaldi, 1968).

Parsons and Mulholland (Parsons et al., 1980, and references cited therein) have, over the last 5 years, highlighted the essential role that urinary tract PoG-GAG plays in preventing the attachment of microorganisms and micro-crystals which form the nidus for stone formation. Analgesic or NSAID suppression of unrothelial PoG-GAG synthesis would, therefore, be expected to result in the increased risk of:-

(i) bacterial fimbriae attachment and colonisation, which in turn would provide a vast supply of free bacteria with the potential for vesico-ureteric reflux infection and bacterial pyelonephritis (Risdon, 1981). In this respect it is perhaps significant that patients who have a high analgesic intake (although not strictly analgesic abusers) also had a high incidence of urinary tract infection (Waters et al., 1973) although other factors may have been the cause. In addition patients who are established analgesic abusers have a very high incidence of urinary tract infection (see 3.1 and Table 3.2 for references).

(ii) The attachment of urinary crystals would be expected to provide the nucleus from which calculi and stones could develop, providing the urine was saturated with "suitable" inorganic material (N.B. this should be less likely if there was a concentrating defect). Blackman et al. (1967) have reported a very high incidence of calculi formation in patients who took large quantities of analgesics, although they were not abusers per se, nor were any diagnosed to have RPN. Patients with RPN have a high incidence of renal calculi and bladder stones (Kincaid-Smith, 1980 and references cited in Table 3.2 and section 3.1).

It is, however, most difficult to separate the formation of renal calculi and stones from the process of urinary tract infection. They
both have a similar nidus and the development of one may predispose to the other.

The role of Tamm-Horsfall glycoprotein in preventing urinary tract infection (Asscher, 1981) and promoting calculi formation and growth (Nordin et al., 1979; Hoyer & Seiler, 1979 and Dawnay, 1981) is still not totally delineated. The recent evidence we have obtained (7.1.4) does, however, suggest that it may play an important role in the development of secondary pathological changes in the cortex, apart from the reported hyaline casts and hyalinisation of glomeruli in humans (Burry, 1968).

Recently the chronic administration of several types of mixed analgesics to Wistar rats over a 72 week period, has been found to cause hypertension (Nanra, R.S., personal communication) with increases in blood pressure of about 40mm Hg. The incidence of hypertension is marked in human analgesic abusers (Table 3.2) with RPN. Hypertension is more common the greater the ingestion of analgesics and the more advanced the lesion. Stewart & Gallery (1976) have suggested that sub-clinical RPN may be associated with a large number of patients who are diagnosed as suffering from essential hypertension. Once again, however, it is not possible to prove that analgesic or NSAID-induced destruction of the medulla, particularly the medullary interstitial cells is the original and only cause of an elevated blood pressure. The secondary cortical changes which follow the RPN may be responsible or, alternatively, urinary tract infection (vide supra) and hypertension are inextricably linked in a clinical situation (see Asscher, 1981).

7.2.5 Towards the Diagnosis of Chronically Induced RPN.

The changes in the urinary excretion of PoG-GAG material after BEA suggests that this may be a selective marker for RPN. The possible value of this approach is supported, by the almost totally ignored work
of Kasanen et al., (1964) who reported that there was an increase in urinary GAG associated with "phenacetin" analgesic abuse and that the concentration of GAG-derived hexosamine almost doubled. These data are, however, difficult to interpret beyond acknowledging that a marked qualitative and quantitative change in urinary PoG-GAG may take place in analgesic abusers. The electrophoretic profile of urinary proteins from analgesic abusers has recently been shown to include marked increases in high and low molecular weight components, a criteria which distinguishes them from normal subjects and from those with other types of renal lesions (Stolte, H., personal communication).

Obviously a great deal of research effort is needed in this direction.

7.2.6 Hypothesis for Future Investigation.

Urothelial and interstitial PoG-GAG could play the most important role in the pathogenesis of RPN, see Fig. 7.1 & 7.2. Chemically induced decrease in its synthesis could predispose to bacterial infection and calculi growth, first in the bladder, and later by reflux infection to the medulla where depressed PoG-GAG would predispose it to both infection and calculi and stones. These factors are inseparably linked, they are self promoting and one could aggravate the other.

Bacteria produce substantial quantities of proteoglycan degrading enzymes (Kennedy, 1979), although there is no data in this respect on those organisms which commonly cause urinary tract infections or bacterial pyelonephritis. Thus bacteria have the potential to destroy medullary PoG-GAG. There is ample experimental (Adriano & Schwarz, 1955 and Hurley & Winner, 1963) and clinical evidence (Tomashefski & Abramowsky, 1981) to show that Candida albicans, and other members of the genus Candida, do cause a papillary necrosis as part of systemic candidosis. These fungi have a predilection for mucoid material, and
Fig. 7.1 The possible interactions between papillotoxins, promoting factors, destruction of the medullary ground substance, formation of renal calculi and loss of urine concentrating function.
Fig. 7.2 The possible sequelae which follow the loss of renal medullary interstitial cells.
would, therefore, on purely teleological grounds be expected to have the necessary enzymic complement to convert the medulla matrix to their own energy and structural requirements.

Both inhibition of synthesis or enzymically digested PoG-GAG would result in the loss of an effective urinary concentrating mechanism.

7.3 POSSIBLE FUTURE RESEARCH DIRECTIONS IN RENAL PAPILLARY NECROSIS

Considered in perspective BEA offers a valuable model for studying the molecular pathogenesis of RPN. Those areas of research which might be followed in an attempt to further clarify the molecular mechanism underlying RPN are suggested below.

7.3.1 The Ultrastructural Changes Which Preceded BEA-induced RPN.

Only one time-course study of ultrastructural changes has thus far been reported for BEA-induced RPN. The only major conclusions reported by Hill et al., 1972, were that the morphological changes ran a parallel course in both blood vessels and in the tubules: all other changes in the early stages were rather non-specific. Unfortunately, however, these observations related to a model that was different to the system we have used for reasons that are far from certain. The possible importance of medullary PoG was obviously not perceived by Hill et al., (1972). Only recently have specific methods been described to fix these macromolecules in situ (Furusato, 1977).

Obviously the ultrastructural changes following BEA have to be reassessed in a time-course study where particular attention is paid to the PoG-GAG ground substance and how changes in it relate to the renal medullary interstitial and other cells as the morphological changes develop. The recent advances in immunohistochemistry, particularly at the ultrastructural level, make it essential to add this technique to the single dimension of morphology. Our own data suggests that THGP is an important marker in the course of RPN. The raising of antibodies (to
be used for immunoperoxidase staining) against the different fractions of PoG-GAG, which most likely occupy the medullary matrix, will give more meaningful results than the general non-specific histochemical methods that are commonly used. Equally, although probably of less significance at this stage, immunohistochemical techniques could be valuable to highlight changes which might not otherwise be apparent. These could include tubular basement membrane, specific areas of the nephron, specific molecules within certain cell types, or sub-population of certain cells.

Assuming that the renal interstitial cells are the vulnerable cell type in the medulla (7.1 and 7.2) it would be necessary to establish that the BEA insult was primarily directed towards them and not, for example, at some adjacent cell which caused (as a secondary consequence) a pathobiological response by the interstitial cells. The cellular specificity of the BEA insult would also have to be studied in vitro (7.3.4).

A most important question that needs to be probed is the exact molecular cause that underlies the increased staining intensity observed in the medullary matrix (5.2) following BEA. In vitro methods (7.3.4) will give an important insight into PoG-GAG kinetics and chemical changes. In addition X-ray microprobe analysis of the matrix in fixed or frozen ultrathin sections, or in freeze fractured tissue masses (using scanning electron microscopy) could be used to give an important idea of changes in water holding capacity, surface exposed -SO_3^- groups and the changes in other anions and cations (Saubermann et al., 1980a, b and Bulger et al., 1980).

7.3.2 The Identity of the BEA Metabolites and Their Distribution and Nephrotoxic Effects

The preliminary data reported above (6.5) suggests that there are 6-
8 urinary BEA metabolites and possibly a similar number of different (?) biliary products.

Despite its simple structure BEA could follow an impressive array of chemical and biological pathways in vivo, based on the limited published information and on theoretical considerations (Fig. 6). Several of these are potential alkylating agents either as they are (e.g. EI) or via metabolic activation, thus it is essential to establish the structural identity of these compounds. This will give an insight into several critical questions such as:-

A) the importance of BEA derived alkylating metabolites,

B) the nature of the higher molecular weight metabolites, (are these degraded products from alkylated macromolecules, and if so what type of macromolecule, or are these compounds alkylated micromolecules, such as lipids, amino acids etc.)

C) the common pathways in the formation of urinary and biliary metabolites,

D) if the metabolism of endogenous molecules were being blocked, e.g. acting as an EA analogue,

E) allowing the chemical synthesis of metabolites so that the nephrotoxicological effects of each compound can be assessed individually,

The possibility that the chemical formation of EI (from BEA) provides the proximate papillotoxin can only be tested properly when the major BEA metabolites have been identified. The definitive test will then impinge on the $^{14}$C labelling pattern of metabolites derived from 2-bromo-[l-$^{14}$C]ethanamine. If the label remains adjacent to the amine exclusively then that metabolite could only have been formed via BEA. The "shift" of any labelling to the C-2 will relate directly to the amount of EI formed and subsequently metabolised.
Recent studies using BEA analogues suggested that the papillotoxic effects of BEA was closely linked to its structure. 2-Chloroethylamine HCl (an established DNA alkylating agent) had no effect over a dose range of 20 to 500mg/kg ip. Whereas 3-bromopropylamine HBr caused focal papillary necrosis at a dose of 500mg/kg ip, 3-bromo-(2-hydroxy)prolylamine HBr caused marked proximal convoluted tubule necrosis at doses of 200mg/kg ip. There are too few compounds to draw any firm structure-activity relationships and data may represent changed pharmacokinetics and distribution, or failure to reach the kidney due to different hepatic metabolism. Structural analogues will, however, be essential when in vitro short-term tests are being assessed (7.3.4).

7.3.3. The Development of Non-invasive Marker Assays for Diagnosing RPN.

The difficulties in diagnosing RPN have remained the major stumbling block for both toxicologists and nephrologists in their attempts to understand and prevent this lesion.

Our interest in studying the changes in and excretion of medullary PoG, GAG and oligosaccharides appears to have been largely vindicated by the histochemical studies (5.2) and by the changed pattern in the polydispersion of urinary uronic acid (6.6.1) and radiolabel distribution (6.6.2), and the approach looks promising for the identification of markers of the lesion.

The medullary ground substance is obviously an area where a great deal of investigations will be required. While the problem will be clarified by ultrastructural and immunohistochemistry, the relevance to RPN will only be established if a change in one or more urinary, blood, bile or expired air metabolites of PoG can be related to the extent of the lesion. These investigations would be futile (in that they might be
impossible to extrapolate to man) unless they were undertaken in the widest context. Here it would be necessary to fully characterise the medullary PoGs and understand the various metabolic pools and the factors which affect them. In this regard the "specific" radiolabelling of the renal PoG, by administering $^{35}$S or $^{14}$C-precursors either into the aorta or directly into the renal artery would be a useful adjunct, and cultured interstitial cells (7.3.4) would be especially valuable, because of the ability to test and control many biological variables.

There may well be other markers which are simpler or more specific determinants of RPN. The renal medullary interstitial cells are unique and characterised morphologically by their inclusion of numerous lipid droplets, which will be released when these cells are necrosed. Thus it should be possible to monitor for arachidonic acid metabolites (2.3.4.3), fatty acids (especially adrenic acid (2.3.4.2) which is confined to interstitial and adrenal cells), or particular phospholipids which appear to be peculiar to the interstitial cells. Several of these compounds have been described to exert anti-hypertensive effects, including renin inhibitor, neutral lipid and the alkyl ethers of phosphatidylcholine. (Muirhead et al., 1981). The latter group of compounds appear destined to be exploited for therapeutic control of hypertension, thus monitoring of phospholipid may, unless methods are very specific, be confined to experimental studies only.

7.3.4 Short-term Tests of Papillotoxic Potential.

Means of assessing the papillotoxic potential of chemicals and drugs are fraught with problems. The lesion is easily overlooked if histopathology is not painstakingly executed, as may often occur in routine toxicological screening. Further, there are species
differences, not all the animals in a group develop the lesion and dosing for many months may be required before any changes occur. Finally, the dose at which compounds are administered may be critical.

The observation (5.3.4) that rats can be made considerably more sensitive to the BEA insult by pre-treating them with chemicals having papillotoxic potential, all within the period of 7 days, suggests an important possible alternative, the possible pitfalls of which have been discussed (5.3.4d). In addition, this system requires a large number of animals, thus it generates a vast amount of histology, and there may be complex unpredictable dose related interactive factors as already discussed (5.3.4d). This approach would be more cost effective, however, if a suitable non-invasive marker for RPN had been developed.

Probably the most promising approach for the immediate future is cell culture techniques. A number of workers have described renal medullary interstitial cells cultures, but the most significant contribution has come from (Kuroda et al., 1979) who showed that non-interstitial cells could not survive a carefully controlled increase in culture media osmolality. The surviving pure interstitial cells which were then cultured at either a high or a normal osmolality were shown to synthesise PGE$_2$.

The culturing of pure interstitial cells offers a unique means of defining the biochemistry and morphology of these cells in response to those factors which might cause perturbations in the normal medulla (e.g. osmolality, pO$_2$, pH, electrolytes, and endogenous messengers such as ADH). Means of assessing the cellular response to these factors should include the metabolism and kinetics of P0G and the full range of lipid materials as well as, perhaps proteins, nucleic acids and intermediates in cellular anabolism and catabolism. These studies
would establish the base line data against which the effects of chemicals with established in vivo papillotoxic potential could be evaluated (BEA, biphenys, analgesics and NSAID) to define the test criteria against which other chemicals might be assessed.

The use of cultured cells would provide an easily controlled cost effective means of screening a large number of chemicals which required only a limited number of test animals.

7.3.5 Factors Which Affect the Development of RPN and the Importance of Therapeutic Papilloprotection

The development of both experimentally induced RPN in animals and particularly analgesic nephropathy in man, are subject to a number of variables which are not well documented. These include genetic factors, diet (type of food, anti-oxidants, vitamins, type of lipid, electrolyte "load", liquid intake), environmental conditions (temperature, humidity), and xenobiotic exposure (type, amount, etc.). These factors might explain the variable, and often irreproducible, development of the lesion in experimental animals, why not all analgesic abusers develop the lesion and the different frequency of RPN within and between countries.

Before a systematic study is undertaken on these factors a specific marker for assessing the development of the lesion must become firmly established.

The long term goal of any such studies would be to delineate those factors which might exacerbate the lesion in humans (at high risk of developing RPN) with a view to minimising them. It is also important to define how chemical intervention could ameliorate or reduce the risk of RPN developing, somewhat akin to pyridoxine preventing peripheral neuritis in isoniazid treated patients. This may be particularly critical if, as appears to be the case, RPN is an inseparable
consequence of analgesic and NSAID action. The limited findings with anti-oxidants and lipotrophic factors discussed above may be a starting point for such studies. Careful differentiation must be made between measures which afford little or no protection against an acutely induced lesion, but would, perhaps, be most successful in preventing those less dramatic functional changes which underly a papillary necrosis that develops over a long period.

7.3.6 Chronically Induced RPN and Extrapolation to Humans with RPN.

Analgesic nephropathy in humans is a chronically induced lesion, thus the extrapolation from the BEA-induced lesion must be considered with due caution until the hypothesis outlined above (7.2) has been verified in a chronically induced experimental system. In the interim, however, much might be learned from comparing the specific urinary determinants of the medulla (i.e. the urinary Pol-GAG, and their breakdown products, and phospholipids and fatty acids, particularly adrenic acid) in patients who are:

I) Analgesic abusers.

II) Prescribed high dose, longterm analgesic and NSAID therapy.

III) Known to have RPN.

IV) Diagnosed to have other types of renal or non-renal disease, and

V) Normal healthy controls.
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