HEPATIC CARCINOGENESIS AND ENZYMIC DEDIFFERENTIATION
FOLLOWING DIETHYLNITROSAMINE ADMINISTRATION TO RATS

By

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SUMMARY

A large body of evidence suggests that neoplasia is associated with a reversal towards a foetal type of cell in terms of morphology, behaviour, antigenic and biochemical properties. This evidence is largely based on the end-point of carcinogenesis, i.e. tumours, so that theories on the mechanisms by which the foetal state arises are speculative. The work reported in this thesis concerns the study of early, as well as late, biochemical and histological changes occurring during hepatocarcinogenesis in comparison with normal liver differentiation and liver regeneration after partial hepatectomy in rats. Enzymes which show phase-specific profiles of activity during development were studied during normal liver differentiation, during chronic and two-stage (phenobarbitone-promoted) diethylnitrosamine-induced hepatocarcinogenesis, in transplantable hepatomas, during liver regeneration and the host liver of tumour-bearing rats.

During chronic hepatocarcinogenesis there were increased activities of characteristic foetal enzymes whereas adult enzyme activities, with the exception of malic enzyme, decreased. These enzyme changes were apparent before histological changes indicative of neoplasia could be detected. Histological similarities with foetal and neonatal hepatocytes also developed in the livers during hepatocarcinogenesis. Rank correlation analysis of the enzyme data reveals that the liver during carcinogenesis first assumes a neonatal enzymic pattern before attaining a foetal enzymic state. In two-stage carcinogenesis experiments enzymic dedifferentiation also occurred, but to a lesser extent, and the focal lesions that developed showed phenotypic heterogeneity. Two transplantable hepatomas studied also exhibited foetal-type enzymic patterns, but contrary to previously reported data no enzymic dedifferentiation was observed in the host liver of tumour-bearing rats.

A similar sequence of enzymic dedifferentiation and histological changes to that during carcinogenesis observed in regenerating liver after partial hepatectomy, but in contrast, the cells retained the capacity to undergo redifferentiation to a normal adult histological
and biochemical pattern.

The results are discussed in relation to the nature of cancer, the mechanisms of carcinogenesis, and their diagnostic potential.
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CHAPTER 1

INTRODUCTION
INTRODUCTION

1.1. The Nature of Cancer

1.1.1. The Characteristics and Incidence of Cancer

Cancer is a disease process which may affect all multicellular organisms and which is characterised by the seemingly uncontrolled multiplication and spread within such organisms of abnormal forms of their own cells. In countries of Western Europe and North America one in four people develop one or more forms of neoplasm and about one in five die from cancer (Roe, 1975). In the United States cancer is the second major cause of death, after heart disease (Cairns, 1978); it is also the second most common cause of death in children (Pitot, 1977a). The overall death-rate from cancer is highest in countries with well developed medical services, presumably because of the reduced risk of death from infectious diseases or other causes.

1.1.2 The History of the Study of Cancer

Though cancer is an increasingly important factor in mortality figures today it is by no means a new disease, bone deformations similar to those produced by bone cancers have been found in ancient Egyptian skeletons. The study of the disease goes back to ancient Greek times; in Hippocratic writings many of the lesions termed karkinos seem to be cancers from the description of their development (Rather, 1978). However, no distinction was made between inflammatory tumours and malignant neoplasms. An effective set of criteria for the recognition of cancer was published only in 1913 by Bayle and Cayol (cited by Rather, 1978). Until the latter part of the last century cancer cells were believed to arise de novo from circulatory blastemas. Even Virchow thought the origins of cancer cells lay in a reservoir of undifferentiated cells in the ubiquitous connective tissue which became pus, tubercle or tumour cells depending on the stimulus. Eventually the idea came about that the sole source of epithelial cancers was normal epithelium, that the tumours arose without cell dissolution
but by the division of the original epithelial cancer cell and that
connective tissue, local or distant, never transforms into epithelial
cancer cells, but that the sole means of local spread is by the
movement of cancer cells into the adjacent tissue and that metastasis
is by the transport of cancer cells via the body fluids (Waldeyer, 1867).

1.1.3. Human Liver Cancer

a) Epidemiology

In contrast to those cancers which are the most common causes
of death in Western Society, liver cell cancer is most common in
tropical countries. Despite its relative infrequency in developed
countries liver cancer is probably one of the most frequent cancers
in man (Linsell and Higginson, 1976). The incidence of the disease is
very high in Mozambique where liver cancer accounts for 65.5% of all
cancers in men and 31% in women (Kew, 1978), and 103.8 men and 30.8
women per 100,000 people are annually reported to have the disease.
In China liver cancer is the most common tumour in people aged 15 to
44 years (Kaplan and Tsuchitani, 1978) and is therefore a major
problem in the most populous area of the world.

b) Factors Affecting Survival

Liver cancer is one of the most malignant neoplasms as far as
prognosis is concerned, the average survival time from detection being
only a few months (Okuda et al., 1978). The liver is the largest organ
in the body and is situated deep within it, so large amounts of normal
tissue can be replaced by malignant growths before the symptoms of
cancer appear (upper abdominal pain, weight loss, anorexia and abdominal
swelling). In addition, the complication of metastasis occurs quite
early in the course of the disease. Surgical resection offers the only
opportunity for effective treatment at the present and this is only
possible in a small percentage of cases and even then the survival rate
is low. A five year survival rate of 19.1% has been claimed in the Far
East, but it has been impossible to achieve this level of success in Africa (Falkson, 1976). Radiotherapy and chemotherapy do not significantly affect survival rates, although 5-fluorouracil and adriamycin may give extended survival (Keppler, 1978).

c) Association With Cirrhosis

Liver cell cancer is frequently associated with macronodular cirrhosis, this type of cirrhosis being most commonly observed in countries reporting a high incidence of liver cancer (Linsell and Higginson, 1976). Nevertheless, the nature of the relationship between cirrhosis and liver cancer is not fully understood. The frequency of occurrence of the two diseases in the liver may be purely coincidental, both the cirrhosis and the cancer may have a common cause, or cirrhosis may cause cancer or in some way modify the body environment to increase the susceptibility of the liver to develop cancer, or, in view of the fact that in areas of high incidence many patients with liver cancer do not have cirrhosis, cirrhosis may follow rather than precede cancer. In areas of low incidence of liver cancer the proportion of patients with the disease in whom cirrhosis is also found is 80 - 90%, whereas in areas of high incidence only 60% of cancer patients have cirrhosis. On the other hand in areas of high incidence the proportion of patients with cirrhosis who develop liver cancer is about 40%, but in areas of low incidence only 5 - 15% of cirrhotic patients develop cancer (Anthony, 1976). This is probably because the commonest cirrhosis in countries of low liver cancer incidence is micronodular, associated with alcohol abuse, rather than the macronodular type associated with liver cancer. Micronodular cirrhosis may develop into the macronodular type, especially after cessation of alcohol consumption and prolonged survival for some years, and this is associated with an increased risk of malignancy. This may account for the fact that liver cancer affects older people in areas of low incidence than in areas of high incidence. In a small minority of cases malignancy arises in alcoholics without cirrhosis. It is unlikely that alcohol per se is carcinogenic, although it may promote the actions of carcinogens (Anthony, 1976).
1.1.4. Environmental Aetiology

Cancer is thought to be largely due to environmental rather than genetic factors. It has been estimated that between 80% and 90% of the total cancer incidence in the human population of the United States is dependent on known or unknown environmental factors (Higginson and Muir, 1973). Probably the first association of lifestyle with tumour development was observed by Ramazzini in 1700 (cited by Boyland, 1980a) who wrote that tumours of the breast were found more often in nuns than other women. Later Hill in 1761 pointed out the association of nasopharyngeal cancer and snuff-taking (cited by Boyland, 1980a) and Pott in 1775 observed that many chimney sweeps develop cancer of the scrotum. These latter two observations were the first to suggest a chemical causation of cancer.

Most liver cancer occurs in parts of the world where alcoholic liver disease is uncommon so other agents must be important in the aetiology of this disease.

a) Hepatitis B Virus

A striking relationship has been observed between the presence of hepatitis B antigen and liver cancer. One of the first controlled studies was in Taiwan where about 15% of the population carry hepatitis B antigen and liver cancer is the most frequent neoplasm. In 80% of the patients with liver cancer, hepatitis B surface antigen was found (Tong et al, 1971). The presence of hepatitis B antigen in liver cancer patients in other areas has also been shown (Anthony, 1976). When hepatitis B viral core antibody (evidence for past or present hepatitis B virus infection) is also measured the association is even more striking. For example, in a study in South Africa where 66% of hepatocellular carcinoma patients were found to be hepatitis B antigen positive, a further 26.5% were found to possess hepatitis B core antibodies (Kew, 1978). Recent studies have shown that this pattern is not only restricted to high incidence areas. A study involving eighty Greek patients with primary hepatocellular carcinoma, one hundred and sixty control individuals and forty with hepatic
metastasis of other tumours, showed that the presence of hepatitis B surface antigen (evidence of active infection) was associated with a significantly increased risk of primary liver cancer, but not metastatic-liver cancer. In addition active hepatitis B virus infection was more common in primary liver cancer patients with cirrhosis (67%) than those without (26%) (Trichopoulos et al., 1978). In Britain a study has shown that 67% of a group of patients with primary liver cancer had hepatitis B core antibody, which is significantly higher than in the normal population (1 - 4%), and 25% of the patients had evidence of persistent infection (Bassendine et al., 1979). It has been estimated that 120 to 176 million people in the world are carriers of hepatitis B (Zuckerman, 1978) and this is undoubtedly an important aetiological factor in the development of liver cancer. However, by no means all people with liver cancer have this viral infection, neither do all hepatitis B patients develop liver cancer. Other factors must therefore contribute to the development of liver cancer.

b) Steroid Hormones

One possible factor, in women at least, may be found in an association between oral contraceptives and liver tumours, which was first suggested by Baum et al (1975). The evidence to date suggests that there is an association between oral contraceptive use and hepatic adenomas and focal nodular hyperplasia (Vana et al., 1977 & 1979). These are considered to be non-malignant tumours though they may cause death by hepatic rupture and intraperitoneal haemorrhage (Feben, 1979). Malignant liver tumours have been found in women taking oral contraceptives (Christopherson and Mays, 1979). However, as hepatocellular carcinomas are the most common liver tumour in the general population the evidence for a positive association between oral contraceptive use and hepatocellular carcinoma remains inconclusive. Nevertheless benign and malignant tumours have been found together in women on oral contraceptives but no such findings have been reported in the absence of oral contraceptive use. In two large studies 63% and 85% of patients had been taking the pill for four years or more, there was a range of onset of the disease from within 6 months of starting
taking oral contraceptives to four years after stopping taking the pill (Sherlock, 1978).

Other hormones such as anabolic steroids have also been implicated in the development of liver cancer (Johnson et al., 1972; Farrel et al., 1975). It has been suggested that steroids may promote liver tumours. This suggestion is based on the observation that corticosterone inhibits the proliferation of rat liver cells and that this inhibition can be inactivated by blocking the cytoplasmic receptors in liver cells for corticosterone with other steroids. High concentrations of steroids may induce liver proliferation and thereby promote transformation or may make hepatocarcinogens effective at lower doses and more rapidly effective (Desser-Wiest, 1979).

b) Vinyl Chloride

Angiosarcoma of the liver is a very rare type of liver tumour but it has been identified as a cause of death in certain workers occupationally exposed to vinyl chloride. This chemical has been found to induce angiosarcoma in rats at concentrations as low as 50 ppm (Maltoni and Lefermine, 1975) and it may also induce liver cell and bile-duct carcinoma (Anthony, 1976).

d) Aflatoxin B₁

Naturally occurring carcinogens may also play an important part in the aetiology of liver cancer. Aflatoxin B₁ is the most frequently encountered and thoroughly studied of the fungal aflatoxins. It is produced by the mould Aspergillus flavus. It has been found in many foodstuffs stored under conditions favourable to fungal growth i.e. warmth and high humidity. Field studies in the tropics have shown a good correlation between areas of high aflatoxin B₁ contamination of foodstuffs and a high incidence of liver cancer (Wogan, 1976).

1.1.5. Nitrosamines

Nitrosamines are a class of compounds that are ubiquitous
contaminant of the environment, the most commonly encountered members being dimethylnitrosamine, diethylnitrosamine and N-nitrosopyrroolidine and give rise predominantly to liver tumours. Originally it was thought that exposure to nitrosamines was largely occupational, but in 1960 a large number of sheep that had fed on fishmeal that had been preserved with nitrite died of acute liver damage. Nitrosamines were detected in the fishmeal and were presumed to have been formed from the nitrite and secondary and tertiary amines in the fish (Magee, 1971). A generalised scheme for the nitrosation of secondary amines is shown below in figure 1.1.

Figure 1.1. \[ 2\text{HNO}_2 \rightleftharpoons \text{N}_2\text{O}_3 + \text{H}_2\text{O} \]

\[
\text{R}_1\text{R}_2\text{NH} + \text{N}_2\text{O}_3 \rightarrow \text{R}_1\text{R}_2\text{N} = \text{N} = \text{O} + \text{NO}_2 \quad \text{Optimum pH 3}
\]

rate = \( k [\text{RR'NH}] [\text{HNO}_2]^2 \)

Nitrosamines are encountered in nitrite or nitrate-preserved foodstuffs; meat, fish etc; cigarette smoke and ambient air (Preussman, 1978). Airborne nitrosamines have been detected as environmental pollutants in the rubber industry (Fajin et al., 1979). Diethylnitrosamine is a contaminant of some cosmetics: face, hand and body lotions, and hair shampoos (Fan et al., 1977). Nitrosamines, though not proven human carcinogens, have been found to be carcinogenic to many experimental animals, for example diethylnitrosamine has been shown to be carcinogenic in twenty different species, including monkeys (Preussman, 1978). Moreover, there are no published reports of any animal species resistant to carcinogenesis by nitrosamines and there is no reason to suppose that man is not also susceptible (Magee, 1971).

Nitrosamines may be formed by the cooking of nitrite-preserved foods (Janzowski et al., 1978). In particular the formation of nitrosopyrroolidine in fried bacon as shown below (Lijinsky, 1976).
There is also much evidence that the nitrosamines can also be formed by the simultaneous ingestion of nitrosatable substances and nitrite. The long-term administration of nitrite and morpholine to rats gave rise to many liver tumours similar to those induced by N-nitrosomorpholine, even at low concentrations (Shank and Newbern, 1976). The nitrosation of food amines in vitro under conditions similar to those found in the stomach, has been demonstrated (Walters et al., 1976) and more recently the formation of nitrosamine in vivo has been shown (Walters et al., 1978). Environmental nitrate comes from many sources such as inorganic fertilizers, decomposition products from plant and sewage wastes and as a product of nitrogen fixation by bacteria. The nitrate may be reduced to nitrite by enzymes in plants and microorganisms present in waste water, food and in intestine and saliva (Olaus and Coulston, 1978). Nitrate and nitrite may also be formed endogenously in humans (Tannenbaum et al., 1978). Sodium nitrite is also used as a preservative to inhibit the growth of Clostridium botulinum and as a colour fixative in cured meat and fish (Miller and Miller, 1974). Many nitrosatable secondary and tertiary amines are natural constituents of food and many drugs for human consumption (Andrews et al., 1980).
1.2. Biological Features of Malignancy

1.2.1. Pathology of Human Liver Tumours

Liver tumours may arise from any of the constituent tissues but most commonly from liver parenchyma (liver cell carcinoma; hepatocellular carcinoma) or the bile duct cells (biliary carcinoma; cholangiocarcinoma) of which hepatocellular carcinomas are the most common in man (Cameron, 1976). Human liver tumours are rarely seen as a "surgical" excision specimen since at autopsy the tumour is usually well advanced and massive. A human hepatocellular carcinoma may present as a massive, solitary, soft and frangible, opaque yellow to grey, bile-stained or haemorrhagic and necrotic tumour. At other times the tumour tissue is distributed throughout the liver as multiple discrete nodules of varying size. Both types may be present in any one liver (Ashley, 1978). A useful early classification was made by Eggel at the turn of the century (cited by Peters, 1976) who divided hepatocellular carcinomas into three types: the nodular, the massive and the diffuse forms. The most common is the nodular type, making up about two-thirds of all tumours, characterised by multiple large nodules which tend to remain discretely delineated from normal liver. Next in frequency of occurrence is the massive type characterised by a large tumour mass which may occupy one entire lobe (more often the right) and infiltrate irregularly into the surrounding liver. This form constitutes about 30% of all primary liver carcinoma and it is most amenable to surgical removal, however it may give few symptoms until shortly before death, most patients dying suddenly and unexpectedly from intraperitoneal haemorrhage (Higgins, 1970). Least common of all being the diffuse type in which the entire liver is composed of very small tumour nodules surrounded by collagen and difficult to distinguish from the cirrhosis from which it always develops. It is often difficult to attribute specimens of human liver cell cancer to one particular category and there have been further classifications since. There is some debate as to whether hepatocellular carcinomas are of unifocal origin with multiple metastasis or arise multifocally from the gross and microscopic appearances, it seems possible that both mechanisms may operate.
The massive and meganodular types probably arise from a single focus and metastasise into other areas of the liver through the portal vein. The diffuse types probably represent the nearly simultaneous development of neoplasia at many sites in the liver (Peters, 1976). Benign tumours of the liver are also known to occur - the adenomas and hepatomas. A characteristic feature of hepatocellular carcinoma is the cytologic similarity of the tumour cells to non-neoplastic liver cells. Occasionally it may be difficult to distinguish between regenerative and neoplastic nodules because the patterns of growth and cellular atypism in areas of hepatic cell regeneration may be so abnormal as to resemble neoplasia more than hyperplasia. Human hepatocellular carcinoma cells may be variable in shape, but are usually polyhedral, either larger or smaller than normal cells and retain a similar or lesser degree of cohesiveness than normal. They usually have a moderate amount of cytoplasm which is often more acidophilic and granular than normal. The nuclei are large, spherical or oval, hyperchromatic and vesicular. There is usually a peripheralization of the chromatin forming a more prominent hyperchromatic membrane at the margin of the nucleus. Nucleoli are large and prominent and mitoses, often abnormal, are common. The degree of anaplasia varies considerably ranging from tumour cells which appear indistinguishable from normal to those that resemble mesenchymal cells. With increasing anaplasia there is a corresponding loss of cohesiveness. There may be a full range of anaplastic change in any one patient. In some liver cell cancers giant cells are seen, these cells may contain a single nucleus, a multilobated nucleus or multiple nuclei, with prominent nucleoli and abundant eosinophilic cytoplasm. Clear cells, which appear so because of an excessive intracellular accumulation of glycogen, may make up to 30 to 100% of some tumours. In some well differentiated tumours fat droplets are seen and this has been taken to mean that the cells are functioning unlike normal hepatocytes, in that they synthesise but do not release fat, but not like truly undifferentiated tumour cells (Peters, 1976). Mallory bodies have been found in some tumours arising from alcoholic cirrhosis long after non-tumour cirrhotic liver no longer contains them, and also very rarely in non-alcoholic patients.

The pattern of growth may also exhibit varying degrees of
differentiation. The most common pattern is a trabecular arrangement. In well differentiated hepatocellular carcinoma the trabeculae consist of linear columns of parenchymal cells one or two cells thick (microtrabecular) or several cells thick (macrotrabecular) separated from the blood sinusoids by widely spaced, flattened and inconspicuous endothelial lining cells. Kupffer cells are usually absent. Less well differentiated tumours may show a 'cobblestone' arrangement of cells growing in solid sheets in a similar pattern to regenerative hyperplasia. An undifferentiated pattern of growth is shown by cells growing in sheets which have such little cohesiveness that they do not even exhibit a 'cobblestone' appearance. The degree of cytological differentiation does not necessarily correlate with the degree of morphological differentiation. It is not unusual to find cytologically undifferentiated cells exhibiting a dedifferentiated growth pattern.

1.2.2. Experimental Hepatocarcinogenesis

Human liver tumours are rarely seen at their early stages so studies on the sequential changes during the pathogenesis of liver cell cancer have to be made in animals. The first induction of liver tumours in rats, after o-aminoazotoluene administration was made in the early 1930's (Sasaki and Yoshishida, 1935). Tumours produced in laboratory rodents are similar in many respects to those appearing in humans: they affect males more frequently than females (Firminger and Reuber, 1961); they are often accompanied by cirrhosis, though not necessarily (Farber, 1976) and they exhibit similar morphological growth patterns and cytological appearance to human tumours. In addition, not only is there an almost identical sequence from fibrotic lesions to angiosarcoma in man following exposure to vinyl chloride and rodents treated with vinyl chloride but also there is a great deal of similarity in appearance between hepatocytic nodular lesions in rats and steroid-induced lesions in humans including cytology, architecture and arrangement of blood vessels (Popper et al., 1977).

a) Preneoplastic Lesions and Tumour Progression

Experimental liver cancer may appear with or without cirrhosis
depending on the dose of the carcinogen (Bannasch, 1968). The development of tumours accompanied by cirrhosis occurs at high dose levels in a relatively short space of time, and the development of tumours in the absence of cirrhosis follows low dosage regimes after a long lag period. In addition, cirrhosis brought about by other means e.g. choline deficiency (Lombardi and Shinozuka, 1979) often increases tumour incidence. The evidence suggests that whilst cirrhosis may accelerate hepatocarcinogenesis in some cases it is not essential for tumour formation. Hepatocellular carcinomas arise from small clusters of cells which increase in size and number. The transition of these original altered hepatocytes into a well defined tumour has been classified in various ways. Because of the overlap of various classifications a workshop committee has suggested a standard system of nomenclature (Squire and Levitt, 1975). In this the use of the following terms has been recommended: foci for small lesions less than one lobule in size; area for lesions as large as or larger than a lobule; neoplastic nodule for lesions equivalent in size to several liver lobules and hepatocellular carcinoma for lesions larger and more irregular than neoplastic nodules. Of the cellular changes in the foci the basophilic cells were thought to have the greatest significance with respect to tumour formation. By the time these foci have grown sufficiently to be termed nodules, the normal liver architecture is lost. The cells themselves are similar to those within the foci and may show various cytoplasmic alterations. Mitoses and varying degrees of nuclear atypia may be observed. The cells may be arranged in solid or jumbled sheets or in irregular plates one or more cells thick, the sinusoids may be dilated or compressed by enlarged hepatocytes. There is a sharp demarcation of the nodule from the surrounding tissue which is often compressed by the expanding nodule. The term neoplastic has been recommended to replace the old term, hyperplastic, when referring to the nodules because experimental evidence shows that these lesions are induced by carcinogens and indicate, at the least, an increased probability for the development of hepatocellular carcinoma. However, it has been suggested recently that nodules are not obligatory precursors to carcinomas and that the origin of carcinomas outside of the nodule may be from altered foci as indicated in figure 1.3. (Williams, 1980).
Hepatocellular carcinoma may compress or extend into the surrounding parenchyma. They may be trabecular and well- to poorly-differentiated. The cells may resemble normal hepatocytes or they may be enlarged or anaplastic with clear, eosinophilic or hyperbasophilic cytoplasm and frequently with enlarged or multiple nuclei. Mitoses are often seen. These liver cell carcinomas develop from hepatocytes (Farber, 1963).

With nearly all hepatocarcinogens, malignant disease does not develop until months after the start of treatment. During this period many cellular changes occur which manifest themselves morphologically and cytologically as already described. Though there may be some slight variation of these changes with different carcinogens, many are common to all carcinogenic regimes. Of these changes those which are intimately involved with tumour formation must be determined, so must the new biological properties acquired by these cells which enable them to become neoplastic.

Ductular proliferation is a common early response to most hepatocarcinogens but hepatocellular carcinoma may develop in the absence of observable ductular proliferation or cirrhosis. Oval cell proliferation is observed under conditions of liver damage and it is not thought to be an essential part of the series of events leading to tumour development though these cells may differentiate into hepatocytes (Dempo et al., 1975).

b) Cellular Changes Occurring Early in Carcinogenesis

Bannasch (1968;1976;1978;1979; Bannasch et al., 1979) has made an
extensive study of the cytoplasmic changes occurring during hepatocarcinogenesis, particularly N-nitrosomorpholine-induced hepatocarcinogenesis, some of which reflect a neoplastic response, others merely a degenerative response. The degenerative changes appear in the centrilobular region of the liver lobule and the degree of toxic change manifested is a reflection of the concentration of the carcinogen. These changes are seen under the light microscope as a reduction in glycogen content of the cells. With low doses of the carcinogen there is only a small band of cells affected around the central vein, and at high doses virtually the whole liver may be affected. The glycogen loss is accompanied by the disaggregation of the rough endoplasmic reticulum (RER) and diffuse cytoplasmic basophilia which may be more or less pronounced. Some cells may be eosinophilic. Often such cells undergo coagulative necrosis. Mitochondria may be more numerous and nearly always have reduced cisternae. These changes reflect toxic damage due to the carcinogen and are reversed between two and four weeks after the removal of the carcinogen. Periportally different changes occur. Glycogen is accumulated above normal levels in clear cells (in which the glycogen is lost during fixation) and acidophilic cells (Bannasch, 1976). If the dose level of the carcinogen is high so as to produce mostly toxic damage the glycogen storage foci do not appear until after the carcinogen is removed. The glycogen is stored in the cytoplasmic matrix or in autophagic vacuoles. Tumours are thought to arise from these cells. With the transition towards tumour formation these glycogen storage cells gradually lose the glycogen with a concomitant increase in cytoplasmic basophilia due to increases in RER and ribosomes. They show transitory fat accumulation. Mitoses become more frequent with increasing basophilia and the cells may become arranged into unusual solid trabecular or tubular patterns. Mitochondrial changes are so varied as to be considered insignificant to tumour formation. Bannasch proposes that the non-specific toxic action of the carcinogen on the centrilobular regions causes regressive alterations leading to necrosis and cirrhosis. On the other hand the periportal changes represent 'summation' effects of the specific carcinogenic action and are due to the alterations leading to cellular transformation to carcinoma. The progression of periportal clear cells to hyperplastic nodules from
which hepatocellular carcinomas develop, accompanied by a change to 'dark' cells as was observed in the first liver carcinogenesis studies (Sasaki and Yoshida, 1935).

The appearance of mitoses in periportal regions has been observed with other carcinogenesis regimes (Firminger, 1955; Novi, 1977). However, Magee and Barnes (1967), whilst observing an increased basophilia in the periportal cells and the development of tumours from clusters of basophilic cells at low doses of dimethylnitrosamine, found that with high doses tumours appeared to develop from nests of cells located centrilobularly. In contrast to Bannasch's study (1968) Novi (1977) reported that with aflatoxin B1 carcinogenesis periportal cells were the most rapidly damaged and centrilobular cells affected least, and that the glycogen storage cells were located centrilobularly. However the periportal cells were induced to become basophilic and phenotypically differentiated (as regards drug metabolism) and ultimately resulted in tumour formation.

Carcinogens also induce nuclear changes such as nuclear enlargement, aneuploidy and hyperploidy early on in the carcinogenesis study but tumours appear to develop from diploid cells (Magee and Barnes, 1967). Many nuclear changes only occur in degenerating cells (Novi, 1977) and revert to normal on removal of the carcinogen (Bannasch et al, 1979). Generally the changes are non-specific and unlikely to be a basic prerequisite for cancer induction. Large lipid vacuoles may be seen in some undifferentiated liver cell carcinomas but this is not a necessary characteristic of hepatocellular carcinoma.

1.2.3. Morphological Dedifferentiation in Liver Tumours

Certain foetal morphological elements are exhibited by the tumour. Foetal hepatocytes are not arranged in the one-cell thick cords characteristic of the adult, but in two cell-thick cords. Some tumours may also show such an arrangement (Farber, 1976). Similarities in the organisational pattern of hyperplastic lesions, developing liver in the perinatal period, regenerating liver after the peak of cell division and hepatocellular carcinomas have been reported (Ogawa et al, 1979a). Ultrastructurally hepatocarcinoma cells induced by diethylnitrosamine
showed morphological similarities with foetal hepatocytes and differences from normal adult hepatocytes in that the smooth endoplasmic reticulum was severely reduced (Bruni, 1973). Human liver tumours have been classified as being either embryonic or foetal forms (Altman, 1978) on the basis of both morphology and cytology. The embryonal-type cells containing glycogen and often fat, with a loosely structured nucleus, are arranged in multiple layers. The foetal-type cells are smaller and more basophilic, with a chromatin-dense nucleus, and are arranged irregularly in larger complexes. Extramedullary haematopoiesis has also been demonstrated in some hepatomas (Rabes et al., 1972a; Enomoto et al., 1978). For further similarities between tumour and foetal or embryonic cells see Table 1.1. (p. 22).
1.3. Biochemical Features of Malignancy

1.3.1. The History of the Study of Cancer Biochemistry

The ultimate reasons for studying the biochemical features of cancer cells is to determine the differences from normal cells that will help in the understanding of the molecular nature of the neoplastic state. With this knowledge therapeutic treatments may be designed on truly rational grounds (Criss, 1975), and in addition, certain biochemical markers might be revealed that could be exploited as diagnostic aids. The characteristic biological properties of cancer cells, i.e. their capacity for autonomous growth and the ability to invade and metastasise, reflect an underlying biochemical disturbance. The question is, what are the basic cellular changes which enable the intracellular activity of the cancer cell to escape the extracellular controls that operate to regulate the activity of normal cells?

Our present views on the biochemistry of cancer have evolved through the modification and extension of earlier ideas. The advances in cancer biochemistry have been contingent upon advances in general biochemistry and the availability of tumours of known pathogenesis. In the early part of this century the only tumours available for experimentation were poorly differentiated and fast growing. Warburg (1931) noticed that such tumours all accumulated lactic acid in the presence of oxygen. He concluded that neoplasia was characterised by a high aerobic glycolysis and arose as a consequence of carcinogens damaging the respiratory mechanisms of cells and thereby causing the adoption of an anaerobic metabolism. An extension of this theory was the convergence theory (Greenstein, 1954) based on the observations of various biochemical parameters, not solely glycolysis. The convergence theory proposed that "tumours tend to converge, enzymatically to a common type of tissue." Both these theories have been found to be inadequate: Waburg's theory because of the discovery of tumours that do not have a high aerobic glycolysis and of some normal tissues that do (Pitot, 1978a); and Greenstein's theory because of the greater phenotypic diversity exhibited by the wide variety of tumours that are available now (Potter, 1964a). Aerobic glycolysis is a characteristic
of the less well-differentiated, faster growing tumour types and it seems that although aerobic glycolysis is not a prerequisite for neoplasia it does correlate with growth rate (Morris, 1965; Weinhouse, 1966; Harrap, 1975). Another early theory was the deletion hypothesis (Miller and Miller, 1947) in which carcinogenesis was thought to result from 'a permanent alteration or loss of proteins essential for the control of growth but not for life.' This hypothesis was based on the observation that normal liver proteins bound carcinogenic azo dyes, but primary hepatocellular carcinomas did not. These proteins are referred to as the $h_2$ proteins; similar proteins in mouse skin bind carcinogenic hydrocarbons in a direct relationship to their carcinogenic activity. The loss of these proteins, however, has been found not to be necessary for carcinogenesis as $h_2$ proteins have been demonstrated in highly differentiated hepatomas (Pitot, 1978a) and azo dye binding has been demonstrated, although at a lower level than in normal liver (Pitot and Cardelli, 1978). In the 1950's Potter (cited by Potter, 1973) suggested that the proteins deleted during carcinogenesis may be those identical or associated with certain catabolic enzymes, a view compatible with Greenstein's hypothesis, as well as some of the biological aspects of neoplasm, such as rapid growth. Many hepatomas available then did lack many catabolic reactions characteristic of normal liver. This hypothesis too has had to be abandoned since further investigation with other hepatomas have shown that there are exceptions to every deletion (Potter, 1973).

1.3.2. The Biochemistry of Transplantable Hepatomas

A major landmark in the study of the biochemistry of cancer was the development of a series of transplantable hepatomas, ranging from slow-growing highly differentiated types to fast-growing poorly differentiated types, by Morris in the late 1950's (Morris, 1965; Morris and Slaughter, 1978). These included the so-called 'minimal deviation' hepatomas which are highly differentiated and slow growing, have normal karyotypes and have a biochemical phenotype qualitatively similar to normal adult liver. Studies of these hepatomas reveal a great diversity in their biochemical phenotype (Wu, 1967). One characteristic they have
in common is an altered, nearly always reduced, response to humoral and hormonal controls (Criss, 1974). For example, there is a loss of feedback control by end product inhibition of cholesterol synthesis not only in poorly differentiated hepatomas but also in the minimal deviation hepatomas (Siperstein, 1965). Defective feedback control is an early acquired phenomenon and is demonstrated in rat liver after only 7 - 14 days following the administration of a variety of hepatocarcinogens (Sabine, 1976). The regulation of cholesterol and fatty acid biosynthesis by cyclic nucleotides is also lost in hepatomas (Bricker and Levey, 1972). Loss of feedback control has similarly been shown in the pathway of haem biosynthesis, δ-aminolevulinic acid synthetase cannot be suppressed by exogenous haem in hepatomas (Sabine, 1976). Other defective metabolic controls have been reported, for example the lack of response of glucose 6-phosphate dehydrogenase and threonine dehydratase to dietary stimuli (Potter, 1964.a). Fasting does not inhibit lipid synthesis in hepatomas as it does in normal liver (Mishkin and Halpern, 1978). An exception to the general rule of decreased responsiveness of enzymes to regulation in hepatomas is shown by tyrosine aminotransferase which is induced by cortisone to a greater degree in a minimal deviation hepatoma (5123) than in normal liver (Pitot and Cardelli, 1978). Furthermore, the metabolic regulation of glucose 6-phosphatase, tyrosine aminotransferase and tryptophan pyrrolase (dioxygenase) has been shown to be retained in hyperplastic nodules (Teebor and Seidman, 1970).

a) Phenotypic Diversity of Transplantable Hepatomas as a Result of Altered Regulatory Control

A study of various minimal deviation hepatomas has shown that although they all exhibit abnormal behaviour in carbohydrate, amino acid, lipid and nucleic acid metabolism, of drug metabolizing enzymes, and the regulation of enzymes by cyclic nucleotides, hormones and dietary stimuli, not all of the hepatomas showed the same relative degree of abnormality for each metabolic characteristic (Goldfarb and Pitot, 1976). There is therefore a great diversity in the biochemistry of tumours and this is analogous to the diversity of normal tissue.
Potter (1973) has suggested that this may be explained by an altered responsiveness to molecular messengers in pathways under regulatory control and feedback loops; since not only can molecular messengers elicit multiple responses and most receptors are responsive to some degree to more than one messenger, but also most intermediary metabolites may be metabolised via a number of alternative pathways and may serve as molecular messengers themselves; furthermore all molecular messengers are subject to metabolism i.e. synthesis, degradation, dissociation, binding and transport. Thus small changes in responsiveness of certain pathways may be amplified to larger changes in many other pathways.

b) Phenotypic Diversity and the Minimal Deviation Concept

Despite the apparent diversity of tumours it is to be hoped that there is some common molecular key to the nature of the cancer cell. The search for a unifying hypothesis led Potter (cited by Potter, 1973) originally to propose the concept of minimal deviation. This concept is an attempt to define the minimum changes necessary to produce cancer by eliminating the non-essential changes. Such non-essential changes, thought to arise when the original transformed cells undergo further changes in metabolism and appearance, would therefore give rise to a wide spectrum of malignant states. For example karyotypic abnormalities, although observed in many tumours, are not found in all of them and therefore belong to the category of non-essential changes. If this concept were true one would expect that preneoplastic nodules would exhibit a greater degree of homogeneity, but this is not the case (Pitot et al., 1963; Kitagawa, 1971). Pitot has suggested that the key to the phenotypic diversity of tumours is as a result of altered translational control, possibly mRNA template stability (Pitot et al., 1974), but there is as yet little direct evidence to support this.

c) Phenotypic Diversity of Tumours Explained by the Molecular Correlation Concept

The molecular correlation concept has also been proposed to explain the enzymic diversity of tumours (Weber and Lea, 1967; Weber, 1972; 1974;
The basis of this concept is the study of key enzymes in metabolism. The phenotype of the tumour being determined by three categories of enzymes:

(i) Progression linked, i.e. the enzyme changes are in parallel with the degree of malignancy;
(ii) Transformation linked, i.e. shared by all neoplasms and connected with malignant transformation itself;
(iii) Coincidental, i.e. random and not connected with the cause of neoplasia.

The first two categories include key enzymes involved in catabolism and biosynthesis which would confer a selective advantage to the tumour cell, e.g. glucose breakdown is increased as is nucleic acid synthesis. In the case of isoenzymes those under regulatory control are replaced by those less responsive to regulatory stimuli to allow metabolism to proceed unfettered. The third category, the coincidental enzyme changes, contribute to the diversity of tumour phenotypes but are of no significance to the neoplastic process itself.

1.3.3. The Concept of Foetalism in Tumours

Another approach to explaining the diversity of tumours is the idea of 'foetalism' i.e. neoplastic cells lose adult differentiated characteristics and acquire foetal, undifferentiated ones so that the biochemical diversity, like the morphological diversity of tumours reflects the degree of dedifferentiation or how far the tumour has regressed down the path to its appropriate foetal tissue counterpart. A striking feature of tumours is the appearance of foetal characteristics. If a list of the properties of cancer cells is drawn up, similarities with certain embryonic cells may be seen (Table 1.1.).

Invasiveness and the ability to stimulate the proliferation of the maternal blood system, whilst not being properties of foetal tissue itself are however characteristic of the embryonic cells - the trophoblasts. In addition the loss of cohesiveness and increased mobility is a property of many tumour cells in common with foetal and embryonic cells.
Table 1.1. Characteristics of Cancer Cells

i) The possession of autoantigens

ii) The ability to escape the immune response of the host

iii) Alterations in the cytoplasmic composition, including isoenzyme ratio changes, the appearance of isoenzymes and other proteins not found in the tissue of origin, or in some instances any adult tissue

iv) Invasiveness and the ability to metastasise

v) Stimulation of host responses, including adequate blood supply and the growth of supporting tissue

vi) Increased rate of growth and cell division in most instances

vii) Progression and the ability to change gradually in a variable and individualistic manner with time from preneoplastic through many stages of neoplastic growth

(Anderson and Coggin, 1974)

Greenstein (1954) observed that, "As a whole, the metabolic behaviour of hepatoma and foetal liver is nearly similar and quite different from that of the nearly similar metabolic properties of resting adult liver and regenerating liver after partial hepatectomy." A recent reevaluation of Greenstein's statement using data from many sources on enzymic and non-enzymic components of foetal, regenerating and resting adult liver and hepatomas has reached the same conclusion (Knox, 1976). The more differentiated, slow-growing tumours being more similar to the adult tissue and the less-differentiated tumours being more similar to the foetal tissue (Knox, 1974). Thus, the apparent diversity of tumour phenotypes reflects the differing degree of differentiation of a spectrum of tumours. As all tissues are derived ultimately from a single cell and since during organogenesis biochemical differentiation causes the gradual divergence of phenotypes of different tissues, one would expect foetal tissues to be more similar to each other than are adult tissues. If tumours represent a progression towards more immature cell forms, then poorly-differentiated
tumours of one tissue would be similar to the poorly-differentiated
tumours of another. Thus, the convergence hypothesis of Greenstein can
be explained in terms of foetalism.

The resurgence of foetal isozymes in tumours has been reported by
many authors (Schapira, 1966; 1973; Criss, 1971; Elford, 1972; Walker and
Potter, 1972; Weinhouse et al., 1972; 1976; Ichihara, 1975; Fishman and
Singer, 1975; Goto et al., 1977; Roth et al., 1977). Another foetal
characteristic of hepatomas is the resurgence of foetal antigens,
particularly alpha-fetoprotein (Abelev, 1971; Alexander, 1972; Baldwin,
1973). The synthesis of alpha-fetoprotein is not merely a reflection of
growth rate, which one might suppose from its transitory appearance in
regenerating liver, because an artificial halting of proliferation does
not prevent its synthesis. Embryonic &-globulin has been shown to
increase in tumours (Watabe, 1971) and carcinoplacental antigens and
isoenzymes (i.e. those found in tumour and placenta but not in normal
adult or foetal tissue) have been shown in both human cancer (Fishman
et al., 1968; Spellman and Pottrell, 1973) and in tumours of experimental
animals (Ichihara, 1976). Various other similarities in factors thought
to play a role in gene regulation and expression, have been observed in
tumours and foetal or embryonic tissues. Certain tRNA molecules have
been found in hepatoma cells and rat foetuses but not in normal or
regenerating liver (Yang, 1971). Furthermore, a study on tRNA methylase
enzymes has shown that the methylating activity of neoplastic liver
enzymes is more similar to foetal liver enzymes than adult liver enzymes.
Changes in non-histone proteins in cancer analogous to foetal changes
have also been described (Yeoman et al., 1976).

The ectopic synthesis of polypeptide hormones and isoenzymes may
also be embryonic in character. The common embryonic origin of the
pituitary and the lung in the endoderm suggests that the production of
pituitary hormones by lung tumours is a reflection of their common embryonic
ancestry (Weinhouse et al., 1976).

The significance of the foetalism of tumours is open to speculation
and the mechanisms involved, unknown. Various hypotheses have been put
forward to explain this phenomenon. It may arise by carcinogenic
selection of stem cells, phenotypically similar to foetal cells, which
instead of undergoing normal differentiation to mature cells, differentiate
into neoplastic cells (Pierce, 1970). Alternatively there may be the formation of embryonic or partially specialised stem cells from adult cells, under the influence of the carcinogen, which then grow to replace dead cells. The stem cells may be blocked at any one of a number of stages of reontogeny (Potter, 1969; Walker and Potter, 1972). Similarly, proposals by Uriel (1976) involve a step-wise retrodifferentiation of normal mature cells to more immature types by a reversal of normal ontogeny, with the phenotype becoming neoplastic by being locked at any one of these steps. The term disdifferentiation has been proposed (Sugimura et al., 1972) as an alternative description of carcinogenesis, emphasising that enzymic forms of tumours may not necessarily be foetal in nature but may be characteristic of a different adult tissue, i.e. implying an alternative route of redifferentiation to that normally followed.

1.3.4. Dedifferentiation in the Host Liver of Tumour-bearing Animals

Enzymic dedifferentiation has been observed in the host livers of tumour-bearing rats (Potter, 1964b; Suda et al., 1966; 1972) and parabiotic experiments suggest that these changes are due to some chemical messenger from the cancer cells inducing the immature state of the host liver (Suda et al., 1966; 1972). The implantation of tumours into young rats prevented the emergence of some enzymes characteristic of the adult liver, and implantation of tumours in adult rats caused an increase of enzymes present in foetal liver and loss of adult-specific ones (Herzfeld and Greengard, 1972). Other host liver effects include a reduction of mixed function oxidase activity as shown by increased hexobarbital sleeping time, decreased cholesterol synthesis, a slight reduction in amino acid metabolism and an increase in 5' nucleotide phosphodiesterase (which is also increased in hepatoma and regenerating liver). Fatty acid metabolism may be increased or decreased depending on the nature of the transplanted tumour (Goldfarb and Pitot, 1976).

1.3.5. Early Changes in Hepatocarcinogenesis

In order that biochemical differences may be exploited as
diagnostic markers for carcinogenesis they must be detectable at an early stage of tumourigenesis. The biochemical changes in preneoplastic altered foci have been investigated by a number of workers. The enzyme changes seen in these foci of altered cells include decreases in glucose 6-phosphatase, adenosine triphosphatase (Moulin and Daoust, 1971; Rabes et al., 1972b; Scherer et al., 1972; Goldfarb, 1973) decreases in ribonuclease (Daoust, 1972; Murthy and Daoust, 1977), deoxyribonuclease (Taper et al., 1971), polycytidilic acid and polyuridylic acid hydrolases (Daoust, 1977). Decreases have also been observed in acid and alkaline phosphatases, succinic dehydrogenase, 5' nucleotidase and β-glucuronidase (Kitagawa, 1971; Kitagawa and Sugano, 1973; Kalengayi and Desmet, 1975; Tatematsu et al., 1977). Increases have been seen in glucose 6-phosphate dehydrogenase (Kalengayi and Desmet, 1975) and ß-glutamyl transpeptidase (Kalengayi et al., 1975; Harada et al., 1976). For some of these enzymes the changes represent the loss of adult enzymes or the increase of enzymes present in the foetal liver (Onoe et al., 1976). In addition, the loss of responsiveness to dietary regulation of serine dehydratase and glucose 6-phosphatase has been demonstrated in preneoplastic nodules (Kitagawa and Pitot, 1975).

Bannasch believes that the first change in preneoplastic cells is the excessive storage of glycogen, which occurs before the enzyme changes take place. The glycogen is then lost together with the loss of some enzymes and the acquisition of others, by progressive alterations related to the phenotypic expression of malignancy (Taper and Bannasch, 1976).

The preneoplastic lesions are also resistant to iron accumulation by dietary overload and in this way they are similar to hepatomas and different from normal liver (Williams et al., 1976). In fact it has been suggested that this is a more sensitive marker for preneoplastic lesions than enzyme changes which are sometimes variable (Hirota and Williams, 1979).
1.4. Enzymic Differentiation in Rat Liver

1.4.1. The Nature of the Enzymic Change

The enzymic complement of adult rat liver does not replace the foetal one gradually, nor does the metabolic behaviour of the liver suddenly switch over at birth. Rather there are three critical periods during the development of metabolic adaptation to physiological needs which result in the replacement of enzymes present in the foetus by ones characteristic of the adult (Greengard, 1971; Snell, 1971). Groups of enzymes show sudden changes in their activity between sixteen and twenty-one days of gestation, during the first post-natal day and in the third post-natal week and are said to belong to the late foetal, neonatal and late suckling clusters respectively (Greengard, 1971).

1.4.2. Changes in Enzyme Activity as a Response to Physiological Stimuli

Before birth the foetus is supplied with glucose and amino acids, but not lipids which cannot traverse the placenta (Vernon and Walker, 1968) and has its nitrogenous waste removed by the mother. Once it has been born it must cope with these requirements itself. The observation that some enzymes arise shortly before birth and others shortly after suggests that the enzymes belonging to the late foetal cluster are required immediately for survival whereas the neonatal cluster enzymes can develop after birth. Urea cycle enzymes rise during the late foetal period (Greengard, 1971) as the deamination of nitrogenous waste is essential. Some deaminases and transaminases e.g. glutamate dehydrogenase and aspartate aminotransferase also make their first appearance at this time. Enzymes responsible for glycogen synthesis are present before birth, leading to massive glycogen accumulation in the late foetal liver (Moog, 1965). The combination of an increase in glycogen phosphorylase and glucose 6-phosphatase with a decrease in phosphofructokinase and fructose diphosphate aldolase pre- and post-natally reflects an increased capacity for the conversion of glycogen to free glucose with decreased capability for the conversion of glycogen to lactate (Burch et al., 1963).
After birth, despite the store of glycogen and the presence of enzymes necessary for its mobilization, the animal becomes hypoglycaemic. The diet of the neonate is rich in amino acids and lipids and low in carbohydrate. Carbohydrates in milk are sufficient for only 10% of the energy needs of the neonate (Vernon and Walker, 1968). Because of this change in diet there is a neonatal increase in enzymes involved in deamination and gluconeogenesis from amino acids. For example serine dehydratase, tyrosine aminotransferase, asparaginase and phosphoenolpyruvate carboxykinase appear and glucose 6-phosphatase shows a further increase. Phosphoenolpyruvate carboxykinase is a key enzyme involved in the regulation of gluconeogenesis and rises rapidly during the first two postnatal days and remains elevated, compared with adult levels, during the suckling period. The changes in enzyme levels are too sudden and too great to be accounted for by an increase in hepatic parenchymal cells and decrease in haematopoietic cells (Greengard et al., 1972). Increases in mitochondrial enzymes e.g. glutamate dehydrogenase in the perinatal period accompany an increase in the number of mitochondria at this time (Moog, 1965). At weaning there is a shift from a high fat, high protein, low carbohydrate diet to one that is carbohydrate rich. There is therefore a need for increased regulation of carbohydrate metabolism, interconversion of amino acids and lipid synthesis.

Glucokinase, a high Km, highly specific enzyme that is strictly regulated (Perez et al., 1964) appears during the late suckling period. Many aminotransferases belong to this cluster too e.g. alanine aminotransferase, ornithine aminotransferase. In response to the high carbohydrate, low fat diet glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase increase (Burch et al., 1963) as do other enzymes involved directly or indirectly with lipogenesis:- ATP citrate lyase, malic enzyme (Vernon and Walker, 1968). ATP citrate lyase, is present in the foetus, which must synthesise lipid, but malic enzyme is not. It is possible that the NADPH required for lipid synthesis is provided in the foetus by glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, both of which are higher in the foetal than the adult liver (Vernon and Walker, 1968). These two enzymes, and pyruvate kinase, remain low in activity during suckling in response to the high fat, low carbohydrate diet.
Some enzymes show a different pattern of expression to the above mentioned ones, i.e. they are present at high levels in the foetus and decline during development. Hexokinase and phosphofructokinase both decrease from high foetal levels with increasing maturity reflecting a decrease in anaerobic glycolysis during maturation (Burch et al., 1963). The activity of thymidine kinase is about 12 times higher in the foetal liver than adult liver and its level drops rapidly around the time of birth (Herzfeld et al., 1976) and this presumably reflects the rapid DNA synthesis required by the growing rat liver.

1.4.3. Influence of Hormones on Developmental Enzyme Changes

The enzymic response of the liver to changes in diet during development is mediated, in part, by hormones. Late foetal changes such as the increase in NADP dehydrogenase activity and glucose 6-phosphatase activity around the 18th day of gestation are associated with the functioning of the thyroid gland, which commences just before this time. The injection of thyroxine enhances the prenatal expression of these enzymes (Greengard, 1969). Hypoglycaemia at birth causes an increased secretion of glucagon, which stimulates the neonatal cluster enzymes - serine dehydratase, tyrosine aminotransferase and glucose 6-phosphatase. Adrenaline also stimulates tyrosine aminotransferase and glucose 6-phosphatase (Greengard, 1969).

After about the 12th postnatal day there is an increased glucocorticoid and thyroxine secretion which is associated with the appearance of some late suckling enzymes. Ornithine aminotransferase is stimulated by hydrocortisone alone, glucokinase is stimulated by hydrocortisone in conjunction with glucose, and tryptophan oxygenase by hydrocortisone and tryptophan administration (Greengard, 1971). Thyroxine stimulates the malic enzyme activity (Murphy and Walker, 1974). Enzymes may be inhibited from appearing by the prevention of hormone secretion (Greengard, 1971) or their precocious appearance may be elicited by hormone injection (Greengard, 1971; Herzfeld and Greengard, 1971). The pattern of change of enzymes during development is shown diagrammatically with examples of representative cluster enzymes in Figure 1.3.
Figure 1.3.

A Late Foetal Cluster
- glucose 6-phosphatase
- glycogen phosphorylase
- glutamate dehydrogenase

B Neonatal Cluster
- phosphoenolpyruvate carboxykinase
- glucose 6-phosphatase
- serine dehydratase
- tyrosine aminotransferase

C Late Suckling Cluster
- glucokinase
- 'malic' enzyme (ATP-citrate lyase)
- glucagon
- thyroxine
- hydrocortisone (insulin)

Age (days)
1.5. Mechanisms of Carcinogenesis

1.5.1. Early Theories on the Genesis of Cancer

The histopathological description of cancer is based largely on the degree of loss of differentiated characteristics and this led the early theorists to propose that the mechanisms of carcinogenesis involved some reappearance of a dedifferentiated or embryonic type of cell. Lobstein in 1829 (cited by Rather, 1978) was the first to draw the analogy between the genesis of tumours and embryological development. The idea that during embryonic development more cells are produced than are necessary and that these cells remain in the adult in their embryonic form capable of rapid proliferation and development into tumours was put forward 60 years later (Conheim, 1889). A similar hypothesis that tumours arose from vagrant stem cells was also proposed (Beard, 1902). The suggestion that malignant behaviour induced by chemical carcinogenesis was analogous to the induction of division in eggs by parthenogenic chemicals was made by Berril (1943).

Alternatively, the presence of aneuploidy in tumours led Boveri (1929) to postulate that tumours arose as a result of chromosomal imbalance caused by multipolar, especially tripolar, mitoses. Such a somatic mutation theory of cancer is very attractive as it explains the heritable change in cell lineage in neoplasia. However, a stable heritable change in cell lineage is not only a characteristic of mutation but also a characteristic of normal differentiation and development, so the debate as to whether cancer is a result of mutational or epigenetic mechanisms, analogous to normal development continues to this day. Both theories have accumulated much circumstantial evidence in their favour but neither have been proven.

1.5.2. Evidence in Favour of the Somatic Mutation Theory of Cancer

a) Karyotypic Abnormalities

Many tumours are characterised by karyotypic abnormalities, however, these changes are not uniform from tumour to tumour, moreover,
some tumours have no detectable abnormalities in the genetic material and many heteroploid cell populations are not malignant (Koller, 1964). Therefore it seems that karyotypic abnormalities are more likely to result from, rather than cause cancer. Nevertheless many tumours do seem to stabilize at a hypotetraploid chromosome number (Wolman and Horland, 1975).

There is an association of chromosomal or genetic disorders with some human cancers. Individuals with Down's syndrome and Kleinfelter's syndrome have an increased susceptibility to leukaemia, and chronic myelogenous leukaemia is associated with the Philadelphia chromosome. Some Mendelian conditions such as Fanconi and Bloom's syndrome, which are autosomal recessive traits with increased rates of chromosomal breakage in vitro and in vivo, predispose the individual to leukaemia and cancer. Some tumours exhibit dominant inheritance patterns e.g. retinoblastoma and intestinal polyposis. Individuals with Xeroderma pigmentosum, a disease in which there is an inherited defective DNA repair system, particularly UV-induced DNA damage, frequently develop skin cancers (Knudson, 1975).

b) Interaction of Carcinogens with DNA

Certain viruses cause neoplastic transformation, the oncogenic viral DNA is incorporated into the host genome (or, in the case of RNA oncoviruses, the viral genetic material is first transcribed into DNA by reverse transcriptase) and constitute a structural genetic alteration. The viral DNA is not only transmitted regularly at cell division but also directs the synthesis of a gene product as demonstrated by the presence of virus specific antigens (Wolman and Horland, 1975). The oncogenic virus may act by rearranging, activating and/or damaging host cell genes to cause transformation to a tumour cell.

Most chemical carcinogens interact with DNA both in vitro and in vivo (Miller and Miller, 1971) and there is a close association between the mutagenic and carcinogenic activity of a wide variety of chemicals (McCann and Ames, 1976). The relationship between somatic mutation by chemicals and neoplastic change in vitro has been demonstrated, though the detection time required for transformation
is longer than the optimal expression time of the somatic mutations (Barrett and Ts'o, 1978). Both carcinogenic chemicals and ionising radiation may cause strand breaks in DNA, and sister chromatid exchange (DiPaolo and Casto, 1977). Furthermore, DNA strand breakage, somatic mutation, sister chromatid exchange and neoplastic transformation have been demonstrated in vitro by a direct perturbation of DNA with a combination of 5-bromodeoxyuridine and UV light (Barrett et al, 1978), though neither treatment alone had this effect.

c) Erroneous DNA Repair

Other postulated mechanisms depend on the infidelity of DNA repair by an error-prone DNA polymerase, produced either by viral or chemical carcinogenesis, which would then increase the mutation frequency leading to new genotypes which might exhibit phenotypes with a selective value for proliferation and ultimately cancer (Sirover and Loeb, 1977).

If carcinogenesis is thought to be a result of mutation fixed by erroneous DNA repair one would expect there to be a relationship between cell replication and tumorigenesis since prereslicative DNA repair is thought to be error-free but post replicative repair is error prone. This is in fact the case. Dividing cells in vitro have been shown to have increased chemically-induced mutation frequencies when compared with non-dividing cells (Berman et al, 1978).

Single or short-term administration of carcinogens that do not induce tumours in normal adult animals do give rise to tumours under conditions of cell replication, such as in young or foetal animals, or during regenerative hyperplasia following surgery or toxic damage (Craddock, 1976). Carcinogens themselves are frequently toxic and may induce regenerative hyperplasia. The possibility that cancer is induced only in those cells which replicate soon after carcinogen treatment would help explain the fact that in liver carcinogenesis, while all or most liver cells are probably affected by the carcinogen only a few give rise to tumours (Craddock, 1978). If the carcinogen dimethylnitrosamine is given to a rat six hours after partial hepatectomy cell division is delayed, and during this period the DNA damage induced by dimethylnitrosamine is repaired and the resulting tumour incidence is low.
On the other hand, dimethylnitrosamine given twenty four hours after partial hepatectomy fails to delay cell division, as the replication enzymes have already been induced, and new DNA is synthesised on a damaged template leading to a high tumour incidence (Craddock, 1978).

d) Probable Target of Mutational Events

If mutational event or events were responsible, at least in part, for carcinogenesis it would be most likely to involve cell regulatory functions rather than specific differentiated functions. Since many mutations are of the missense or nonsense type, the expected result would be a loss of regulatory function. Such changes would be expected in functions controlling cell division, repair of DNA, formation and regulation of cell surface components and antigens, loss of rate-limiting metabolic regulators so as to confer a selective advantage to the mutated cell (Wolman and Hordland, 1975). Comings (1973) has proposed that there are multiple structural genes coding for transforming factors which can release the cell from its normal growth constraints. These are suppressed in adult cells by diploid pairs of regulatory genes but may be expressed at certain stages of the cell-cycle and during embryogenesis. The regulatory genes may become non-functional as a result of double mutations induced by chemicals or radiation or disturbed by the incorporation of viral DNA. In heritable tumours only a single mutation would be required as the proposal is that one of the regulatory genes is already inactivated under such circumstances.

1.5.3. Evidence for Epigenetic Mechanisms of Carcinogenesis

a) Similarity of Cancer Development with Normal Development and Ageing

Despite such a large body of evidence in favour of a somatic mutation theory of cancer there is also much evidence to oppose such a theory and this has been summarised by Nery (1976). The alternative theory, that carcinogenesis does not involve a mutation but results from a process similar to normal differentiation, is supported by much
Cellular differentiation, like neoplastic transformation, represents a stable heritable change and the extreme cases of terminal differentiation are irreversible (Braun, 1975). It has been suggested that similarities exist between the development of cancer and the process of ageing (Pitot, 1978b) and that these two phenomena may be related.

The majority of proteins are transcribed from unique sequence DNA, most of which is unexpressed at any given time. Different parts of the genome are transcribed in different organs and at different times during development. The malignant phenotype has been proposed to arise from the coexpression in the adult cell of a gene set or sets normally expressed during early development (Manes, 1974). The discovery that some chromatin proteins of tumour cells are foetal antigens has led to the suggestion that some of the "switches" involved in gene activation for tumour induction may be foetal or oncodevelopmental. This has led Busch (1976) to postulate that mRNA for the protein products involved in growth, invasiveness and metastasis are produced by interaction of foetal gene derepressors with the genome. This then results in malignancy in the adult due to the absence of the gene inhibitors present during embryonic development.

b) Induction of Retrodifferentiation and Redifferentiation in Normal and Tumour Cells

That the retrodifferentiation and redifferentiation of the genome is possible has been shown by Gurdon (1963). He transplanted nuclei from the gut of Xenopus tadpoles into enucleated Xenopus eggs and found that about 25% of them developed into tadpoles. If DNA damage is responsible for neoplasia one would not expect the resultant tumours to have the same totipotential nature. However, similar experiments have been carried out: normal tadpoles have developed from enucleated frog ova containing nuclei transplanted from frog renal cancer cells (McKinnel et al., 1969). The implication of these results is that the genetic information in the cancer cell nucleus was effectively reprogrammed by cytoplasmic factors in the frog egg that regulate nuclear gene activity during normal development. A similar situation is
illustrated by the study of teratocarcinomas, strange tumours most commonly found in the gonads, composed of multiple kinds of somatic tissues, representing each of the three germinal layers, which may be arranged into recognisable organs. Intermingled with these differentiated tissues are highly malignant embryonal carcinoma cells, so called because of their resemblance to embryonic epithelium (Pierce, 1974). The embryonal carcinoma cells are multipotential and will form new teratocarcinoma cells, complete with the various somatic tissues, when injected into isologous hosts (Pierce, 1967). Teratocarcinoma cells can also undergo complete differentiation to normal adult animals, which exhibit no neoplasms, after injection into blastocysts (Minz and Illmensee, 1975). However, aneuploid teratocarcinoma cells injected into blastocysts cause teratocarcinomas and other neoplasms to develop in the neonatal and adult chimera (Pitot, 1977b). These studies suggest that cells which express a neoplastic phenotype may possess a normal genotype and may revert to a normal phenotype under appropriate conditions e.g. when the environment is comparable to that found in the developing foetus. Alternatively the tumour cell may undergo changes causing an altered genotype incapable of reversion to the normal situation. Certain human tumours may undergo a reversion to a normal phenotype e.g. neuroblastoma cells may spontaneously revert to ganglioneuroma cells and can also be induced to revert in vitro (Pierce et al, 1978).

c) Non-mutagenic Carcinogens

Another argument in favour of an epigenetic approach to the possible mechanisms of carcinogenesis is the existence of non-mutagenic carcinogens. It is difficult to imagine how the implantation of plastic films into animals induces tumours by interaction with DNA (especially when the carcinogenic response seems to be partly determined by the texture of the film, and powdered plastic fails to elicit this response). Rather the film may act by causing an unremitting stimulus for growth (Kaplan, 1964).
d) Modification of Gene Expression in Neoplasia

Many investigations have revealed that transcriptional and translational events are modified in a wide range of animal and human neoplasms. However, the interpretation of these differences is impeded by the present inadequate knowledge of the regulation of gene expression in normal tissues (Harrap, 1975). Broadly speaking, in cancer the normal gene activity may be misprogrammed by directly regulating the gene function to turn on, or off, or modulate the synthesis of specific RNA species, or by modifying the translation of RNA into specific proteins, or by regulation of the topographic distribution or function of proteins (Markert, 1968). The situation that the carcinogen may interact with a repressor molecule(s) in a Jacob and Monod-type model causing abnormal gene expression resulting ultimately in malignancy has been envisaged (Monod and Jacob, 1961; Pitot and Heidelberg, 1963). Non-histone proteins are thought to be important in gene regulation and expression (Busch, 1977) and differences have been found in the non-histone protein complement of neoplastic, regenerating, embryonic and normal tissues (Chiu et al, 1976; Ruoslahti et al, 1977).

Changes in RNA species have also been observed in tumour cells and Pitot (1974) has suggested that changes in the half-life of mRNA may be responsible for differentiation and that neoplasia may result from altered mRNA stability. Posttranscriptional control by tRNA is thought to be important during embryogenesis, differentiation, virus infection and other regulatory activities, including the rate of protein synthesis. Different isoaccepting tRNA species are found in tumour and embryonic tissues from normal adult tissues, and tRNA methyltransferase activity is altered, representing a partial reexpression of the foetal phenotype (Yang, 1971; Pillinger and Wilkinson, 1971; Griffin, 1975; Kuchino and Borek, 1976).

1.5.4. The Two-Stage Model of Carcinogenesis

The two theories, mutational and epigenetic, of the mechanisms of carcinogenesis may be partly reconciled by the two-stage model of carcinogenesis. In this model a rapid mutagenic event of initiation
induces the cancer genotype which remains unexpressed unless a promoting stimulus is applied. Promotion is thought to be via epigenetic mechanisms and requires prolonged promoting stimulus. The two-stage carcinogenesis model was first demonstrated in mouse skin (Berenblum and Shubik, 1947; 1949). The painting of mouse skin with a subcarcinogenic dose of carcinogenic hydrocarbons, when followed by repeated application of croton oil, a non-carcinogenic hyperplastic agent, resulted in multiple tumour formation even if there was a considerable time interval between the carcinogen and croton oil applications. No tumours arose in mice treated with the same dose of carcinogen alone, croton oil application alone, or if the croton oil application preceded the carcinogen. Promoters may act by triggering gene activation (Boutwell, 1974) or by inhibiting differentiation (Diamond et al., 1978a).

Multi-stage models of carcinogenesis have since been demonstrated in other tissues besides skin: thyroid gland, liver, mammary gland, forestomach, thymus, subcutaneous tissues, kidney, lungs, colon, pancreas and also cells grown in tissue culture (reviewed by Berenblum, 1979).

Of particular interest to the present study is the promotion of hepatic carcinogenesis. Phenobarbitone, when administered to acetamidofluorene-treated rats was found to increase the incidence of hepatic tumours (Peraino et al., 1971) and the treatment with phenobarbitone could be delayed by thirty days without decreasing its enhancing effect (Peraino et al., 1973). Phenobarbitone also enhances diethylnitrosamine-induced hepatocarcinogenesis (Weisburger et al., 1975) and azo-dye hepatocarcinogenesis (Kitagawa and Sugano, 1977).

Hepatocarcinogenesis, initiated by various carcinogens may be promoted by agents other than phenobarbitone. Diethylnitrosamine-induced hepatic carcinogenesis has been found to be enhanced by DDT (dichlorodiphenyltrichloroethane) and polychlorinated biphenyls (Nishuzumi, 1976; 1979; Peraino et al., 1975). The enhancement of acetamidofluorene-induced carcinogenesis has been shown with DDT, butylated hydroxytoluene (Peraino et al., 1978), polychlorinated biphenyls and 3-(3,5, dichlorophenyl)-5,5-dimethylloxazoline-2,4-dione (Ito et al., 1978). The latter two compounds being administered in conjunction with partial
hepatectomy. Rapid production of hyperplastic nodules has been found with a single i.p. injection of diethylnitrosamine followed, after two weeks basal diet, by a diet containing acetamidofluorene for a short period, during which the rats underwent partial hepatectomy (Solt et al., 1977b). Other chemicals: 3 methyl-4-(dimethylamine)azobenzene, dimethyl-nitrosamine, diethylnitrosamine, DL-ethionine and quinoline may be substituted for acetamidofluorene in the above model with similar effect (Tatematsu et al., 1977). In such a model the acceleration of tumour promotion is thought to be because hepatocytes initiated by diethylnitrosamine are resistant to the cytotoxicity of the second carcinogen and are therefore capable of proliferating in response to partial hepatectomy whereas normal cells are inhibited from dividing in such a cytotoxic environment (Solt et al., 1977b).
1.6. Action of DiethylNitrosamine as a Carcinogen

1.6.1. Metabolism of DialkylNitrosamines

Direct acting carcinogens will give rise to tumours at the site of their injection; carcinogens requiring metabolic activation form cancers at distal sites. The nitrosamines belong to the latter class (Druckrey, 1973). The metabolism of carcinogens is primarily directed towards deactivation, most of the administered carcinogen is eventually excreted as non-carcinogenic derivatives and usually only a small proportion of the metabolism leads to the formation of proximate or ultimate carcinogens (Miller and Miller, 1974). The proposed sequence of events is shown in Figure 1.4. (Miller and Miller, 1976a).

Figure 1.4.

The dialkylNitrosamines undergo a series of reactions leading to the final reactive metabolite and various deactivated metabolites. Dimethylnitrosamine was the first to be studied; formaldehyde was found to be formed by liver microsomes and the remaining monomethylnitrosamine rapidly breaks down to give diazomethane or carbonium ion depending on the pH. The latter is thought to be the carcinogenic alkylating agent (Magee and Barnes, 1967). In view of the very short half-life of this electrophile it is possible that it is produced transiently during a concerted reaction among the substrate, the enzyme system and the target.
Figure 1.5. Activating Metabolic Pathway of Dimethylnitrosamine According to the $\alpha$-hydroxylation Hypothesis
molecule (Weisburger and Williams, 1975). Research in various laboratories has led to the α-hydroxylation hypothesis being currently accepted as the mechanism of metabolic activation of the dialkylnitrosamines as illustrated in figure 1.5, for dimethylnitrosamine (Lai and Arcos, 1980).

The metabolism of nitrosamines is rapid, e.g. if 50 mg dimethylnitrosamine/kg body weight is injected into the rat only 30% is recoverable after 6 hours and none after 24 hours. Experiments with hepatectomised rats indicate that the metabolism occurs mainly in the liver.

The first critical rate limiting step is hydroxylation of the α-carbon, the putative α-hydroxylated alkynitrosamine is unstable and on hydrolysis yields the corresponding aldehyde and monoalkynitrosamine. The latter molecule is highly unstable and undergoes spontaneous non-enzymatic breakdown to the alkylating intermediate.

The enzyme system responsible for the oxidative demethylation of dimethylnitrosamine, DMN-demethylase, requires NADPH and oxygen and can be inhibited by carbon monoxide and therefore appears to be a typical cytochrome P₄₅₀-dependent microsomal mixed function oxidase. Microsomes from dimethylnitrosamine treated rats demethylate dimethylnitrosamine faster than controls if high substrate concentrations are used, but slower than controls if the concentration of dimethylnitrosamine is low. It seems that there are at least two liver cytochrome P₄₅₀ species involved, one with a high affinity, operating at low concentrations of dimethylnitrosamine, and the other with a low affinity, and only the latter is inducible (Remmer, 1978). These two enzymes have been termed DMN-demethylase I (low Km) and DMN-demethylase II (high Km). DMN-demethylase I, the enzyme regarded to be responsible for the metabolic activation of dimethylnitrosamine in vivo, has been extensively studied (for references see Lai and Arcos, 1980).

Diethylnitrosamine undergoes a similar metabolic reaction sequence to dimethylnitrosamine. The first step, catalysed by DEN-deethylase is an α-oxidation yielding acetaldehyde and monoethylnitrosamine which breaks down to give the reactive ethonium ion.

Despite the similarities of metabolic activation of the nitrosamines there are differences in their biological activity during ontogeny,
e.g. deethylation, in contrast to demethylation, is possible at early stages of prenatal development and diethylnitrosamine is carcinogenic as early as the fifteenth gestational day in the rat (Druckrey, 1973).

1.6.2. Effect of Inducers and Inhibitors of the Cytochrome P<sub>450</sub> Monooxygenase System on Nitrosamine Metabolism

A positive correlation between deethylase activity and the relative susceptibility to diethylnitrosamine carcinogenesis of various organs of the rat and hamster has been demonstrated (Montesano and Magee, 1971). The induction of DEN-deethylase by phenobarbitone and inhibition by 3-methylcholanthrene together with the finding that phenobarbitone increases, whereas 3-methylcholanthrene decreases the microsome catalysed binding of diethylnitrosamine to DNA also suggests that deethylating activity is relevant to carcinogenesis (Arcos et al., 1976). In contrast to this suggestion however, is the finding that inducers of the cytochrome P<sub>450</sub> monooxygenase system e.g. phenobarbitone, 3-methylcholanthrene, α-hexachlorocyclohexane or polychlorinated biphenyls cause a reduction in the number, and delayed time of onset, of diethylnitrosamine-induced tumours, and inhibitors of the microsomal monooxygenase system, halothane and SKF 525A enhanced the development of hepatomas. Phenobarbitone and 3-methylcholanthrene cause an increase in formaldehyde formation from dimethylnitrosamine and decreased CH<sub>3</sub> bound to microsomal proteins, whilst halothane and SKF 525A produce the opposite effect. Such an inverse relationship of formaldehyde formation with carbonium ion formation and tumour induction might suggest that there is a cytochrome P<sub>450</sub>-dependant reaction catalysing the oxidative demethylation to form formaldehyde and a P<sub>450</sub>-independent stage leading to the formation of alkylating metabolites. However, experiments with P<sub>450</sub> inhibitors in vitro show an inhibition of formaldehyde formation and alkylation to the same extent suggesting that cytochrome P<sub>450</sub> is necessary both for activation and deactivation (for references see Kunz et al., 1978). Furthermore phenobarbitone or 20-methylcholanthrene, despite stimulating DMN-demethylase do not stimulate the formation of <sup>14</sup>C<sub>2</sub> from <sup>14</sup>C dimethylnitrosamine (Phillips et al., 1975).
1.6.3. Alkylation of Cellular Macromolecules

The growth of tumours in a clonal fashion seems to require at least a quasi-permanent alteration in the phenotype of the cells. It is likely therefore that the ultimate carcinogenic derivatives must interact with one or more of the informational macromolecules of the cell: DNA, RNA or protein. The electrophilic derivatives of the nitrosamines are capable of reacting with nucleophilic centres in both nucleic acids and proteins, not all of which are critical. The potential sites of electrophilic attack are shown in table 1.2. (Magee et al, 1976; Singer, 1977).

Table 1.2. Sites of Alkylation in Informational Macromolecules

<table>
<thead>
<tr>
<th>Nucleic Acids</th>
<th>Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>N-1, N-3, N-6, N-7</td>
</tr>
<tr>
<td>Cytosine</td>
<td>N-3, N-4, 0-2</td>
</tr>
<tr>
<td>Guanine</td>
<td>N-1, N-2, N-3, N-7, 0-6</td>
</tr>
<tr>
<td>Thymidine</td>
<td>N-3, 0-2, 0-4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine</td>
<td>S</td>
</tr>
<tr>
<td>Histidine</td>
<td>N-1, N-3</td>
</tr>
<tr>
<td>Methionine</td>
<td>S</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0-3</td>
</tr>
</tbody>
</table>

Experimental data on various carcinogens and the degree of interaction with these macromolecules in target tissues has not proved definitive, and it is not possible to conclude from any of these correlations that a reaction with a specific macromolecule is of key importance (Miller and Miller, 1974). However, in view of the fact that many proteins exist as multiple copies and there is a low degree of alkylation of proteins by nitrosamines, which is apparently random
and the same arguments applying to RNA, most emphasis has been placed on the alkylation of DNA (Pegg, 1977). Alkylated nucleic acids do occur naturally and some of the alkylated bases produced by carcinogens do have natural counterparts: 7-methylguanine, 1-methyladenine and 3-methylcytosine. However, carcinogens may alkylate a portion of DNA that would not normally contain a methylated base (Pegg, 1977).

1.6.4. The Postulated Promutagenic Lesion

Experiments with carcinogenic alkylating agents, not necessarily nitrosamines, have strongly suggested that the $O^6$ position of guanine is one critical target. Although 7-alkylguanine is the major DNA product (Magee and Farber, 1962), it is also produced by non-carcinogenic alkylating agents (Abbott and Saffhil, 1977). $O^6$-alkylation of deoxyguanine was first suggested to be important in mutagenesis by Loveless (1969). $O^6$-methylguanine has subsequently been found to be mistranscribed in vitro both by RNA polymerase (Gerchman and Ludlum, 1973) and DNA polymerase (Abbott and Saffhil, 1977). $O^6$-methylguanine is stable at neutral pH and is enzymatically excised (Nicoll et al., 1975) and its persistence in target organs, because of slower rates of excision, has been shown to correlate well with the organotrophy of the carcinogen (Goth and Rajewsky, 1974; Nicoll et al., 1975; Margison et al., 1976b; Kleihues and Margison, 1976; Kleihues et al., 1978). However, $O^6$-methylguanine has been shown to accumulate in non-target tissues in rats treated with dimethylnitrosamine (Margison et al., 1977).

Since repair of non-replicating DNA is usually faithful and post replicative repair error-prone, the persistence of $O^6$-alkylguanine in target tissues could explain the organotrophy of certain carcinogens (Miller and Miller, 1976b). It seems likely that cancer is initiated during S phase of the cell-cycle. There is no difference in the methylation of DNA by dimethylnitrosamine between normal and regenerating liver either in terms of the extent or persistence of methylation, therefore it is probable that post-replicative repair is important (Craddock, 1978). Chronic feeding studies with diethylnitrosamine showed that the duration of dosing necessary to cause a maximum increase in DNA replication correlated with the critical minimum feeding time for the induction of
cancer (Craddock, 1978). Dimethylnitrosamine has also been shown to accelerate cell proliferation in vitro (Takayama and Inui, 1968).

1.6.5. The Acute Toxicity of Diethylnitrosamine

The acute toxicity of diethylnitrosamine is characterised histologically within twenty-four to seventy-two hours by an increase in the number of acidophilic cells and a loss of glycogen, particularly centrilobularly. This is followed by centrilobular necrosis, usually haemorrhagic with macrophage and neutrophil infiltration. Whereas dimethylnitrosamine causes nuclear enlargement diethylnitrosamine does not elicit this response. In chronic experiments, however, the cells may become changed in size leading to nodule formation. Ultrastructurally there is a marked increase in smooth endoplasmic reticulum (SER), ribosomal detachment from the rough endoplasmic reticulum (RER) with conspicuous polysomes and free ribosomes, all presumably related to degranulation of the RER. The Golgi apparatus is usually dilated, composed of apparently empty vesicles. Mitochondria may be decreased with a loss of matrix granules and there may be some swelling. There is an increased fat content. "Coated vesicles" appearing to originate from the plasma membrane, but occasionally located deep in the cytoplasm may be seen (Svoboda and Reddy, 1975). The toxic effects of diethylnitrosamine are similar in many respects to those caused by dimethylnitrosamine and are believed to be related to their metabolites, since the toxicity is manifest not at the site of administration but at the site of their metabolism, the liver. It is thought that neither the parent compound nor products of side reactions are toxic, but that the toxic compound arises from the direct oxidative demethylation pathway (Heath, 1962). Probably cell death results from a varying degree of alkylation of cellular components (Magee and Lee, 1964). There is correlation of DMN-demthylase activity, cytoplasmic basophilic granulation and degree of cell necrosis in various areas of the liver i.e. they are more pronounced centrilobularly and in the left lobes of the liver (Lawson and Pound, 1974). Reducing the microsomal hydroxylating activity by feeding a protein-free diet protects the rat from the hepatotoxic effects of dimethylnitrosamine but the effects cannot
be restored by the administration of inducers such as phenobarbitone or DDT (McLean and Verschuuren, 1969). In contrast to $\text{CCl}_4$ (another hepatotoxin, requiring metabolism by the mixed function oxidase system and which produces centrilobular damage) the toxicity of dimethylnitrosamine is enhanced by partial hepatectomy, is inhibited by phenobarbitone and is toxic to the same extent in foetal, newborn and adult rats. $\text{CCl}_4$ toxicity is reduced by partial hepatectomy, enhanced by phenobarbitone and is only effective in mature rats (Nayak et al., 1975). These workers suggested that phenobarbitone did not induce DMN-demethylase and that acute toxicity was due to a reduction in demethylation. Other studies have shown that phenobarbitone does enhance DMN-demethylase although it does not increase the production of $\text{CO}_2$ from dimethylnitrosamine suggesting that the metabolism of dimethylnitrosamine, and presumably other dialkynitrosamines, is not ascribable solely to the action of microsomal demethylase associated with the mixed function oxidase system (Phillips et al., 1975). Further work on inhibitors of DMN-demethylase suggesting that DMN-demethylase may not be involved in the acute toxicity of dimethylnitrosamine has been reported (Friedman and Sanders, 1976). Others (Abanobi et al., 1977) have shown that an inhibitor of dimethylnitrosamine-induced strand breaks in liver DNA also inhibits liver cell necrosis, and that this inhibitor also supresses in vitro the microsomal-mediated oxidative demethylation of dimethylnitrosamine.
1.7. Research Proposals

1.7.1. The Experimental Approach

The work described in this thesis was concerned with the study of the development of liver cancer in relation to normal hepatic differentiation. In order that dedifferentiation patterns of gene expression during carcinogenesis could be studied and, additionally, that some insight into the biochemical nature of the developing tumours could be obtained, enzymes were investigated that exhibit both developmental phase-specific patterns of activity and also are important in intermediary metabolism (Table 1.3). To this end the enzymes were assayed during normal hepatic development, at intervals during hepatocarcinogenesis, in transplantable hepatomas and in the host livers of tumour-bearing rats. The enzymes were also assayed at various times after partial hepatectomy to ascertain what changes characterised hyperplasia and to what extent tissue regeneration was accompanied by dedifferentiation.

1.7.2. The Aims of the Study

The aim of these studies was to determine if the progression to malignancy is accompanied by a step-wise regression to a foetal-like state, passing through progressively more immature states.

Hitherto most studies concerning the biochemistry and/or the foetal nature of tumours have been carried out on transplantable tumours or occasionally primary tumours. Both these situations represent end-points of the carcinogenic process and therefore hypotheses as to the nature of the genesis of the tumours must be speculative. Furthermore, whereas transplantable hepatomas are undoubtedly a useful tool for the study of neoplasms, they do not exactly resemble the normal hepatic situation. The liver receives 70% of its blood via the hepatic portal vein which drains the splanchnic visceral tissues. Experiments with dogs show that the pancreatic hormones, largely insulin and glucagon, exert "hepatotrophic" effects. These effects include maintenance of cell size and liver glycogen and regulation of cyclic AMP concentration
and enzyme levels. Transplantable hepatomas, on the other hand, are not subject to these conditions in that they are situated subcutaneously, usually on the flank or back of the host animal, and they receive an arterial blood supply which is thought to perfuse at only 5% of the rate of the normal liver blood supply (Goldfarb and Pitot, 1976).

Some of the changes observed may therefore result from adaptation to an abnormal environment and transplantation.

1.7.3. Diagnosis of Pre-cancerous Lesions

The studies that have been made concerning the sequence of events leading to tumour formation have been mostly histological or histochemical, the aim being to determine the earliest changes occurring during carcinogenesis, presumably with a view to exploitation for diagnostic purposes. In these studies little or no attempt has been made to investigate the mechanisms of carcinogenesis, whether there is an overriding foetal theme to the changes or whether the changes merely reflect a non-specific response.

Because of these ambiguities the work reported here has been mostly concerned with the development of primary liver tumours induced chemically. The carcinogen used has been diethylnitrosamine and this was chosen because it gives rise specifically to hepatocellular carcinomas (Druckrey, 1967), as well as being a very potent carcinogen with low toxicity when compared with many other carcinogens (Magee and Barnes, 1967; Scherer et al., 1972; Weisburger and Williams, 1975).

Moreover, by studying several enzymes, simultaneously, that have characteristic activities at different times during development, a more comprehensive picture of the state of biochemical differentiation of the neoplastic or preneoplastic lesions can be constructed. In contrast, if only one or two parameters were studied (e.g. the comparison of the isozymes of a particular enzyme in normal adult, foetal or tumour tissues), the changes observed might be phenomenal and not necessarily characteristic of an overall reconstruction of genetic expression towards a foetal pattern.

By studying a spectrum of enzymes during the course of tumour formation, changes occurring early on in the process might be identified
and could be utilized in the search for early diagnostic markers. The phenotypic diversity of tumours and preneoplastic lesions makes it more likely that a battery of markers, rather than any single one, are needed to positively identify precancerous conditions.

1.7.4. Short-term Carcinogenicity Testing

If the biochemical changes which characterise the early stages of the development of cancer can be identified it may be possible to develop short-term \textit{in vivo} tests for carcinogens. Providing sufficiently differentiated cell-cultures could be established, it may even be possible to develop a similar short-term test \textit{in vitro}.

In this investigation a study has also been made of carcinogenesis initiated by a short-term exposure to diethylnitrosamine, followed by a prolonged promotion phase using phenobarbitone. The aim was to try and detect and separate the biochemical changes that occur as a result of initiation and those which are brought about during the promotion phase. If such enzymatic changes could be identified, then it may be possible to devise short-term screening tests \textit{in vivo} for initiators and promoters along the lines already proposed for complete carcinogens (Tatematsu et al., 1977).
<table>
<thead>
<tr>
<th>ENZYME</th>
<th>PATTERN OF EXPRESSION</th>
<th>ASSOCIATED FUNCTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymidine kinase</td>
<td>Very high in the foetus, declines after birth</td>
<td>DNA synthesis</td>
</tr>
<tr>
<td></td>
<td>(Machovich and Greengard, 1972)</td>
<td></td>
</tr>
<tr>
<td>Hexokinase</td>
<td>High in the foetus, declines after birth</td>
<td>Glycolysis (low Km isozyme of glucokinase)</td>
</tr>
<tr>
<td></td>
<td>(Burch et al, 1963)</td>
<td></td>
</tr>
<tr>
<td>Glucose 6-phosphate</td>
<td>Present in foetus, declines during suckling, rises again</td>
<td>Pentose phosphate shunt (lipogenesis and</td>
</tr>
<tr>
<td>dehydrogenase</td>
<td>after weaning</td>
<td>nucleic acid synthesis)</td>
</tr>
<tr>
<td></td>
<td>(Vernon and Walker, 1967)</td>
<td></td>
</tr>
<tr>
<td>Glutamate dehydrogenase</td>
<td>late foetal rise, continues after birth</td>
<td>Amino acid metabolism</td>
</tr>
<tr>
<td></td>
<td>(Herzfeld et al, 1976)</td>
<td></td>
</tr>
<tr>
<td>Aspartate aminotransferase</td>
<td>Appears late foetally, rises to approx. adult values</td>
<td>Amino acid metabolism and gluconeogenesis</td>
</tr>
<tr>
<td></td>
<td>soon after birth (Herzfeld &amp; Greengard, 1971)</td>
<td></td>
</tr>
</tbody>
</table>
Table 1.3. Continued

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>PATTERN OF EXPRESSION</th>
<th>ASSOCIATED FUNCTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose 6-phosphatase</td>
<td>Appears late foetally. Reaches a maximum neonatally then declines to adult levels (Vernon &amp; Walker, 1967)</td>
<td>Gluconeogenesis</td>
</tr>
<tr>
<td>Phosphoenolpyruvate carboxykinase</td>
<td>Sudden increase to maximum postnatally then declines to adult levels (Vernon &amp; Walker, 1967)</td>
<td>Gluconeogenesis</td>
</tr>
<tr>
<td>Glucokinase</td>
<td>Appears in late suckling period (Greengard, 1971)</td>
<td>Glycolysis (high Km isozyme of hexokinase)</td>
</tr>
<tr>
<td>Malic enzyme</td>
<td>Appears in late suckling period (Vernon &amp; Walker, 1967)</td>
<td>Lipogenesis</td>
</tr>
</tbody>
</table>
CHAPTER 2

MATERIALS AND METHODS
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2.1. Animal Experimentation

Wistar Albino rats from the breeding colony of the Animal Unit, University of Surrey were used in all experiments, except for transplantable hepatoma studies, which were carried out on Chester Beatty Hooded Strain of rats, now inbred at the Animal Unit, University of Surrey. Male animals were used for all experiments, except for those on foetal animals (sex not determined) and on pregnant and non-pregnant female controls.

Animals were housed in plastic cages with a bedding of sawdust, wood chips or, in the case of animals receiving carcinogen and the control animals for these studies, 'Sterelit' (J.C. Lee and Company, Chertsey, Surrey). The animals were allowed food (Laboratory diet 1; Spratts Patent Ltd., Barking, London) and water ad libitum. They were kept under conditions of twelve hours light, twelve hours darkness.

Suckling rats were fully weaned by separation from their mothers at 21 days post partum. Control adult male rats were in the weight range 200 g to 300 g.

2.1.1. Carcinogen Treatment

Cages of animals receiving diethylnitrosamine (Eastman Kodak Company, Kirkby, Liverpool) were housed in a large perspex cabinet (5' x 4' x 3') with an activated charcoal-filtered air inlet and an activated charcoal-filtered air outlet driven by a suction pump. The rats were allowed to acclimatise for one week, during which time their water consumption was monitored. The animals were weighed and diethylnitrosamine was added to their drinking water at a concentration such that the animals received 10 mg diethylnitrosamine/kg body weight/day. Initially this was 80 ppm but was increased to over 100 ppm as the water consumption per kg body weight decreased. This dose was expected to give a mean hepatoma induction time of 14 weeks (Druckrey, 1967).

In studies on cancer initiation and promotion, animals were given phenobarbitone (Sigma Ltd., Poole, Dorset) mixed in powdered
diet (Laboratory diet 2; Spratts Patent Ltd.) to a final concentration of 500 ppm. Stale food was renewed twice or thrice weekly.

For studies on the inhibition of normal differentiation by tumour promotors, sodium phenobarbitone (British Drug Houses, B.D.H., Poole, Dorset) was administered daily by oral intubation to suckling rats from ten days post partum until weaning at twenty-one days post partum. A dose of 58.67 mg/kg body weight was used as this was calculated to correspond to the dose used in the tumour promotion experiments. After weaning, the rats received sodium phenobarbitone in the drinking water at a concentration of 400 ppm.

2.1.2. Transplantable Tumours

Two transplantable tumours, originally induced by ethionine as described previously (Reid, 1970) were passaged by subcutaneous implantation on the flanks of male Chester Beatty hooded rats. Late passages of UA (passage No. ≈220) and WDA (passage No. ≈75) hepatomas were donated by Dr. E. Reid (Robens Institute of Industrial and Environmental Health and Safety, University of Surrey). The UA tumour was a fast-growing (time between implantations of twenty-one days), poorly differentiated hepatoma and the WDA tumour was a slower-growing (time between implantations of twenty-eight days), well differentiated hepatoma. These tumours were studied and compared with livers from the tumour-bearing rats and from control adult male hooded rats.

2.1.3. Partial Hepatectomies

Partial hepatectomies (65 - 70%) were performed on adult Wistar rats in the weight range 150 - 250 g by the technique of Higgins and Anderson (1931). All operations were carried out under ether anaesthesia. The abdomens of the animals were shaved with an Oster Animal Clipper (John Oster Manufacturing Company, Milwaukee, Wisconsin, USA) and washed with a dilute Wescodyne antiseptic solution (Ciba-Geigy, Horsham, Sussex).

A median-line incision of about 5 cm was made in the skin just
below the diaphragm. The skin was loosened from the abdominal muscles using round-ended scissors. An incision of 1.5 to 2.0 cm was made through the body wall into the peritoneal cavity, the upper limit being at the xiphoid cartilage. The median and left liver lobes were expelled by gentle pressure on the sides of the rib-cage and abdomen of the animal. The lobes were ligated with Mersilk suture thread (Holborn Surgical Instrument Company Ltd., London) and removed. The peritoneum and abdominal muscles were closed with a Mersilk suture and the skin closed with Michel suture clips (Holborn Surgical Instrument Co. Ltd.). Animals were allowed a 20% sucrose solution instead of water for the first post-operative day. Sham-operated control animals underwent similar operative procedures except that the liver lobes were not ligated and removed.

2.1.4. Killing Methods

Adult rats were killed by cervical dislocation, neonatal and late foetal rats were killed by decapitation and fourteen to sixteen days gestation foetal rats died on removal from the uterus.
2.2. Histological Techniques

All histological stains were purchased from R. A. Lamb Ltd. (London) and routine chemicals from British Drug Houses (Poole, Dorset) unless indicated otherwise.

Pieces of tissue were fixed in 10% neutral buffered formalin (Lillie, 1954) for several days. After fixation small pieces of tissue, 2 - 3 mm in thickness, were processed in a Histokinette (British American Optical, Slough, Bucks.); that is they were dehydrated in graded alcohols, cleared in toluene and impregnated with wax at 56°C. The tissue was then placed in plastic moulds and covered in molten wax. After hardening, the sections were cut from the block at a thickness of 7μ using an American Optical Spencer 820 Rotary microtome and mounted on glass slides.

2.2.1. Haematoxylin and Eosin

Wax sections were routinely stained with haematoxylin (Ehrlich acid) and eosin (C.I. No. 45380) using the method of Smith and Bruton (1977) after the original method of Ehrlich (1886) as follows: sections were de-waxed and brought to water, stained in haematoxylin for fifteen minutes, rinsed in tap water, differentiated in 1% acid alcohol for 5 - 10 seconds, blued in tap water for 10 minutes, stained in 1% eosin, rinsed in tap water, dehydrated through graded alcohols, cleared in xylene and finally mounted in DPX.

2.2.2. Staining for Glycogen

The presence of glycogen was demonstrated in wax sections using periodic acid and Schiff's reagent as described by Culling (1974). The staining procedure was as follows: sections were brought to water, oxidised in 1% periodic acid for 5 minutes, washed in tap and then distilled water, treated with Schiff's reagent for 30 minutes, washed in tap water, counterstained in light green (alcoholic) for 5 - 10 seconds, dehydrated, cleared and mounted in DPX. A diastase control was run simultaneously i.e. duplicate sections were incubated in 0.1%
diastase for 30 minutes at 37°C to remove the glycogen prior to oxidation in periodic acid.

2.2.3. Staining for Lipid

Staining for neutral fat was carried out on unprocessed, fixed tissue. Sections 10μ thick were cut from small pieces of tissue using a sledge microtome (Reichert, Austria) fitted with a Mectron Thermostage (Frigister Ltd.). Floating sections were stained with Oil Red O (C.I. No. 26125) using a modified Lillie and Ashburn isopropanol Oil Red O method of Culling (1974) as follows: sections were washed well in water and placed in the stain (6 ml Oil Red O and 4 ml distilled water, left to stand for 10 minutes, filtered and used within 1 hour) in a sealed container for 10 - 15 minutes, rinsed in 60% alcohol to clear the background, washed in water, the nuclei were stained lightly in Harris's Haematoxylin for 2 minutes, washed in water, differentiated in 1% HCl in 70% alcohol, washed in water, blued in borax, washed in water, dried on a clean glass slide and mounted in glycerine jelly.

2.2.4. Staining for DNA

Quantitative DNA estimations were made in wax sections stained using the Feulgen reaction of Bancroft (1975) after an original method of Feulgen and Rossenbech (1924) as follows: sections were brought to water rinsed in 1N HCl at room temperature for 1 minute, incubated in 1N HCl at 60°C for 8 - 10 minutes, rinsed in 1N HCl for 1 minute, rinsed in distilled water, incubated in Schiff's reagent for 45 minutes, given three 2 minute rinses in bisulphite solution (5 ml 10% potassium metabisulphite and 5 ml 1N HCl and 90 ml water), rinsed in tap water, dehydrated, cleared and mounted in DPX.

Colour intensity was measured in a Vickers M85 scanning microdensitometer (Vickers Instruments, Haxby Road, York) at 570 nm using a 100 X oil-immersion objective.
2.3. Enzyme Histochemistry

2.3.1. Preparation of the Tissue

The liver was removed rapidly from the animal after death and a portion removed. This was cut into small pieces using crossed scalpel blades and dropped into a jar of hexane cooled to -70°C in a bath of alcohol and crushed solid CO₂ (Distillers Company Carbon Dioxide Ltd., Distillery Road, Hammersmith, London) as described by Chayen et al. (1973). Such rapid freezing prevents ice crystals forming (Altman, 1972). Originally very small pieces of about 3 mm³ were used as suggested in the original method (Chayen et al., 1973), however, larger pieces of about 8 mm x 5 mm were used in later experiments as these maintained a greater degree of tissue preservation. The frozen tissue was transferred to pre-cooled glass vials and stored in crushed solid CO₂ until it was cut.

The frozen tissue was mounted on to pre-cooled metal blocks using Tissue Tek II OCT compound (R.A. Lamb, London) in a cryostat (Bright Instrument Co. Ltd., Huntingdon, England) maintained at -30°C to -20°C. Sections of 10μ thickness were cut in the cryostat with a microtome blade cooled to -70 to -50°C, and freeze-dried onto alcohol-cleaned slides at room temperature, as described by Chayen et al (1973). The slides were stored in the cryostat for up to 48 hours unless indicated otherwise.

2.3.2. Histochemical Assays

Glucose 6-Phosphatase (E.C. 3.1.3.9.) was assayed by the method of Wachstein and Meisel (1956) as modified by Chayen et al. (1973). Slides were incubated in a reaction mixture containing 2.27 mM glucose 6-phosphate, monosodium salt (Sigma Ltd., Poole, Dorset) and 2.5 mM lead nitrate in 100 mM acetate buffer pH 6.5 at 37°C for 15 minutes. Lead phosphate formed at the site of the reaction was visualised by conversion to lead sulphide (dark brown) on incubation in 0.5% (v/v) ammonium sulphide solution for 1 minute. Duplicate control slides, for non-specific phosphatase action, were incubated with sodium β-glycerophosphate (Sigma) in place of the glucose 6-phosphate. Slides were
mounted in glycerine jelly.

Dehydrogenase Enzymes

To inhibit the diffusion of dehydrogenase enzymes from the tissue section into the reaction mixture polyvinyl alcohol, PVA (initially grade I, Sigma, later molecular weight 14,000 type, B.D.H.) was incorporated into the reaction mixture to give a final concentration of 20%, as had been recommended previously (Altman, 1972). Phenazine methosulphate, PMS (N-methylphenazonium methosulphate, B.D.H.) was added to the reaction mixture (0.1 mg/ml) to allow the demonstration of dehydrogenase enzymes independent of endogenous NADH or NADPH-tetrazolium reductases. The coloured formazan produced by the reduction of the tetrazolium salt was prevented from re-oxidising initially by gassing the reaction mixture with N\textsubscript{2} and later by the addition of 10 mM NaN\textsubscript{3} (Rieder et al., 1978).

The preparation of the reaction mixture and the incubation of the tissues for all dehydrogenase enzymes was similar and is outlined below. Suitable amounts of the appropriate buffer (see later) and tetrazolium salt, neotetrazolium chloride (Sigma), were mixed and heated gently until the tetrazole had dissolved. The mixture was cooled, filtered and mixed with PVA in appropriate quantities. The mixture was heated gently until the PVA had completely dissolved and allowed to cool to about 40°C. To this mixture aliquots of stock solutions of PMS, NaN\textsubscript{3}, NADP (Cambrian Chemicals, Croydon), NAD (Boehringer Corporation (London) Ltd., Lewes, East Sussex), glucose 6-phosphate, sodium malate (NaOH-neutralised malic acid, Sigma) and monosodium glutamate (Sigma) were added as appropriate, to give final concentrations as indicated later, and stirred well.

Glass rings of internal diameter 1 cm. and height 4 mm. were greased on their upper and lower surfaces and sealed onto the slide encircling the tissue. Reaction mixture was poured into the rings and the whole assembly covered with a glass coverslip. The slides were incubated in an oven at 37°C and then rinsed under a warm running tap before mounting in glycerine jelly.
Glutamate Dehydrogenase (E.C. 1.4.1.2.) was assayed as described by Chayen et al. (1973). The reaction mixture was composed of 50 mM potassium phosphate buffer, 20% (w/v) PVA, 91 mM monosodium glutamate, 3 mM NAD, 10 mM NaN₃ and 0.1 mg/ml PMS. The sections were incubated for 30 mins, duplicate sections were incubated in substrate-free reaction mixture as controls.

NADP-Specific Malate Dehydrogenase "Malic" Enzyme (E.C. 1.1.1.40) was assayed as described by Reider et al. (1978) using a reaction mixture of 50 mM tris buffer pH 7.4 + 20% (w/v) PVA, 5 mM MgCl₂, 0.8 mM NADP, 10 mM NaN₃, 100 mM sodium malate and 0.1 mg/ml PMS. Incubations were for 30 - 45 minutes.

Glucose 6-Phosphate Dehydrogenase (E.C. 1.1.1.49) was assayed using a reaction mixture similar to that used for malic enzyme substituting 10 mM glucose 6-phosphate for malate as described by Reider et al. (1978). Incubations were for 30 - 45 minutes. Tissues incubated in substrate-free reaction mixture were blanks for both malic enzyme and glucose 6-phosphate dehydrogenase.

Control and test tissues were always assayed simultaneously.

γ-Glutamyl Transpeptidase (E.C. 2.3.2.2.) was assayed as described by Rutenburg et al. (1969). Frozen sections were air-dried and incubated in a reaction mixture containing 2.5 mg N-γ-1-glutamyl 4-methoxy-2-napthylamide (a gift from Dr. R. Legg, BIBRA, Carshalton, Surrey, originally from Bachem Fine Chemicals Inc., California, USA), 10 mg Fast blue BB salt (diazotised 4'-amino-2',5'-diethoxybenzaniilide; Raymond A. Lamb, London), 22.3 mM glycylglycine and 40 mM sodium phosphate buffer pH 6.7 in physiological saline. After incubation at 25°C for 10 minutes the sections were rinsed in physiological saline and the colour allowed to develop in 0.1 M CuSO₄ for 2 minutes. The sections were counterstained in 1% Aqueous methyl Green (Raymond A. Lamb) and mounted in glycerine jelly. Photomicrographs of the sections were taken within 36 hours as the colour faded with time.

Photographs of the various histological and histochemical slides
were taken using a Vickers M15 microscope fitted with a 35 mm Kodak camera and an automatic exposure unit.
2.4. Hepatic Enzyme Assays

2.4.1. Chemicals

All routine chemicals were of Analar grade from B.D.H., L-aspartic acid was also obtained from this source. In addition to chemicals mentioned previously bovine serum albumin (Cohn fraction V) for protein standardisation, adenosine triphosphate (disodium salt), inosine diphosphate (monosodium salt) and thymidine were obtained from Sigma Ltd. (Poole, Dorset). All other enzymes, coenzymes and substrates were obtained from Boehringer Corporation. The ancillary enzymes used were: glucose 6-phosphate dehydrogenase (E.C.1.1.1.49) from yeast (1 mg/ml) suspension in ammonium sulphate (350 U/mg) and malate dehydrogenase (E.C.1.1.1.37) from pig heart mitochondria (5 mg/ml) solution in 50% glycerol (1200 U/mg). The coenzymes and substrates used were: reduced nicotinamide adenine dinucleotide (NADH); phosphoenolpyruvate (monopotassium salt) and α-ketoglutaric acid neutralised with KOH.

2,5-diphenyloxazole (PPO) and 1,4-bis-[2-(5 phenyloxazoyle)]-benzene (POPOP) were of scintillation grade obtained from Packard Instrument Inc. (Downes Grove, Ill. 60515, USA). For non-aqueous scintillant 5g PPO and 0.5g POPOP were dissolved in 1 litre of toluene. For counting aqueous samples 5g PPO and 0.5g POPOP were dissolved in 667 ml toluene and made up to 1 litre with Metapol or Synperonic NX detergent obtained from Durham Chemical Distributors Ltd. (Birtley, Tyne and Wear).

DE81 discs 2.4 cm diameter were obtained from Whatman Ltd. (Springfield Mill, Maidstone, Kent).

2.4.2. Preparation of the Tissue

The liver, or hepatoma tissue, was excised rapidly after death from a pre-weighed animal and dropped into ice-cold homogenising medium: 0.15M KCl, 0.2mM KHCO₃ (Le Page, 1948) blotted dry and weighed. Livers
from foetal (whole litters) and neonatal animals (5 - 10 animals) were pooled. After the removal of some tissue for histochemical and/or histological examination, the remaining tissue was reweighed and homogenised in 2 volumes of homogenising medium using a Potter-Elvehjem homogeniser (Potter and Elvehjem, 1936). Aliquots of the 33% (w/v) homogenate were further diluted in homogenising medium to give a 10% homogenate. Some of the 10% homogenate was further diluted 10-fold in distilled water and this 1% tissue extract was then sonicated for 3 X 10 seconds with a Soniprobe Type 1130 A (Daw Instruments Ltd., London) sonating probe. The remaining 33% homogenate was centrifuged for 1 hour at 100,000g at 4°C in an MSE superspeed 50 centrifuge. All subsequent low speed sedimentsations, as required for certain enzyme assays, were performed using a Beckman J6 centrifuge or MSE minor bench centrifuge.

2.4.3. Biochemical Enzyme Assays

**Glucose 6-Phosphatase (E.C. 3.1.3.9.)**

Glucose 6-phosphatase was assayed at 37°C in the 10% homogenate using the method of Nordlie and Arion (1966) with a reaction mixture containing 25mM glucose 6-phosphate and 25mM tris-maleate buffer, pH 6.7 (Gormori, 1948). The reaction was started by the addition of 200 µl of 10% homogenate and stopped after ten minutes by the addition of 10% trichloroacetic acid. The inorganic phosphate liberated was measured by comparison with zero time blanks (i.e. trichloroacetic acid added before the homogenate) and a standard phosphate solution using the acid ammonium-molybdate-aminonaphthalsulphonic acid colour reaction of Fiske and Subbarow (1925) as modified by Rowsell (Snell, 1971).

**Thymidine Kinase (E.C. 2.7.1.21)**

Thymidine kinase was assayed in the 100,000g supernatant fraction at 37°C by the method of Weber et al (1978) except that tris-HCl buffer pH 8.0 was used at a final concentration of 100mM (Machovich and
Greengard, 1972). The reaction mixture was made up as follows:

<table>
<thead>
<tr>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymidine 10mM 1.0 Ci</td>
<td>50 µl</td>
</tr>
<tr>
<td>ATP 50mM</td>
<td>50 µl</td>
</tr>
<tr>
<td>MgCl₂ 12.5mM</td>
<td>100 µl</td>
</tr>
<tr>
<td>Tris-HCl pH 8.0 250mM</td>
<td>200 µl</td>
</tr>
<tr>
<td>Sample</td>
<td>100 µl</td>
</tr>
<tr>
<td>Total</td>
<td>500 µl</td>
</tr>
</tbody>
</table>

Incubations were for 30 minutes, the reaction was stopped by placing the tubes in boiling water for 3 minutes. Duplicate test incubations and a single zero time blank incubation was run for each sample. Aliquots of 50 µl of the mixture were pipetted onto Whatman DE81 filter paper discs which specifically retained the resultant [2-¹⁴C] thymidine monophosphate. The discs were counted in non-aqueous scintillant using a LKB-Wallac 1210 and compared with 50 µl of 0.1mM (0.01 µCi) [¹⁴C]thymidine dried onto DE81 discs (approx. 1,000 cpm).

**Phosphoenolpyruvate Carboxykinase (E.C. 4.1.1.32)**

Phosphoenolpyruvate carboxykinase was assayed in the 100,000g supernatant at 30°C by measuring the incorporation of H¹⁴CO₃ into oxaloacetate by the method of Ballard and Hanson (1967) as follows:

<table>
<thead>
<tr>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphoenolpyruvate 25mM</td>
<td>50 µl</td>
</tr>
<tr>
<td>NaH¹⁴CO₃ 0.5M</td>
<td>100 µl</td>
</tr>
<tr>
<td>Inosine diphosphate 25mM</td>
<td>50 µl</td>
</tr>
<tr>
<td>NADH 3mM</td>
<td>200 µl</td>
</tr>
<tr>
<td>Malate dehydrogenase 1200 units/ml</td>
<td>10 µl</td>
</tr>
<tr>
<td>Dithiothreitol 100mM</td>
<td>20 µl</td>
</tr>
</tbody>
</table>

*The discs were washed in ice-cold 1mM ammonium formate for 30 min, washed twice in 1mM ammonium at room temperature for 10 min, then rinsed in gently running tap-water and finally rinsed in distilled water and air-dried.*
\[
\begin{array}{ccc}
\text{MnCl}_2 & 10 \text{ mM} & 200 \mu l & 2.0 \text{ mM} \\
\text{Imidazole buffer pH 6.0} & 0.5 \text{ M} & 200 \mu l & 100.0 \text{ mM} \\
\text{Sample} & & 4 \mu l \\
\text{Water} & & 166 \mu l \\
\text{Total} & & 1000 \mu l \\
\end{array}
\]

Incubations were started by the addition of the 100,000 g supernatant and stopped after 15 minutes by the addition of 1 ml 2N HCl and excess \(^{14}\text{C}O_2\) removed by passing \(CO_2\) through the mixture. Incubations without phosphoenolpyruvate were run in parallel as blanks. Aliquots of 0.5 ml were added to 4.0 ml aqueous scintillant in minitubes (Nuclear Chicago Division, G.D. Searle Ltd., High Wycombe, Bucks.) and compared with 0.5 ml 2.5 mM NaH\(^{14}\text{CO}_3\) (0.1 µCi) standard (approx. 70,000 cpm) using a LKB-Wallac 1210.

All other enzymes were assayed by linking them, either directly or via coupling enzymes to NAD(P)H production or utilization. The reactions were followed in silica cuvettes of 1 cm light-path and 1 ml volume at 340 nm using a Gilford 250 spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio, USA) fitted with a constant temperature housing maintained at 30°C and a chart recorder.

**Glucokinase (E.C. 2.7.1.2.) and Hexokinase (E.C. 2.7.1.1.)**

Glucokinase and hexokinase were assayed in parallel in the 100,000 g supernatant fraction by the method of Sharma et al (1963) after an original glucokinase method of Di Pietro and Weinhouse (1960). The reaction mixture contained glycylglycine buffer pH 7.5, MgCl\(_2\), KCl, NADP and ATP at final concentrations of 50 mM, 10 mM, 133 mM, 0.7 mM and 6.7 mM respectively and containing 5 units glucose 6-phosphate dehydrogenase in a final volume of 750 u. To each cuvette 10 µl supernatant was added to start the reaction and the rates measured after 5 minutes incubation. Hexokinase was measured in the presence of 0.5 mM glucose and glucokinase and hexokinase activity together were measured in the presence of 100 mM glucose, and glucokinase activity calculated by subtraction.
NADP-Specific Malate Dehydrogenase, 'Malic' Enzyme (E.C. 1.1.1.40)

Malic enzyme was assayed by the method of Hsu and Lardy (1969) in the 100,000g supernatant in a reaction mixture made up as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malate 10mM</td>
<td>50 μl</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>NADP 10mM</td>
<td>50 μl</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>MnCl₂ 120mM</td>
<td>50 μl</td>
<td>6.0 mM</td>
</tr>
<tr>
<td>Triethanolamine buffer 0.2M pH 7.5</td>
<td>250 μl</td>
<td>50.0 mM</td>
</tr>
<tr>
<td>Water</td>
<td>590 μl</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>10 μl</td>
<td></td>
</tr>
<tr>
<td>Total volume</td>
<td>1,000 μl</td>
<td></td>
</tr>
</tbody>
</table>

Malate was omitted from the blank incubations.

Glucose 6-Phosphate Dehydrogenase (E.C. 1.1.1.49)

Glucose 6-phosphate dehydrogenase activity was measured in the 100,000g supernatant fraction by the method of Langdon (1966). The reaction was started by the addition of 10 μl sample to the following reaction mixture:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose 6-phosphate 175mM</td>
<td>10 μl</td>
<td>1.75 mM</td>
</tr>
<tr>
<td>MgCl₂ 75mM</td>
<td>100 μl</td>
<td>7.5 mM</td>
</tr>
<tr>
<td>NADP 10mM</td>
<td>20 μl</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>Tris-HCl buffer 0.2M pH 7.5</td>
<td>200 μl</td>
<td>40 mM</td>
</tr>
<tr>
<td>Water</td>
<td>660 μl</td>
<td></td>
</tr>
</tbody>
</table>

Glucose 6-phosphate was omitted from the blank incubations.
Glutamate Dehydrogenase (E.C. 1.4.1.2.)

Glutamate dehydrogenase was assayed in the 1% sonicated homogenate as described by Herzfeld (1972) after the method of Wergedal and Harper (1964). The reaction mixture was made up as follows:

<table>
<thead>
<tr>
<th></th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-ketoglutarate</td>
<td>60mM</td>
<td>50 µl</td>
</tr>
<tr>
<td>NADH</td>
<td>3mM</td>
<td>50 µl</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>1.75M</td>
<td>200 µl</td>
</tr>
<tr>
<td>Potassium phosphate buffer</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>Water</td>
<td>590 µl</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>10 µl</td>
<td></td>
</tr>
</tbody>
</table>

The reaction was started by the addition of the sample. Blank incubations were carried out in the absence of α-ketoglutarate.

Aspartate Aminotransferase (E.C. 2.6.1.1.)

Aspartate aminotransferase was measured as described by Herzfeld and Greengard (1971) after an original method of Karmen (1955) in the 1% sonicated homogenate. The reaction product, oxaloacetate, was reduced by exogenous malate dehydrogenase and followed by the re-oxidation of NADH in the following reaction mixture:

<table>
<thead>
<tr>
<th></th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-aspartate</td>
<td>0.8M</td>
<td>100 µl</td>
</tr>
<tr>
<td>α-ketoglutarate</td>
<td>60mM</td>
<td>110 µl</td>
</tr>
<tr>
<td>NADH</td>
<td>3mM</td>
<td>50 µl</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>500 units</td>
<td>10 µl</td>
</tr>
<tr>
<td>Water</td>
<td></td>
<td>720 µl</td>
</tr>
<tr>
<td>Sample</td>
<td></td>
<td>10 µl</td>
</tr>
</tbody>
</table>
Blank incubations were carried out in the absence of L-aspartate and reactions were started by the additions of the sample.

2.4.4. Protein Estimation

Protein in the 100,000g supernatant was measured by the Biurette method described by Yatzides (1977) and standardised with dried bovine serum albumin.
2.5. DNA Alkylation Studies

Animals receiving diethylnitrosamine in the drinking water for 1, 5 and 10 weeks were given normal drinking water for 24 hours prior to a single intraperitoneal injection of 10mg/kg di[\textsuperscript{14}C]ethylnitrosamine; [\textsuperscript{14}C]DEN, (synthesised from di[\textsuperscript{14}C]ethylamine hydrochloride, 55mCi/mmol; Radiochemical Centre, Amersham, Bucks.) or 2mg/kg di[\textsuperscript{14}C]methyl-nitrosamine; [\textsuperscript{14}C]DMN.

The [\textsuperscript{14}C]DEN was diluted to a specific activity of 6.7mCi/mmol (1 weeks pretreatment experiment) 16.0mCi/mmol (5 weeks pretreatment) or 8.16mCi/mmol (10 weeks pretreatment) with unlabelled DEN. The [\textsuperscript{14}C]DMN was diluted to a specific activity of 9.9mCi/mmol (1 weeks pretreatment), 25.2mCi/mmol (5 weeks pretreatment) or 24.5mCi/mmol (10 weeks pretreatment) with unlabelled DMN. The animals were killed 12 hours later, the livers removed and immediately frozen on solid CO\textsubscript{2}. DNA was extracted from the liver and alkylated bases were analysed as described previously (Margison et al., 1979). The method involved extraction of DNA from the liver using a phenol procedure (Margison et al., 1976b) followed by hydrolysis in 0.1N HCl at 70°C for 30 minutes. After adjusting the pH to 2.8 with 0.1N NaOH, normal and alkylpurines were separated on columns of Sephadex G-10 eluted with 0.05M ammonium formate in 0.2% Na\textsubscript{3}PO\textsubscript{4} (pH 6.75). Normal purines were determined spectrophotometrically and alkylated purines by liquid scintillation counting.
CHAPTER 3

RESULTS
RESULTS

3.1. Hepatic Differentiation

3.1.1. Change in Liver Histology During Development

a) The Adult Liver

Hepatocytes make up about 80% of the volume of the liver, Kupffer cells and endothelial cells account for a further 16% of the volume and bile ductule cells and connective tissue account for the remainder (Greengard et al., 1972). The hepatocytes have an eosinophilic cytoplasm and basophilic nucleus and are arranged in cords or plates radiating from the vessels. The sinusoidal spaces between the plates are lined with the Kupffer cells. Architecturally the cells are organised in lobules drained by a central vein with portal tracts at the periphery of the lobule (Plate 1.1). Functionally, the centre of the liver acinus is the portal (or afferent) vessel the acini being drained at their periphery by the central vein (terminal hepatic venule). The zones of the functional liver acinus have been defined by Rappaport (1963). Zone 1 (corresponding to the periportal region) surrounds the afferent vessels and receives oxygen-, substrate- and hormone-rich blood. Zone 2 is an intermediate or transitional area and zone 3 (corresponding to the centrilobular region) receives oxygen-, substrate- and hormone-depleted blood.

Hepatocytes with a large central glycogen storage vacuole are called 'plant' cells because of their appearance (Plate 1.2). 'Plant' cells are predominantly located in zone 1, and occasionally in zone 2, in the fed animal. In fixed tissue the vacuoles did not show a positive reaction with Periodic acid-Schiff's reagent (PAS) due to the leaching out of the glycogen during the fixation and processing of this tissue. Thus, the glycogen deposition appears to be predominant in zone 3, (Plate 1.3). A number of different fixatives were explored without any improvement in glycogen retention. In unfixed frozen tissue the PAS reaction appears stronger in zone 1 (Plate 1.4) supporting the hypothesis that glycogen leaches out of the 'plant' cells during

PLATE 1.2 Normal adult liver 'Plant' cells. Fixed tissue: H & E 400X mag.
PLATE 1.3 Normal adult liver. Glycogen deposition appears greatest centrilobularly. C.v., central vein P.t., portal tract. Fixed tissue: PAS 40X mag.

PLATE 1.4 Normal adult liver. Unfixed frozen tissue, glycogen deposition appears greatest periportally; P.t., portal tract; C.v., central vein PAS 100X mag.
fixation and processing.

Little fat deposition is normally seen in the adult and it is usually distributed evenly throughout the tissue (Plate 1.5). However, occasionally, quite heavy fat deposits may be seen, either located periportally (Plate 1.6) or, less frequently, located centrilobularly. In fixed tissue the fat was seen as cytoplasmic droplets, but in frozen tissue it was distributed in small droplets located at the cell membrane.

Enzyme histochemical studies of normal adult liver reveal a positive reaction for glucose 6-phosphatase which is greater in zone 1 (Plate 1.7). Glucose 6-phosphate dehydrogenase (Plate 1.8) and malic enzyme (Plate 1.9) are located predominantly in zone 3, and glutamate dehydrogenase is more evenly distributed throughout the tissue with a bias towards zone 3 (Plate 1.10).

b) The Foetal Liver

During differentiation and development the rat liver underwent several changes in histological appearance. The liver rudiment of the 14-day foetus bore little resemblance to the adult organ. There is no lobular architecture or trabecular cellular organisation, rather the cells are amorphously arranged and the tissue is permeated by large blood spaces (Plate 1.11). Haematopoietic tissue was abundant in the tissue especially around the blood spaces. The cells were small with finely vacuolated basophilic cytoplasm. The nuclei were rounded or oval and usually hypochromatic with hyperchromatic margins and one or two prominent hyperchromatic nucleoli (Plate 1.12). The nuclear/cytoplasmic ratio was high and mitoses were frequent. Large fat droplets were scattered evenly throughout the tissue (Plate 1.13).

By the seventeenth gestational day the foetal liver showed signs of vascularisation, with clusters of vascular epithelial cells appearing and some large vessels developing (Plate 1.14). There was, however, no evidence of lobular architecture or a plate-like arrangement of the hepatocytes. The cytoplasm of the hepatocytes was basophilic with large vacuoles similar to the 'plant' cells of the adult. PAS
PLATE 1.5 Normal adult liver. Neutral fat deposition is low and scattered throughout the liver. Fixed tissue: ORO 25X mag.

PLATE 1.6 Normal adult liver localised fat deposits: P.t., portal tract. Fixed tissue: ORO 25X mag.
PLATE 1.7 Normal adult liver. Glucose 6-phosphatase predominantly located in zone 1. P.t., portal tract; C.v., central vein. 40X mag.

PLATE 1.8 Normal adult liver. Glucose 6-phosphate dehydrogenase predominantly located in zone 3. P.t., portal tract. 40X mag.
PLATE 1.9 Normal adult liver. Malic enzyme predominantly located in zone 3. P.t., portal tract. 40X mag.

PLATE 1.10 Normal adult liver. Glutamate dehydrogenase, bias towards zone 3. 40X mag.
PLATE 1.11  Liver rudiment of fourteen-day foetus. Sheets of hepatocytes permeated by blood spaces, b.s.; Abundant haematopoietic tissue, H. Fixed tissue: H & E 100X mag.

PLATE 1.12  Liver rudiment of fourteen-day foetus. Hepatocytes are small and basophilic. Hypochromatic nuclei with hypochromatic margins can be seen, N.; haematopoietic cells, H. Fixed tissue H & E 400X mag.
PLATE 1.13  Liver rudiment of fourteen-day foetus. Large fat droplets scattered throughout tissue. Fixed tissue: ORO 40X mag.

PLATE 1.14  Liver of seventeen-day foetus. Vascularisation starting but no evidence of lobular architecture characteristic of adult liver. Abundant haematopoietic tissue. Fixed tissue H & E 40X mag.
reaction failed to show the presence of glycogen in these cells, presumably because of leaching out, but abundant glycogen deposits were seen in some areas. The nuclei were darker than at fourteen days gestation, there was a high nuclear/cytoplasmic ratio and many mitotic figures were seen. Very little fat could be detected in the tissue. Haematopoietic cells made up about 25% of the total cell volume.

One day prior to birth (the twentieth gestational day) the liver had become more vascularised and in some areas the hepatocytes seemed to be arranged in two or three cell-thick plates or islands of cells (Plate 1.15). The hepatocytes had basophilic cytoplasm and there was a higher nuclear/cytoplasmic ratio than in the adult. Some nuclei were enlarged and there was a greater number of cells in mitosis than observed in the earlier foetal livers. Quite heavy deposits of glycogen (Plate 1.16) could be seen and a substantial amount of fat was present in the form of droplets scattered evenly throughout the tissue. Haematopoietic tissue was slightly reduced compared with seventeen days gestation.

Overall, the changes from day fourteen to one day before birth include the development of vascular tissue, the increase then slight decrease in haematopoietic tissue, increasing numbers of mitoses and increased cytoplasmic glycogen and fat.

c) Changes After Birth

In the liver of the newborn animal (less than eighteen hours post partum) the haematopoietic cells were confined to small clusters (Plate 1.17). In some areas the hepatocytes were arranged in cords or plates two cells thick, sometimes one cell thick, with dilated sinusoids. The pattern of vascularisation was very similar to that seen in the adult liver.

The hepatocytes had lost the vacuolation of the cytoplasm seen before birth, presumably because of a mobilisation of the glycogen store. Loss of glycogen was also indicated by a poor PAS reaction (Plate 1.18). The cytoplasm was more eosinophilic than in foetal hepatocytes and slightly more than in the adult. The nuclear margin
PLATE 1.15 Liver of twenty-day foetus. Vascularisation well-developed in some areas are arranged in two cell-thick plates. Haematopoietic tissue slightly reduced. Fixed tissue: H & E 40X mag.

PLATE 1.16 Liver of twenty-day foetus. Glycogen deposits are seen. Fixed tissue: PAS 40X mag.
PLATE 1.17  Newborn rat liver. Haematopoietic tissue greatly reduced
cytoplasm of hepatocyte is more eosinophilic than in the foetal liver.
Fixed tissue: H & E 100X mag.

PLATE 1.18  Newborn rat liver. Absence of glycogen deposits. Fixed
tissue: PAS 40X mag.
and nucleoli were more hyperchromatic than in adult or foetal hepatocytes. The nuclear/cytoplasmic ratio remained higher than in the adult.

Moderately heavy fat deposits were seen localised predominantly in zone 2 and zone 1 (Plate 1.19).

At five days post partum there was still some residual haematopoietic tissue but this was restricted to very small clusters of cells. The architecture of the liver resembled closely that of the adult (Plate 1.20). The cells were arranged in cords, mostly one cell thick but sometimes two cells thick, separated by sinusoids that were occasionally slightly dilated. Many macrophages and/or Kupffer cells could be seen.

The hepatocytes were very similar in appearance to adult hepatocytes but with a slightly elevated nuclear/cytoplasmic ratio and some contained hypochromatic nuclei with hyperchromatic margins and nuclei, giving them an 'empty' appearance (Plate 1.21).

There was a moderately strong PAS reaction, especially in zone 1 (Plate 1.22). Fat deposition was localised in vascular areas, possibly corresponding to zone 3 (Plate 1.23).

By ten days post partum the hepatocytes resembled very closely the adult hepatocytes, though the nuclear/cytoplasmic ratio was still elevated. The architecture and vascularisation pattern was indistinguishable from the adult except that in some areas the bile ducts were very pronounced and thick walled with some bile duct proliferation (Plate 1.24). Glycogen deposits appeared to be slightly reduced compared to five days post partum and fat deposits were concentrated mainly around the portal tracts. Some nuclei were 'empty' looking but the majority were similar to adult hepatocytes nuclei. More mitoses could be seen, scattered more or less evenly throughout the tissue, than in either the newborn or the five-day old rat liver.

In the liver of the fifteen-day old rat very few haematopoietic cells were seen. The architectural arrangement of the hepatocytes was identical to that of the adult. Bile ducts were still very pronounced and multiple with heavy vascularisation peripherally (Plate 1.25). Some nuclei had an 'empty' appearance and a few were hyperchromatic but the vast majority appeared indistinguishable from the
PLATE 1.19  Newborn rat liver. Presence of fat deposits. Fixed tissue: ORO 40X mag.

PLATE 1.20  Five-day old rat liver. Hepatocytes arranged in cords one or two cells thick, some dilated sinusoids. Fixed tissue: H & E 100X mag.
PLATE 1.21 Five-day old rat liver. The nuclei of some hepatocytes are hypochromatic with hyperchromatic margins and nucleoli. Fixed tissue: H & E 400X mag.

PLATE 1.22 Five-day old rat liver, glycogen deposition in zone 1. Fixed tissue: PAS 40X mag.
PLATE 1.23 Five-day old rat liver. Fat deposition near some vascular areas. Fixed tissue: ORO 40X mag.

PLATE 1.24 Ten-day old rat liver. Bile duct proliferation. Fixed tissue: H & E 100X mag.
PLATE 1.25  Fifteen-day old rat liver. Heavy vascularisation periportally. Fixed tissue: H & E 25X mag.

PLATE 1.26  Weanling rat liver. Normal liver architecture, portal vascularisation still prominent. Fixed tissue: H & E 40X mag.
Figure 3.1.1 Changes in Relative Liver Weight During Development

Error bars represent mean ± SEM (3-6). Broken line represents adult value.

Figures on abscissa:
- F, 14 - 16 days gestation;
- LF, 19 - 20 days gestation;
- NB, Newborn, one day post-partum;
- 5D, 10D and 15D, neonate 5, 10 and 15 days post-partum respectively;
- EW, early weaning;
- W, mid-weaning; and LW, late weaning.
adult nuclei.

The PAS reaction indicated that glycogen was predominantly centrilobular in location. Fat deposition was more marked periportally.

In the weanling animal all the residual haematopoietic tissue had disappeared and the livers were virtually indistinguishable from those of adult rats. A few mitotic figures could still be seen and the bile ducts were still prominent though this was much less pronounced than at earlier ages, (Plate 1.26).

The presence of fat vacuoles was restricted to relatively few cells and the glycogen deposits were reduced, presumably reflecting the change in diet from high-fat milk to low-fat solid food.

To summarise the changes occurring from birth to weaning: there was initially a loss of glycogen, which was restored by five days post partum; there was a loss of haematopoietic tissue and the lobular architecture characteristic of the adult liver was acquired; marked vascularisation occurred periportally and this was associated with bile duct proliferation; little mitotic activity was seen up to five days post partum but by ten days the mitoses were abundant, the mitotic rate declined thereafter.

3.1.2. Changes in Liver Weight During Development

The changes in liver weight in relation to body weight during development are shown in figure 3.1.1. These changes are in good agreement with the findings of other workers (Winick and Noble, 1965; Vernon and Walker, 1968). The liver of foetal rats was large in relation to body size and reached a peak shortly before birth. Part of this increase is accounted for by the storage of glycogen seen histologically (section 3.1.1.a.). There was a sudden decrease in liver weight during the first postnatal day which reflects the loss of glycogen seen histologically (section 3.1.1.a.). The liver weight continued to decrease in relation to body weight because of reduced growth rate compared with the rest of the body, so that it reached a trough at ten days post partum. The mitotic activity of the hepatocytes, as judged histologically, was increased at ten days post partum. This presumably contributed to the observed increase in relative liver
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<th>19-20 days Gestation</th>
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<th>5 days post partum</th>
<th>10 days post partum</th>
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<td>74.5 ± 5.9***</td>
<td>51.0 ± 6.1***</td>
<td>26.7 ± 2.1**</td>
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<td>7.37 ± 1.18*</td>
<td>11.1 ± 3.3</td>
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<td>0.24 ± 0.69***</td>
<td>0.371 ± 0.061***</td>
<td>0.368 ± 0.041***</td>
<td>0.489 ± 0.019***</td>
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<td>2.13 ± 0.14***</td>
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<td>197 ± 26</td>
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<td>0.10 ± 0.02***</td>
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<td>0.041 ± 0.10±</td>
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<td>0.071 ± 0.04**</td>
<td>0.281 ± 0.09***</td>
<td>2.64 ± 0.9*</td>
<td>1.01 ± 0.17</td>
<td>12.3 ± 4.9</td>
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<td>Soluble Protein</td>
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<tr>
<td>23.6 ± 4.2***</td>
<td>24.9 ± 4.5**</td>
<td>28.8 ± 2.5*</td>
<td>31.3 ± 0.8**</td>
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<td>29.1 ± 1.1**</td>
<td>30.0 ± 1.5*</td>
<td>31.7 ± 1.0*</td>
<td>38.7</td>
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Values are given as mean ± SEM - the figure in parenthesis indicate the number of observations, either from individual animals or from pooled tissue. The upper value is units/g liver; the lower value is units/100 g body weight. Statistically significant differences from normal adult values are given by: +, p<0.4; *, p<0.05; **, p<0.01; ***, p<0.005. ND = not detectable.
Figure 3.1.2  Changes in Enzyme Activities During Development

Error bars represent mean ± SEM.
Closed circles, solid line represent units/g liver expressed in normalised liver units (L.U.); Open circles, broken line represent units/100g body weight converted to L.U.

Figures on abscissa as in Fig. 3.1.1

(i) TK; Thymidine kinase
(ii) HK; Hexokinase
(iii) G6PDH; Glucose 6-Phosphate dehydrogenase
(iv) GDH; Glutamate dehydrogenase
(v) AAT; Aspartate aminotransferase
(vi) G6Pase; Glucose 6-phosphatase
(vii) PEPCK; Phosphoenolpyruvate carboxykinase
(viii) GK; Glucokinase
(ix) Malic; Malic enzyme
Figure 3.1.2

(iii)

G6PDH L.U.

2.0

1.0

0.0

F LF NB 5D 10D 15D EW W LW Adult
weight from this time point. The relative liver weight of the weanling animal was approximately the same as for the adult.

These changes must be taken into account if the physiological effectiveness of the hepatic enzyme activities to the whole animal is being considered. For this reason the activities of the enzymes are expressed not only as units/g liver but also as units/100g body weight.

3.1.3. Enzyme Changes During Development

The nine enzymes studied show specific patterns of expression during development (see table 1.1 in the Introduction). The results obtained are shown in Table 3.1.1., and Fig. 3.1.2.(i - ix) shows the activities of the enzymes during development, expressed in normalised liver units (i.e. LU; the value in adult liver is taken as 1.0 LU) to facilitate comparison with normal adult levels.

For some of the enzymes (glucokinase, glucose 6-phosphate dehydrogenase and malic enzyme) the change in activity was large during the weaning period so three weaning values are given: early weanling (within the first twelve hours of weaning); weanling (one day after weaning); and late weanling (greater than one day after weaning). The change in activity of the other enzymes was negligible during this period and only one weanling value is given.

In general the results were as expected from the published data. Thymidine kinase was present at very high levels in the foetus, declined rapidly around birth and then continued to decline gradually from five days post partum to adulthood. Likewise hexokinase was elevated in the foetal liver, declined towards birth, and near adult levels were attained in the postnatal period.

Glucose 6-phosphate dehydrogenase activity was significantly higher in the foetal liver than the adult liver, declined after birth to significantly lower than adult values and increased to adult values on weaning.

Lower than adult levels of aspartic aminotransferase were observed in the liver of the rat at fourteen to sixteen days gestation. When expressed in units/g tissue the activity increased steadily, before and after birth to reach a maximum value at ten days post partum.
followed by a decrease to adult levels on weaning. If the results are
expressed in terms of body weight the peak occurs shortly after birth.

Glutamate dehydrogenase was virtually absent until the late
foetal time point when the activity was 10 - 20% of the adult value.
There was a sharp rise in activity after this time and on the first
postnatal day the activity was about 0.8 LU/g tissue (0.6 LU/100 g
body weight). The activity remained fairly constant during the
postnatal period and adult values are not achieved until after weaning.

The activity of glucose 6-phosphatase increased rapidly during
the late foetal period and peaked in the first few postnatal days,
at a level above adult values. This reflects an increased capacity
for gluconeogenesis and glycogen metabolism. The maximum, in terms
of units/g tissue, occurred at five days post partum and then the
activity fell steadily to near adult values by weaning. When expressed
in terms of body weight the results indicate peak activity during the
first postnatal day.

Phosphoenolpyruvate carboxykinase, another gluconeogenic enzyme,
exhibited a similar pattern of activity to glucose 6-phosphatase.
Activity was negligible before birth but there was a sudden rise at
birth and a maximum, greater than twice the adult levels then decreased
rapidly to adult levels on weaning.

Both glucokinase and malic enzyme were undetectable before fifteen
days post partum, but showed a sudden increase on weaning. For malic
enzyme maximal activity (about 1.6 IU) was observed during late weaning.

The soluble protein content was significantly reduced in the
foetus compared to adult values but increased after birth to reach
adult values after weaning.
3.2. Changes During Diethylnitrosamine-induced Hepatocarcinogenesis

3.2.1. Experimental Protocol

Rats were given diethylnitrosamine in the drinking water as described in the methods section and were killed at various time intervals after the start of the treatment. The livers were examined histologically and biochemically to investigate the possibility that the development of tumours is accompanied by a step-wise retro-differentiation.

In a preliminary experiment animals were killed at six weeks (when no tumours were formed) at eleven weeks (at the beginning of tumour formation) and at fifteen weeks (when large tumours were present but animals were becoming moribund). Biochemical investigations revealed that large changes in enzymic activity had occurred by six weeks although histological changes were minor. A second experiment was therefore carried out and animals were killed after two, four, six, eight, ten and eleven weeks, and their livers examined. The results of this second experiment will be described in detail with brief reference to the first experiment.

3.2.2. Morphological and Histological Changes During Diethylnitrosamine-Induced Hepatocarcinogenesis

After two weeks of diethylnitrosamine treatment the liver was indistinguishable from normal liver (Plate 2.1). Periportal there were 'plant' cells from which the glycogen had leached out during fixation. This resulted in glycogen deposits appearing heaviest centriflobularly. In one of the four animals however, bands of cells, about three cells thick, around the central veins had reduced glycogen content (Plate 2.2.). These cells had slightly condensed and eosinophilic cytoplasm and the nuclei were slightly hypochromatic.

Essentially the histological appearance of the liver after four weeks was normal (Plate 2.3). A few eosinophilic cells could be seen around the central vein and in these cells there was a depletion of the glycogen content. The degree of centriflobular eosinophilia and

PLATE 2.3  Liver of rat after four weeks diethylnitrosamine treatment. Normal appearance with no architectural disturbance. Fixed tissue: H & E 25X mag.

glycogen loss varied, from a band of cells about three cells thick round the central veins of one animal to a few isolated cells around the central vein in the other three animals (Plate 2.4). No excessive deposition of fat was observed in the livers of treated rats (Plate 2.5). A few isolated cells in the centrilobular areas were pyknotic and mitotic figures were occasionally seen (Plate 2.6).

For the first four weeks the macroscopic appearance of the liver was normal but by six weeks the surface of the liver had acquired a 'grainy' appearance, due to the multiple small indentations, though it was otherwise unchanged.

Histologically various changes occurred at six weeks. In half of the animals there was an increase in the number of 'plant' cells which occurred in zone 2 as well as zone 1. In one of the four animals there was a focus of cells with large glycogen-storage vacuoles and cells undergoing hydropic degeneration (Plate 2.7). In this focus mitotic figures were present and some enlarged nuclei could be seen. Some of the cells in the focus gave a very positive PAS reaction, others appeared to have reduced glycogen content. Generally, the glycogen content of the hepatocytes of the treated animals was reduced slightly with more marked reduction in a few cells around some central veins (Plate 2.8).

There was an increase in fibrous tissue around the portal tracts in half of the animals and the bile ducts became more prominent (Plate 2.9). Enlarged nuclei could be seen occasionally in hepatocytes in zone 3, and mitotic figures, randomly distributed throughout the tissue were more common than normal. Increased fat deposits in zones 1 and 2 occurred in one animal (Plate 2.10) but the other three animals had normal amounts.

By eight weeks the surface of the liver had become more uneven, with larger, multiple invaginations. Fibrosis was apparent, but without gross disturbance of the architecture, in most of the animals. In one of the four animals the fibrosis had increased to such an extent that the normal liver architecture was disturbed and the liver was cirrhotic (Plate 2.11). The nodules so-formed varied in cell-type; some contained glycogen-storage cells, others eosinophilic or small and basophilic cells. Bile duct proliferation was evident in several areas. Some large and/or
PLATE 2.5  Liver of rat after four weeks diethylnitrosamine treatment. No excessive fat deposition. Fixed tissue: ORO 25X mag.

PLATE 2.6  Liver of rat after four weeks diethylnitrosamine treatment. Evidence of cytotoxicity and regeneration. P., pyknosis; m., mitosis. Fixed tissue: H & E 100X mag.
PLATE 2.7 Focus of 'Plant' and hydropic cells in liver of rat after six weeks treatment with diethylnitrosamine. Fixed tissue: H & E 100X mag.

PLATE 2.9  Liver of rat treated with diethylnitrosamine for six weeks. Bile ducts becoming more prominent. Fixed tissue: H & E 40 X mag.

PLATE 2.10  Liver of rat treated with diethylnitrosamine for six weeks. Increased fat deposition in zones 1 and 2. P.t., portal tract; C.v., central vein. Fixed tissue: ORO 25X mag.

PLATE 2.12 Mitotic figures observed in cirrhotic liver shown in Plate 2.11. Fixed tissue: H & E 400X mag.
hyperchromatic nuclei, some 'empty' looking nuclei and several mitotic nuclei could be seen (Plate 2.12). Most of the nodules gave a positive, if slightly reduced, PAS reaction but some eosinophilic nodules were deficient (Plate 2.13). Fat accumulation was observed in some of the nodules and along many of the bands of fibrous tissue (Plate 2.14).

In other animals at this time-point, where the nodularity was less marked, some bile duct proliferation could be seen. Foci of enlarged 'plant' cells or 'clear' cells (Banasch, 1968) were preferentially located in zones 1 and 2. In the centrilobular regions some eosinophilic cells and cells with enlarged nuclei were observed. A few isolated pyknotic cells occurred, mostly in the centrilobular area. There were several mitotic figures scattered throughout the tissue. Glycogen deposits appeared reduced generally and particularly around the central veins.

After ten weeks of diethylnitrosamine treatment the surface of the liver was covered in numerous nodules about 2 - 4 mm in diameter, many of them pale-coloured. Some small cysts were also evident on the surface and the liver was harder in texture than normal. The posterior right lateral lobe was the most nodular, the anterior right lateral and caudate lobes being slightly less nodular, and the median and left lobes were the least nodular.

Cirrhosis was apparent in most of the tissue and there was some bile duct proliferation. The cirrhosis, like the macroscopic nodularity, was most evident in the posterior right lateral lobe. In the cirrhotic areas the hepatocytes were apparently not arranged in cords but appeared as an amorphous mass. In some non-cirrhotic areas cords were observed, together with dilated sinusoids. Foci and nodules of clear cells, hydropic cells, eosinophilic cells and small basophilic cells made up most of the tissue with only about 25% of the tissue being composed of normal-looking hepatocytes (Plate 2.15). Many of the nodules were hyperplastic, but the hyperplasia was not restricted to any particular cell-type although hepatocellular carcinomas are thought to arise principally from the basophilic cells (Squire and Levitt, 1975). Abnormal mitoses were seen (Plate 2.16) and various types of abnormality occurred in the nuclei: large, hyperchromatic and 'empty'-looking (Plate 2.17). In one of the four animals a large hyperplastic nodule
PLATE 2.13  Glycogen has been lost in some of the nodules in the liver shown in Plate 2.11. Fixed tissue: PAS 25X mag.

PLATE 2.14  Heavy fat deposition in some of the nodules in the liver shown in Plate 2.11. Fixed tissue: ORO 25X mag.
PLATE 2.15 Liver of rat treated for ten weeks with diethylnitrosamine. Most of the tissue is abnormal and nodular. Fixed tissue: H & E 25X mag.

PLATE 2.16 Liver of rat after ten weeks diethylnitrosamine treatment. Abnormal mitoses. Fixed tissue: H & E 400X mag.
PLATE 2.17  Liver of rat treated for ten weeks with diethylnitrosamine. Enlarged nuclei with prominent nucleoli and hyperchromatic margins: 'empty' looking. Fixed tissue: H & E 400X mag.

PLATE 2.18  Preneoplastic nodule containing clear cells and compressing the surrounding tissue, in liver of rat treated for ten weeks with diethylnitrosamine. Fixed tissue: H & E 25X mag.
composed of enlarged 'clear' cells and smaller fat-loaded cells, which was compressing the surrounding tissue, was probably preneoplastic (Plates 2.18, 2.19). Very little glycogen was observed in the cells in this nodule (Plate 2.20). Elsewhere in this liver, and in the livers of the other animals, the glycogen content of the hepatocytes was reduced, particularly in the hyperplastic areas and the hydropic and 'clear' cell foci. It was frequently difficult to distinguish between enlarged 'plant' cells or 'clear' cells and cells undergoing hydropic degeneration, so the reduced PAS reaction seen in the hydropic or 'clear' cells may represent either a true glycogen depletion or the leaching out of excessive glycogen stores during fixation and processing.

A further week of treatment with diethylnitrosamine caused an increase in overall liver size and nodularity (Plate 2.21) with the nodules becoming increasingly more hyperplastic. The cellular changes were much the same as at the ten-week stage.

If the carcinogen administration was stopped after eleven weeks and the animals examined one week later then much of the fibrosis regresses. However, a few hyperplastic nodules remain, some probably representing small hepatocellular carcinomas (Plate 2.22).

In the preliminary carcinogenesis experiment referred to above the diethylnitrosamine treatment was prolonged. Some animals became moribund between thirteen and fourteen weeks after the beginning of the treatment but the majority did not become moribund until fifteen to sixteen weeks of treatment. All the animals at this stage had large multiple liver tumours, often with free-floating intraperitoneal metastases, and in one case a lung metastasis was found (Plate 2.23). The tumours were mostly composed of small basophilic cells with a high nuclear/cytoplasmic ratio. Tumours often arose in association with bile duct proliferation (Plate 2.24) and frequently they were undergoing haemorrhagic necrosis at the centre of the tissue. The tumour cells were deficient in glycogen (Plate 2.25). There were in addition to the basophilic cells, areas of liver composed of eosinophilic cells which also had greatly reduced glycogen content. Some areas of 'clear' cells could also be seen (Plate 2.26). The eosinophilic cells were sometimes arranged in cords which were one-, two- or more cells thick with dilated sinusoids, or arranged in islands of cells. Many nuclear changes were observed: there were large and abnormally shaped nuclei; hyperchromatic
PLATE 2.19  Same tissue as Plate 2.18. Heavy fat deposition.  
Fixed tissue: ORO 25X mag.

PLATE 2.20  Same tissue as Plate 2.18. Glycogen depletion.  
Fixed tissue: PAS 25X mag.
PLATE 2.21  Gross morphology of liver taken from a rat treated with
diethylnitrosamine for eleven weeks.

PLATE 2.22  Liver of rat treated for eleven weeks with diethylnitrosamine
followed by a further week with no treatment. Small hepatocellular
carcinoma composed largely of clear cells. Fixed tissue: H & E 25X mag.
a) 25X mag.

b) 400X mag. showing numerous mitoses

PLATE 2.23 Lung metastasis from hepatocellular carcinoma arising in a rat after fifteen weeks of diethylnitrosamine treatment.
Fixed tissue: H & E.
PLATE 2.24  Hepatocellular carcinoma, composed of basophilic hepatocytes, arising in association with bile duct proliferation in liver of rat treated for fifteen weeks with diethylnitrosamine. Fixed tissue: H & E 100X mag.

PLATE 2.25  Hepatocellular carcinoma in rat treated with diethylnitrosamine for fifteen weeks, largely deficient in glycogen. Fixed tissue: PAS 40X mag.
PLATE 2.26  Hepatocellular carcinoma in rat treated with diethylnitrosamine for fifteen weeks. Area of eosinophilic and clear cells.
Fixed tissue: H & E 100X mag.
nuclei; and 'empty'-looking nuclei often with prominent, multiple nucleoli. Mitotic figures were frequently observed. Some fatty areas could be seen but these were not usually associated with the basophilic cells.

In summary there were several changes in the liver induced by chronic diethylnitrosamine administration. There was an increase in cytoplasmic eosinophilia and decrease in glycogen content centrilobularly, which varied in extent from one animal to another. This reflected the toxicity of the treatment and occasionally a few pyknotic cells could be seen near the central veins. Mitoses occurred, scattered throughout the tissue with no obvious lobular location. Fibrosis developed to a varying extent ranging from slight fibrosis around the vessels to cirrhosis. Bile duct proliferation was common among the more fibrotic livers. Various types of cellular atypia arose: 'plant' cells increased and appeared to develop into either clear cells or cells undergoing hydropic degeneration; eosinophilic cells and small, basophilic cells. Some large and/or hyperchromatic nuclei developed and many nuclei had an 'empty' appearance, with prominent nucleoli. Fat accumulation was noted in the hyperplastic and (pre) neoplastic nodules, developing after eight weeks of treatment, but this was not seen in the final tumours, composed of small basophilic cells.

3.2.3. Enzyme Histochemistry of the Liver During Diethylnitrosamine-induced Hepatocarcinogenesis

Liver tissue was examined histochemically after six and ten weeks of diethylnitrosamine treatment. There was little difference in the activities of glucose 6-phosphate dehydrogenase and glutamate dehydrogenase between treated and control rats at six weeks (Plates 2.27 and 2.28; c.f. Plates 1.8 and 1.10). There was, however, a noticeable reduction in glucose 6-phosphatase, particularly centrilobularly, where glycogen was depleted (Plate 2.29).

Histochemical analysis of a preneoplastic nodule in the liver of a rat treated for ten weeks showed a very marked increase in glucose 6-phosphate dehydrogenase (Plate 2.30) an increase in malic enzyme activity (Plate 2.31), a reduction in glutamate dehydrogenase (Plate 2.32) and
PLATE 2.27 Liver of rat treated with diethylnitrosamine for six weeks. No obvious effect on glucose 6-phosphate dehydrogenase activity. 40X mag.

PLATE 2.28 Liver of rat after six weeks diethylnitrosamine treatment. Glutamate dehydrogenase activity similar to control. 40X mag.
PLATE 2.29 Glucose 6-phosphatase depletion, especially centrilobularly in liver of rat treated for six weeks with diethylnitrosamine. P.t., portal tract; C.v., central vein. 40X mag.

PLATE 2.30 Preneoplastic nodule in rat liver after ten weeks diethylnitrosamine treatment showing greatly elevated glucose 6-phosphate dehydrogenase. 40X mag.
PLATE 2.31  Same tissue as Plate 2.30. The nodule, N, has increased malic enzyme activity. 40X mag.

PLATE 2.32  Same tissue as Plate 2.30. The nodule, N, has decreased glutamate dehydrogenase activity. 40X mag.
a virtual absence of glucose 6-phosphatase in the nodule (Plate 2.33). The nodule was composed of enlarged cells with enlarged nuclei, some of which were undergoing mitosis (Plate 2.34). The nodule was similar to that shown in Plates 2.18 to 2.20.

3.2.4. Changes in Liver Weight During Diethylnitrosamine-induced Hepatocarcinogenesis

The changes in liver weight relative to body size during diethylnitrosamine treatment are shown in figure 3.2.1. The liver size decreased in relation to body weight during the first eight weeks. However, as the animals were still growing at this time, it did not represent actual tissue loss but rather an inhibition of growth for the first four weeks. There may have been some tissue loss between four and eight weeks. The decrease in relative liver weight presumably reflects the hepatotoxicity of diethylnitrosamine. The liver began to increase in size after eight weeks and this was associated with an increased number of mitoses. Foci of cells loaded with glycogen also appeared at this time and this may have contributed to the increase in liver weight.

The increase in hyperplasia seen at ten weeks was reflected in a rapid increase in liver size between ten and eleven weeks. The growth during this period resulted in the relative liver weight of the animals treated for eleven weeks being greater than control values. From the data obtained in the initial carcinogenesis experiment, the relative liver size continued to increase so that it was nearly half as much again as the normal relative liver weight.

3.2.5. Enzyme Changes During Diethylnitrosamine-induced Hepatocarcinogenesis

The changes in the enzyme activities during diethylnitrosamine administration are shown in Table 3.2.1. The activities of the enzymes expressed as normalised liver units, after comparison with control animals assayed at the same time, are shown in Figure 3.2.2.(i - ix).

After two weeks of carcinogen treatment there was a reduction in
PLATE 2.33 Same tissue as Plate 2.30. The nodule, N, has greatly reduced glucose 6-phosphatase activity. 40X mag.

PLATE 2.34 Mitoses and enlarged cells and nuclei in the nodule, N, shown in Plates 2.30 - 2.33.
Figure 3.2.1 Changes in Relative Liver Weight During Diethylnitrosamine-Induced Hepatocarcinogenesis

Error bars represent mean ± SEM.
The broken line represents the normal adult value.
### TABLE 3.2.1. Changes in Enzyme Activities During Diethylnitrosamine-induced Hepatocarcinogenesis

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>11</th>
<th>(a)</th>
<th>11 + 1 week clean water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymidine Kinase</td>
<td>8.59 ± 3.27</td>
<td>38.7 ± 4.5**</td>
<td>8.23 ± 0.21***</td>
<td>11.8 ± 2.9*</td>
<td>4.85 ± 1.16</td>
<td>17.5 ± 21.5*</td>
<td>17.2 ± 3.2*</td>
<td>7.7 ± 14.3</td>
</tr>
<tr>
<td></td>
<td>36.5 ± 6.6</td>
<td>138 ± 16</td>
<td>25.6 ± 0.7</td>
<td>38.5 ± 9.6</td>
<td>15.8 ± 3.9</td>
<td>103 ± 113</td>
<td>102 ± 25</td>
<td>31.3 ± 50.7</td>
</tr>
<tr>
<td>Hexokinase</td>
<td>0.29 ± 0.012</td>
<td>0.305 ± 0.027*</td>
<td>0.323 ± 0.028*</td>
<td>0.581 ± 0.05*</td>
<td>0.26 ± 0.06*</td>
<td>0.52 ± 0.52**</td>
<td>0.6 ± 0.07**</td>
<td>0.39 ± 0.39</td>
</tr>
<tr>
<td></td>
<td>1.08 ± 0.48</td>
<td>1.13 ± 0.10</td>
<td>0.99 ± 0.08</td>
<td>1.63 ± 0.11</td>
<td>1.57 ± 0.32</td>
<td>2.6 ± 3.48</td>
<td>4.3 ± 0.96</td>
<td>1.45 ± 1.45</td>
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<tr>
<td>Glucose 6-Phosphate</td>
<td>45.0 ± 1.1*</td>
<td>60.3 ± 2.1*</td>
<td>65.1 ± 7.1</td>
<td>60.8 ± 13.3</td>
<td>40.6 ± 2.6*</td>
<td>12.9 ± 17.7</td>
<td>16.1 ± 21.6</td>
<td>12.9 ± 17.7</td>
</tr>
<tr>
<td></td>
<td>202 ± 4</td>
<td>216 ± 9.0</td>
<td>202 ± 24</td>
<td>184 ± 37</td>
<td>133 ± 6</td>
<td>82.8 ± 84.6</td>
<td>47.4 ± 47.6</td>
<td>51.6 ± 64.4</td>
</tr>
<tr>
<td>Aspartate Amino-Transferase</td>
<td>145 ± 7</td>
<td>167 ± 9</td>
<td>203 ± 23</td>
<td>172 ± 25</td>
<td>109 ± 3</td>
<td>53.1 ± 56.3*</td>
<td>93.0 ± 11.5</td>
<td>63.3 ± 91.7</td>
</tr>
<tr>
<td></td>
<td>660 ± 31</td>
<td>660 ± 37</td>
<td>452 ± 137</td>
<td>525 ± 85</td>
<td>358 ± 24</td>
<td>254 ± 362</td>
<td>588 ± 89</td>
<td>271 ± 323</td>
</tr>
<tr>
<td>Glucose 6-Phosphatase</td>
<td>12.9 ± 1*</td>
<td>14.6 ± 0.9</td>
<td>17.8 ± 1.1*</td>
<td>10.6 ± 0.7*</td>
<td>7.71 ± 0.49***</td>
<td>4.07 ± 6.78*</td>
<td>8.35 ± 1.38*</td>
<td>19.7 ± 21.5</td>
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<td></td>
<td>58.0 ± 4.8</td>
<td>47.2 ± 3.6</td>
<td>54.8 ± 3.5</td>
<td>31.9 ± 2.3</td>
<td>23.6 ± 2.6</td>
<td>22.9 ± 38</td>
<td>52.7 ± 2.3</td>
<td>69.6 ± 90.6</td>
</tr>
<tr>
<td>Phosphoenol-Pyruvate Carboxykinase</td>
<td>1.82 ± 0.16</td>
<td>1.37 ± 0.21</td>
<td>1.89 ± 0.20*</td>
<td>1.75 ± 0.18*</td>
<td>1.72 ± 0.13*</td>
<td>0.873 ± 0.886*</td>
<td>0.79 ± 0.09*</td>
<td>2.93 ± 4.79</td>
</tr>
<tr>
<td>Glucokinase</td>
<td>1.45 ± 0.19</td>
<td>1.40 ± 0.10**</td>
<td>1.01 ± 0.08***</td>
<td>0.89 ± 0.23*</td>
<td>0.949 ± 0.109**</td>
<td>0.51 ± 0.6*</td>
<td>0.85 ± 0.06*</td>
<td>0.85 ± 0.06*</td>
</tr>
<tr>
<td></td>
<td>6.50 ± 0.89</td>
<td>5.02 ± 0.35</td>
<td>3.13 ± 0.24</td>
<td>2.72 ± 0.71</td>
<td>3.1 ± 0.2</td>
<td>2.86 ± 3.26</td>
<td>5.23 ± 0.7</td>
<td>3.99 ± 3.99</td>
</tr>
<tr>
<td>Malic Enzyme</td>
<td>1.61 ± 0.16</td>
<td>1.36 ± 0.19</td>
<td>1.13 ± 0.12</td>
<td>2.95 ± 0.37*</td>
<td>1.63 ± 0.21</td>
<td>1.95 ± 3.2</td>
<td>1.66 ± 0.16*</td>
<td>0.99 ± 0.87</td>
</tr>
<tr>
<td></td>
<td>7.19 ± 0.60</td>
<td>4.92 ± 0.78</td>
<td>3.51 ± 0.41</td>
<td>11.5 ± 1.36</td>
<td>6.98 ± 1.19</td>
<td>12.5 ± 18.7</td>
<td>11.3 ± 1.7</td>
<td>1.69 ± 1.69</td>
</tr>
</tbody>
</table>

Values are given as mean ± SEM. The figures in parenthesis indicate the number of animals studied. The upper value is units/g liver, the lower value is units/100g body weight.

Statistically significant differences from control animals assayed at the same time are: *p < 0.05; **p < 0.01; ***p < 0.005.

(a) Data from initial carcinogenesis experiment.
Figure 3.2.2 Changes in Enzyme Activities During Diethylnitrosamine-Induced Hepatocarcinogenesis Compared to Reversed Differentiation

Left hand graph:

Error bars represent mean ± SEM. Figures on abscissa represent the number of weeks of diethylnitrosamine treatment. Closed circles, solid line represent units/g liver expressed in liver units (L.U.); open circles, broken line represent units/100g body weight converted to L.U.

Right hand graph:

A reversal of figure 3.1.2 (i - ix) vertical scale same as left hand graph (u/g liver).

(i) TK; Thymidine kinase
(ii) HK; Hexokinase
(iii) G6PDH; Glucose 6-phosphate dehydrogenase
(iv) GDH; Glutamate dehydrogenase
(v) AAT; Aspartate aminotransferase
(vi) G6Pase; Glucose 6-phosphatase
(vii) PEPCK; Phosphoenolpyruvate carboxykinase
(viii) GK; Glucokinase
(ix) Malic; Malic enzyme
all the enzymes, except for hexokinase and thymidine kinase. These changes were not significant at the 95% confidence level, though the reduction in glutamate dehydrogenase and glucose 6-phosphatase were significant at the 90% level.

Thymidine kinase and hexokinase activities continued to increase, and the activities of the other enzymes to decrease, with a further two weeks of treatment. At this four-week stage the increase in thymidine kinase and decrease in glucokinase were significant.

By six weeks after the start of the carcinogen treatment, the reductions in glucose 6-phosphatase, phosphoenolpyruvate carboxykinase and glucokinase were significant, as was the elevation of thymidine kinase.

The pattern of enzyme changes altered after eight weeks of treatment. Glucose 6-phosphate dehydrogenase and malic enzyme had significantly elevated activities rather than reduced activities (Figure 3.2.2. iv, vi). This coincided with the more pronounced histological changes including increased fat deposition. The elevation of hexokinase and the reduction of glucose 6-phosphatase, phosphoenolpyruvate carboxykinase and glucokinase were all significant at this stage. Although glutamate dehydrogenase and aspartate aminotransferase were reduced in activity, this was not statistically significant.

The trend of the changes in enzyme activities found at eight weeks continued throughout the duration of the rest of the experiment, and a similar pattern was found in the initial carcinogenesis experiment, in the liver of rats treated for fifteen weeks, malic enzyme, although remaining increased in activity after more than eight weeks of treatment was not significantly different from controls, however. Soluble protein content was unchanged throughout the experiment (data not shown).

The changes observed histochemically in the nodules agree well with the changes in enzyme activity seen in the biochemical examination of whole liver. It is likely therefore that the changes observed biochemically represent changes in the nodules themselves. The changes in the nodules are probably greater than those observed in whole livers because of tissue dilution effects.

In order to compare these changes in enzyme activities with those changes occurring during normal differentiation two approaches have been made.
Firstly, by comparing changes in each individual enzyme during diethynitrosamine administration (Figure 3.2.1 - ix) with the changes observed in the same enzyme during development (Figure 3.1.2, i - ix). If there is a retrodifferentiation of expression of enzyme activity the changes observed in the carcinogenesis experiment should be similar to a reversal of those changes taking place during development. [To facilitate this comparison Figures 3.1.2 (i - ix) have been redrawn, in reverse, adjacent to Figure 3.2.2 (i - ix)]

By comparing the results in this way it can be seen that a certain qualitative but not necessarily quantitative, similarity exists between the pattern of expression during carcinogenesis and a reversal of normal differentiation for the following enzymes: thymidine kinase; hexokinase; glucokinase; glutamate dehydrogenase and glucose 6-phosphate dehydrogenase. The changes observed in the activities of aspartate aminotransferase and particularly glucose 6-phosphatase and phosphoenolpyruvate carboxykinase during carcinogenesis bore little resemblance to a reversal of differentiation and for malic enzyme the pattern of expression during carcinogenesis was the opposite of that expected from a reversal of differentiation.

3.2.6. Correlation Analysis of the Enzyme Data

The second approach is by a statistical analysis of the results using Spearman's rank correlation analysis, the principle and applications of which have been described by Knox (1976). Briefly, the activities of the enzymes in Liver Units are ranked for each developmental stage and each stage of carcinogenesis, the rank correlation coefficient (Rs) and Students t-values being calculated as follows:

\[ Rs = 1 - \frac{6 \sum d^2}{(n-1)n(n+1)} \]

\[ t = \frac{Rs \sqrt{\frac{n-2}{1-Rs^2}}}{d.f. = n-2} \]

where \(d\) is the difference in rank of each observation between the two situations (differentiation and development) and \(n\) is the number of observations.
TABLE 3.2.2. Spearman's Rank Correlation Analysis of Enzyme Profiles During Diethylnitrosamine-induced Hepatocarcinogenesis Compared With Enzyme Profiles During Development

<table>
<thead>
<tr>
<th>Developmental Stage</th>
<th>WEAKS ON DIETHYLNITROSAMINE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Rs</td>
</tr>
<tr>
<td>Foetal</td>
<td>.5125</td>
</tr>
<tr>
<td>Newborn</td>
<td>.5125</td>
</tr>
<tr>
<td>5 days</td>
<td>.5458</td>
</tr>
<tr>
<td>10 days</td>
<td>.3167</td>
</tr>
<tr>
<td>15 days</td>
<td>.2833</td>
</tr>
<tr>
<td>Weanling</td>
<td>.35</td>
</tr>
</tbody>
</table>

Statistical significance: +, p<0.1; *, p<0.05; **, p<0.01; ***, p<0.005.
The results obtained from a comparison of the profile of enzyme activities at stages during differentiation and diethylnitrosamine-induced hepatocarcinogenesis are shown in Table 3.2.2.

After two weeks diethylnitrosamine treatment there was no significant correlation with any of the stages of differentiation, although the greatest similarity was seen with the five day post partum stage. There was a significant correlation between the enzyme profile of weanling liver and that of the liver of animals receiving diethylnitrosamine for four weeks. A correlation between the enzyme profile of animals treated for four weeks and that of the five-day old animals was also significant (p<0.1). From six weeks of diethylnitrosamine treatment onwards there was a significant correlation with the foetal liver enzyme profile, and no significant correlation with enzyme profiles at any other stage of hepatic differentiation.
3.3. Two-stage Diethylnitrosamine/Phenobarbitone-induced Hepatocarcinogenesis

3.3.1. Experimental Protocol

A study was made of the changes occurring during the promotion of carcinogen-initiated tumorigenesis by a non-carcinogenic agent. The aim was to compare the changes observed during chronic diethylnitrosamine treatment with those accompanying treatment with a short-term dose of diethylnitrosamine followed by the long-term administration of pheno-barbitone. It was to be hoped that those changes due to the continuous intake of diethylnitrosamine could thus be distinguished from those associated with the carcinogenic process per se.

Many initiation-promotion studies with liver additionally involve partial hepatectomy which has the disadvantage of introducing another variable. For this reason animals were treated as described by Kitagawa and Sugano (1978). Rats were divided into three groups: those which received diethylnitrosamine (0.01% in the drinking water) for one week and thereafter received a diet containing 0.05% phenobarbitone (group 1); a control group of animals which received diethylnitrosamine (0.01% in the drinking water) and were given basal diet thereafter (group 2); and a second control group which was given fresh water and basal diet for three weeks and thereafter received a diet containing 0.05% phenobarbitone (group 3). The animals were twenty-three days old at the start of the experiment.

Animals were killed at the end of the first week for liver histology and histochemistry. Histological, histochemical and biochemical examination of the livers of animals from each of the three groups were made at eight, sixteen, twenty-four and thirty-two weeks after the start of the experiment.

3.3.2. Changes in Histology and Histochemistry During the Experiment

a) After the First Week

Animals which had received diethylnitrosamine for one week had a
reduced number of 'plant' cells compared with control animals. The cells in the centrilobular regions appeared to have paler cytoplasm than controls and some of these cells were vacuolated. The vacuoles seen with haematoxylin and eosin were probably fat vacuoles as staining with Oil Red O was heaviest around the central veins. Lipid deposition was not significantly greater in the treated tissue than the control tissues.

Despite the reduction in the number of plant cells, there did not seem to be a significant decrease in glycogen content in the treated animals. PAS staining was carried out on unfixed frozen sections and the periportal regions were the most positive.

No focal loss of glucose 6-phosphatase could be seen in the treated animals, though there may have been a slight reduction of activity in the centrilobular regions. No differences could be seen in the staining for glucose 6-phosphate dehydrogenase, glutamate dehydrogenase and malic enzyme activities between the two groups of animals.

There were marked differences in the staining for y-glutamyltranspeptidase however. In control animals this enzyme was restricted to bile ductule cells (Plate 3.1). In the treated animals several foci of y-glutamyltranspeptidase-positive hepatocytes could be seen associated with vascular areas (Plate 3.2). It was not possible to determine whether the y-glutamyltranspeptidase-positive areas were centrilobular or periportal because of the low staining intensity. However, y-glutamyltranspeptidase activity is often associated with cell damage and since most of the toxic effect of diethylamino was manifest in the centrilobular areas this might be the expected localisation of y-glutamyltranspeptidase.

b) After Eight Weeks

Centrilobular cell enlargement, a characteristic of chronic phenobarbitone administration, was seen in animals in both groups 1 and 3 (Plate 3.3). In the fixed tissue there seemed to be no difference in glycogen deposition between the three groups. Lipid deposition was variable between animals of the same group and it was therefore impossible
PLATE 3.1 Control rat liver. γ-glutamyltranspeptidase is restricted to bile ducts. P.t., portal tract. 25X mag.

PLATE 3.2 Rat liver after one week of treatment with diethylnitrosamine. Foci of hepatocytes staining for γ-glutamyltranspeptidase. 25X mag.
PLATE 3.3 Liver of group 1 animal at eight weeks. Centrilobular cell enlargement. C.v., central vein; P.t., portal tract. Fixed tissue: H & E 40X mag.

PLATE 3.4 Glucose 6-phosphatase activity in liver of group 2 animal at eight weeks. Essentially normal distribution. C.v., central vein; P.t., portal tract. 40X mag.
to attribute any effect on lipid deposition to any of the treatments. In the frozen tissue however it appeared that the glycogen content was marginally reduced in groups 1 and 3 compared with group 2, and that fat deposition in groups 1 and 3 was greater than in group 2 (being higher in the centrilobular regions than the periportal ones).

In both groups 1 and 3 the staining for glucose 6-phosphatase activity was reduced in the centrilobular regions, compared to group 2. In group 2 the difference in staining between zones 1 and 3 was less obvious than in groups 1 and 3 (Plates 3.4, 3.5). No difference in glutamate dehydrogenase, glucose 6-phosphate dehydrogenase and malic enzyme activities could be detected histochemically between the groups. In group 2 animals, γ-glutamyltranspeptidase activity found to be located exclusively in the portal tracts (Plate 3.6) but in group 3 animals (Plate 3.7) and, more frequently, in group 1 animals (Plate 3.8) γ-glutamyltranspeptidase was found apparently in non-portal areas.

c) After Sixteen Weeks

Again, centrilobular cell enlargement was observed in groups 1 and 3, with no change in group 2 animals. In group 1 animals some cells, especially those in zone 3, had a waxy appearance and some were finely vacuolated, although not apparently necrotic. 'Plant' cells were located preferentially in the periportal regions of the livers of animals from all groups and there seemed little difference in the PAS reaction between groups.

In sections of unfixed frozen tissue no 'plant' cells could be seen (as the glycogen does not leach out during the preparation of the tissue) and in general PAS staining was heavier, especially in zone 1. Some areas of increased glycogen deposition were seen in the livers of group 1 animals. Examination of frozen tissue sections revealed that the vacuolated cells seen in the centrilobular regions had reduced glycogen content. From their appearance, and the fact that clusters of fat-loaded cells are seen centrilobularly (Plate 3.10), it is likely that the vacuoles were due to fat deposition. Lipid accumulation in the livers of group 1 animals was seen periportal as well as centrilobularly and overall was heavier than in the livers of
PLATE 3.5 Liver of group 3 animal at eight weeks. Glucose 6-phosphatase reduced centrilobularly. 40X mag.

PLATE 3.6 Liver of group 2 animal at eight weeks. $\gamma$-glutamyltranspeptidase activity restricted to portal tissue. 25X mag.
PLATE 3.7  γ-glutamyltranspeptidase activity in liver of group 3 animal at eight weeks in non-portal area, g. 25X mag.

PLATE 3.8  γ-glutamyltranspeptidase activity in liver of group 1 animal at eight weeks in non-portal area, g. 25X mag.
PLATE 3.9  Liver of group 1 animal at sixteen weeks. Vacuolated cells deficient in glycogen. Unfixed frozen tissue; PAS 100X mag.

PLATE 3.10  Fat loaded cells seen centrilobularly in liver of group 1 animal at sixteen weeks. P.t., portal tract. Unfixed frozen tissue: ORO 40X mag.
group 2 animals. In group 3 lipid accumulation was also heavier than in group 2 animals, and the deposition seemed slightly heavier in zone 1 than zone 3. In group 2 animals no fat-loaded cells could be seen and lipid deposition was heaviest in zone 1 (Plate 3.11).

In groups 1 and 3 there was a greater difference in glucose 6-phosphatase activity between zone 1 (greatest activity) and zone 3 (least activity) than in group 2. This was due to reduced activity in zone 3. Some small foci of cells with reduced glucose 6-phosphatase activity were developing in the livers of group 1 animals.

Glutamate dehydrogenase activity showed normal lobular zonation in group 2 animals and most of group 3 animals. In group 1 animals however there seemed to be slightly higher activity in zone 1 compared to zone 3. Foci of slightly reduced glutamate dehydrogenase activity could be seen in half the group 1 animals and in some cases these areas appeared to correlate with fat accumulation.

The activity of glucose-6-phosphate dehydrogenase was much higher in zone 3 than zone 1 in all animal groups. Animals from group 1 showed some small foci of higher than normal activity. In some cases this appeared to correlate with increased glycogen deposition and with the absence of heavy fat deposition, but in other animals the increased glucose 6-phosphate dehydrogenase activity was in areas of increased fat deposition and reduced glucose 6-phosphatase activity.

Malic enzyme also exhibited increased activity in zone 3 compared with zone 1 in all animals from all groups. In group 1 animals some small foci of increased activity were developing which on occasion correlated with other observed histochemical alterations, in particular, fat deposition.

Positive staining for \(\delta\)-glutamyltranspeptidase was only seen in the bile duct cells in animals from groups 2 and 3 (Plate 3.12). In group 1 animals, however, several \(\delta\)-glutamyltranspeptidase-positive foci were seen (Plate 3.13). These foci did not necessarily correlate with the other histochemical alterations seen in the livers of animals from this group.
PLATE 3.11  Liver of group 2 animal at sixteen weeks. Lipid deposition greatest in zone 1, no centrilobular fat-loaded cells. C.v., central vein; P.t., portal tract. Unfixed frozen tissue: ORO 40X mag.

PLATE 3.12  Liver of group 3 animal at sixteen weeks, $\gamma$-glutamyltranspeptidase activity is restricted to bile ducts. P.t., portal tract; C.v., central vein. 25X mag.
PLATE 3.13 Liver of group 1 animal at sixteen weeks. γ-glutamyl transpeptidase positive foci. 25X mag.
d) After Twenty-four Weeks

Foci of hydromic degeneration were seen in the livers of all of the group 1 animals (Plate 3.14). Hydromic degeneration was also observed in the livers of some animals from groups 2 and 3, but fewer areas of the liver were affected. Animals in groups 1 and 3 had centrilobular cell enlargement and haematoxylin and eosin staining of unfixed, frozen sections had a mottled appearance because of cellular hypochromic material in zone 3 (Plate 3.15). In group 1 the hydromic degeneration was sometimes accompanied by white cell infiltration, and occurred most frequently in zone 2 and zone 3. Animals from group 1 had foci of both basophilic and eosinophilic cells (Plate 3.16). In general no basophilic or eosinophilic foci could be seen in any of the animals from groups 2 or 3. One of the animals in group 1 had developed a small low-grade hepatocellular carcinoma with adenoid structures, cirrhosis and bile duct proliferation (Plate 3.17). The normal architecture had been lost. Some of the hepatocytes in this tumour were finely vacuolated, others had large vacuoles and there were some greatly enlarged cells. All of the hepatocytes had enlarged 'empty' looking nuclei. Bile duct proliferation and a glycogen-rich focus was also seen in the liver of this animal.

A PAS-positive reaction was seen in all the animals, with heavier glycogen deposits in the periportal regions.

There were some foci of vacuolated cells in group 1 animals. In several areas of the liver these vacuolated foci were also deficient in glucose 6-phosphatase and had increased glucose 6-phosphate dehydrogenase and malic enzyme activities (Plates 3.18 - 3.21). The enzyme changes did not always correlate with alterations in glycogen deposition (Plate 3.22) although some foci of altered enzyme activity had increased glycogen deposition. Foci of hepatocytes staining for 6-glutamyltranspeptidase activity were also seen but these did not always correlate with other enzymic alterations (Plate 3.38). In one of the four animals in group 1, a glycogen-deficient focus of basophilic cells was also found to have reduced glucose 6-phosphatase, glucose 6-phosphate dehydrogenase, glutamate dehydrogenase, malic enzyme and 6-glutamyltranspeptidase activities.
PLATE 3.14  Liver of group 1 animal at twenty-four weeks. Focus of hydropic degeneration. Fixed tissue: H & E 100X mag.

PLATE 3.16 Liver of group 1 animal after twenty-four weeks.

Fixed tissue: H & E 100X mag.
PLATE 3.17  Small hepatocellular carcinoma with bile duct proliferation and adenosemorphism developing in liver of group 1 animal after 24 weeks. Fixed tissue: H & E 25X mag.

PLATE 3.18  Liver of group 1 animal at twenty-four weeks. Focus of vacuolated cells. C.v., central vein; P.t., portal tract. Fixed tissue: H & E 40X mag.
PLATE 3.19  Same tissue as Plate 3.18. Vacuolated cells have reduced glucose 6-phosphatase activity. C.v., central vein; P.t., portal tract. 40X mag.

PLATE 3.20  Same tissue as Plate 3.18. Glucose 6-phosphate dehydrogenase activity is elevated in the area corresponding to the vacuolated cells. C.v., central vein; P.t., portal tract. 40X mag.
PLATE 3.21  Same tissue as Plate 3.18. Malic enzyme activity is increased in the area of vacuolated cells. C.v., central vein; P.t., portal tract. 40X mag.

PLATE 3.22  Same tissue as Plate 3.18. Glycogen deposition is not significantly altered in the area of vacuolated cells. Unfixed frozen tissue: PAS 40X mag.
PLATE 3.23  Same tissue as Plate 3.18. γ-glutamyltranspeptidase activity is not significantly elevated in the area of vacuolated cells though foci of increased activity, g, are seen. C.v., central vein: P.t., portal tract. 25X mag.

PLATE 3.24  Same tissue as Plate 3.18. Fat-loaded cells seen in area of vacuolated cells. C.v., central vein: P.t., portal tract. Unfixed frozen tissue: ORO 40X mag.
In groups 2 and 3 the glycogen deposition was normal being heavier periportally. The difference in PAS staining between zone 1 and zone 3 was more marked in group 3. Fat deposition was heavier in group 3 animals than group 2 and on the whole occurred centrilobularly. Animals in group 1 also had heavier fat deposition than those in group 2 and a few foci of heavy fat deposition were seen (Plate 3.24) which were frequently associated with areas of increased glucose 6-phosphate dehydrogenase and malic enzyme activities.

In both groups 2 and 3, the pattern of enzyme activity was normal: glucose 6-phosphatase activity was greater in zone 1 than in zone 3 (especially marked in group 3 animals); γ-glutamyltranspeptidase was restricted to bile duct cells; and the other enzymes studied showed greatest activity centrilobularly. Group 1 animals had areas of altered enzyme activities; viz., reduced glucose 6-phosphatase activity, increased glucose 6-phosphate dehydrogenase and malic enzyme activities and hepatocytes staining positively for γ-glutamyltranspeptidase. No changes in glutamate dehydrogenase activity was seen. Usually changes in one enzyme were accompanied by other histochemical changes, including glycogen and fat deposition, as mentioned earlier, but it was unusual to find all the changes in one focus.

The tumour in the group 1 animal showed considerable bile duct proliferation and had compressed the surrounding tissue. Markedly increased γ-glutamyltranspeptidase activity was seen not only in the bile ducts but also in the majority of the hepatocytes (Plate 3.25). Most of the tumour was glucose 6-phosphatase deficient except for a few cells near the periphery of the tumour. Glycogen deposition showed the same distribution as glucose 6-phosphatase activity (Plates 3.26, 3.27). Glutamate dehydrogenase was also reduced especially towards the centre of the tumour (Plate 3.28). The tumour hepatocytes showed a very high glucose 6-phosphate dehydrogenase activity (Plate 3.29) and malic enzyme activity whilst being higher than the periportal regions of normal tissue was not as high as the activity in normal centrilobular areas (Plate 3.30). No fat deposition was seen in the bile duct cells and fat deposition seemed reduced in the hepatocytes compared with normal hepatocytes. However, the cells around the periphery of the tumour had increased fat deposition (Plate 3.31).
PLATE 3.25 Same tissue as Plate 3.17. ß-glutamyltranspeptidase activity is elevated in the hepatocytes as well as associated with the bile duct cells in the hepatocellular carcinoma. 25X mag.

PLATE 3.26 Same tissue as Plate 3.17. The tumour is glucose 6-phosphatase deficient, 25X mag.
PLATE 3.27  Same tissue as Plate 3.17. Glycogen has been lost in the tumour. Unfixed frozen tissue: PAS 25X mag.

PLATE 3.28  Same tissue as Plate 3.17. Glutamate dehydrogenase activity is reduced, especially at the centre of the tumour. 25X mag.
PLATE 3.29  Same tissue as Plate 3.17. Tumour has elevated glucose 6-phosphate dehydrogenase activity. 25X mag.

PLATE 3.30  Same tissue as Plate 3.17. Tumour hepatocytes have greater malic enzyme activity than normal hepatocytes in zone 1 but less activity than normal hepatocytes in zone 3 (c.f. Plate 1.19) 25X mag.
PLATE 3.31  Same tissue as Plate 3.17. Fat deposition is seen at the periphery of the tumour but not at the centre. Unfixed frozen tissue: ORO 25X mag.

PLATE 3.32  Tumour arising in a group 1 animal after thirty-two weeks showing multiple cell-thick trabecular arrangement of hepatocytes. Fixed tissue: H & E 100X mag.
After Thirty-two Weeks

Six of the remaining fourteen animals in group 1 had developed tumours at this stage. Five of these were large and the final one was about 7mm in diameter. In contrast to chronic diethyl-nitrosamine treatment these tumours developed in the left and median lobes of the liver. The tumours were of varying size and type. In two tumours bile duct proliferation was seen but this was not observed in the other tumours. In the larger tumours some areas showed a trabecular arrangement of the cells (Plate 3.32) in other areas the cells were arranged in jumbled sheets and in one tumour cirrhosis was seen. Various cellular changes were observed: eosinophilic and basophilic cells were seen and hydropic degeneration, karyolysis (often associated with fat vacuolation), karyolysis, pyknosis and haemorrhagic necrosis (Plate 3.33) in some areas of some tumours were seen. Cell and nuclear enlargement were often seen and most of the nuclei had an 'empty' appearance. Mitoses were frequently seen (Plate 3.34). Both glycogen and lipid deposition were significantly reduced in the tumour tissue though some large areas of fat deposition, were seen in the tumours (Plates 3.35, 3.36). The enzyme changes observed histochemically in the tumour tissue were the same as those seen in the tumour arising after twenty-four weeks. The non-tumour liver tissue in the group 1 animals was on the whole histologically and histochemically similar to the livers of the group 1 animals after twenty-four weeks with a slight increase in the amount of hydropic degeneration and fat deposition.

One of the group 2 animals had a pre-neoplastic nodule which was positive for 8-glutamyltranspeptidase, had markedly increased glucose 6-phosphate dehydrogenase and slightly increased malic enzyme activity, slightly reduced glutamate dehydrogenase activity and was deficient in glucose 6-phosphatase and glycogen. In another animal from group 2 an area of the liver was seen with enlarged sinusoidal spaces. The nuclei of the hepatocytes in this area were hyperchromatic. A third animal from this group had a focus of basophilic cells with 'empty-looking' nuclei. Mitoses were not seen in either of these areas. The remaining six livers from animals in group 2 were similar histo-
**PLATE 3.33** Haemorrhagic necrosis observed in the tumour in Plate 3.32.
Fixed tissue: H & E 100X mag.

**PLATE 3.34** 'Empty' looking nuclei and mitotic figure in the tumour shown in Plate 3.32. Fixed tissue: H & E 400X mag.
PLATE 3.35  An area of cells containing large vacuoles seen in one part of the tumour shown in Plate 3.32. H & E 100X mag.

PLATE 3.36  Same tissue as Plate 3.35 the vacuoles are due to excessive fat deposition. Fixed tissue: ORO 100X mag.
logically and histochemically to those examined at twenty-four weeks.

On the whole, the livers from animals in group 3 examined after thirty-two weeks appeared similar histologically and histochemically to those examined at twenty-four weeks. Some increase in the hydropic degeneration was observed and this was most common centrilobularly. In some cases there was necrosis in these hydropic areas. In two of the seven animals examined there was some slight bile duct proliferation.

f) Summary

Various changes were observed histologically and histochemically in the livers during the course of the two-stage carcinogenesis. Firstly, group 2 animals showed little change from normal livers although one animal developed a pre-neoplastic nodule at thirty-two weeks. Centrilobular cell enlargement was induced in all animals in groups 1 and 3 by chronic phenobarbitone feeding. This was associated with an increased fat deposition, and a decreased glycogen deposition in the centrilobular areas. Hydropic degeneration developed in these animals, particularly group 1 animals, usually centrilobularly. In group 3 no γ-glutamyltranspeptidase positive hepatocytes were seen except at eight weeks.

In group 1 animals foci of altered histochemistry were first detected at sixteen weeks these included an increase in fat deposition, an increase or decrease in glycogen deposition, a decrease in glucose 6-phosphatase and glutamate dehydrogenase activities and an increase in the activities of glucose 6-phosphate dehydrogenase, malic enzyme and γ-glutamyltranspeptidase. Often several of these changes were observed in the same focus but it was unusual to find alterations in all the histochemical criteria in one focus. Tumours with various cellular alterations arose in the livers of about half of the animals in group 1 after thirty-two weeks and they were deficient in glycogen and, on the whole, fat. They had lost glucose 6-phosphatase and glutamate dehydrogenase activity and had elevated glucose 6-phosphate dehydrogenase, malic enzyme and γ-glutamyltranspeptidase activities.
3.3.3. Changes in Liver Weight During Two-stage Carcinogenesis

Liver weight relative to body weight declined with age in all the animals (Fig. 3.3.1). Chronic feeding with phenobarbitone caused an increase in liver weight and this was most marked in the animals which had been pretreated with diethylnitrosamine. In the animals which had no pretreatment (group 3 animals), the increase in liver weight due to phenobarbitone was gradually reduced and at the twenty-four week stage these animals had normal-sized livers. The livers of the group 1 animals remained increased in size.

3.3.4 Changes in Enzyme Activity During the Experiment

The changes in enzyme activities observed during the two-stage experimental hepatocarcinogenesis are shown in Table 3.3.1 and in normalised liver units in Fig. 3.3.2. Some variation in enzyme activities were observed probably partly due to experimental variation and partly due to dietary variances or other non-specific disturbances of the animals. On the whole, the enzyme activities recorded in the group 2 animals were similar to control values. This is in good agreement with the normal histological appearance of the livers of these animals. The greatest alteration in enzyme activity was observed in the group 1 animals with group 3 animals usually showing intermediate changes. Thymidine kinase activity in group 1 animals was increased, but this was not significant, and decreased significantly in group 3 animals at eight weeks indicating that the increase in liver weight in these two groups was probably a hypertrophic rather than a hyperplastic response. This would confirm the histological finding of centriflobular cell enlargement in these two groups without increased mitotic rate. Thymidine kinase was significantly elevated in the group 1 animals at later stages of the experiment corresponding to the appearance of neoplastic tissue. Thymidine kinase was also elevated in group 3 animals at thirty-two weeks but this was not significant because of the wide inter-animal variation. This may have been because of liver regeneration in areas of hydropic degeneration seen histologically.
Figure 3.3.1 Changes in Relative Liver Weight During 2-Stage Hepato-Carcinogenesis, Diethylnitrosamine Treatment and Phenobarbitone Treatment

Error bars represent mean ± SEM.
Group 1; Closed circles, solid line.
Group 2; Open circles, broken line.
Group 3; Triangles, dotted line.
TABLE 3.3.1: Enzyme Changes During Two-stage Hepatocarcinogenesis

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>8 Weeks (U/g liver)</th>
<th>Group 1 (h)</th>
<th>Group 2 (h)</th>
<th>Group 3 (h)</th>
<th>16 Weeks (U/g liver)</th>
<th>Group 1 (h)</th>
<th>Group 2 (h)</th>
<th>Group 3 (h)</th>
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<tr>
<td>Thymidine kinase</td>
<td>1.36 ± 0.2</td>
<td>6.92 ± 1.05</td>
<td>1.01 ± 0.23</td>
<td>4.18 ± 1.00</td>
<td>0.839 ± 0.162*</td>
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<td>Hexokinase</td>
<td>0.213 ± 0.031</td>
<td>1.08 ± 0.15</td>
<td>0.251 ± 0.026</td>
<td>0.136 ± 0.005**</td>
<td>0.219 ± 0.017</td>
<td>0.286 ± 0.014*</td>
<td>0.181 ± 0.021</td>
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<td>Glucose 6-Phosphate</td>
<td>7.17 ± 1.56</td>
<td>1.40 ± 0.38</td>
<td>1.35 ± 0.26</td>
<td>5.59 ± 1.08</td>
<td>2.42 ± 0.94***</td>
<td>2.12 ± 0.71*</td>
<td>1.15 ± 0.23</td>
<td>1.85 ± 0.16***</td>
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<tr>
<td>Dehydrogenase</td>
<td>29.1 ± 4.8</td>
<td>169 ± 26</td>
<td>32.2 ± 3.0</td>
<td>133 ± 12</td>
<td>28.1 ± 7.1</td>
<td>38.6 ± 1.5</td>
<td>56.3 ± 4.2**</td>
<td>51.0 ± 4.0*</td>
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<tr>
<td>Glutamate</td>
<td>90.4 ± 13.9</td>
<td>462 ± 76</td>
<td>126 ± 23</td>
<td>523 ± 99</td>
<td>110 ± 26</td>
<td>62.3 ± 4.1**</td>
<td>84.4 ± 2.5</td>
<td>74.4 ± 6.0*</td>
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<td>Aspartate</td>
<td>20.6 ± 1.5</td>
<td>105 ± 5</td>
<td>28.4 ± 2.4*</td>
<td>117 ± 15</td>
<td>19.9 ± 3.3</td>
<td>12.7 ± 0.7***</td>
<td>18.2 ± 1.9</td>
<td>16.3 ± 1.1*</td>
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<tr>
<td>Glucose 6-Phatasease</td>
<td>0.702 ± 0.037***</td>
<td>3.57 ± 0.16</td>
<td>1.32 ± 0.13</td>
<td>5.43 ± 0.49</td>
<td>0.72 ± 0.055**</td>
<td>0.885 ± 0.109*</td>
<td>1.92 ± 0.07*</td>
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<td>Carboxykinase</td>
<td>1.86 ± 0.14</td>
<td>9.44 ± 0.57</td>
<td>2.39 ± 0.11</td>
<td>9.87 ± 0.52</td>
<td>2.11 ± 0.13</td>
<td>1.55 ± 0.18*</td>
<td>1.96 ± 0.08</td>
<td>2.09 ± 0.23</td>
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<td>Glucokinase</td>
<td>3.01 ± 0.53*</td>
<td>15.3 ± 2.7</td>
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<td>13.7 ± 5.3</td>
<td>4.21 ± 0.69**</td>
<td>3.79 ± 0.83*</td>
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<td>Malic enzyme</td>
<td>40.3 ± 0.8</td>
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<td>41.2 ± 1.1</td>
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Values are mean ± SEM, the upper value is U/g liver; the lower value is U/100g body weight.
Figures in parenthesis indicate the number of animals studied.
Statistically significant differences from normal adult values (not assayed at the same time) are:
+ p<0.1, * p<0.05, ** p<0.01, *** p<0.005.
NM = not measured
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<th>Group 2 (4)</th>
<th>Group 3 (4)</th>
<th>Group 1 (4)</th>
<th>32 Weeks</th>
<th>Group 2 (4)</th>
<th>Group 3 (4)</th>
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<td>Thymidine kinase</td>
<td>4.82 ± 1.72*</td>
<td>1.39 ± 0.56</td>
<td>1.24 ± 0.44</td>
<td>7.56 ± 2.61***</td>
<td>1.89 ± 0.74</td>
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<td>Glucose 6-Phosphate</td>
<td>19.7 ± 7.18</td>
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<td>4.36 ± 1.82</td>
<td>10.1 ± 3.52</td>
<td>15.8 ± 5.6</td>
<td>15.7 ± 6.9</td>
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<td>Glutamate</td>
<td>0.295 ± 0.092*</td>
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<td>0.292 ± 0.025*</td>
<td>0.178 ± 0.031</td>
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<td>0.688 ± 0.07</td>
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<td>Aminotransferase</td>
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<td>319 ± 25</td>
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<td>Glucose 6-Phosphatase</td>
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<td>Carboxykinase</td>
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<td>Glucokinase</td>
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<td>Glutamate</td>
<td>1.13 ± 0.10***</td>
<td>1.48 ± 0.09*</td>
<td>1.46 ± 0.12*</td>
<td>1.74 ± 0.26</td>
<td>1.89 ± 0.03</td>
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<td>Malic enzyme</td>
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<td>Soluble Protein</td>
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Values are mean ± SEM, the upper value is U/g liver; the lower value is U/100g body weight.

Figures in parenthesis indicate the number of animals studied.

Statistically significant differences from normal adult values (not assayed at the same time) are:

* p = 0.01, * p = 0.05, ** p = 0.01, *** p = 0.005.

NM = not measured
Figure 3.3.2 Enzyme Changes During 2-Stage Hepatocarcinogenesis, Diethylnitrosamine and Phenobarbitone Treatment

Error bars represent mean ± SEM of values /g liver converted to liver units (L.U.).
Group 1; closed circles, solid lines.
Group 2; open circles, broken lines.
Group 3; triangles, dotted lines.

T represents value obtained for tumours developing in livers of group 1 animals. H represents values for uninvolved host liver of tumour-bearing liver of group 1 animals.

(i) TK; Thymidine kinase
(ii) HK; Hexokinase
(iii) G6PDH; Glucose 6-phosphate dehydrogenase
(iv) GDH; Glutamate dehydrogenase
(v) AAT; Aspartate aminotransferase
(vi) G6Pase; Glucose 6-phosphatase
(vii) PEPCK; Phosphoenolpyruvate carboxykinase
(viii) GK; Glucokinase
(ix) Malic; Malic enzyme
(vi) AAT L.U.

(vii) G6Pase L.U.

(viii) PEPCK L.U.

(ix) Malic L.U.
Glucose 6-phosphate dehydrogenase was not significantly altered in the group 1 animals at eight weeks but thereafter the activity increased significantly. In group 3 animals, however, this enzyme was significantly elevated at eight weeks and though at later time points the activity decreased it was still higher than normal. Similarly, malic enzyme activity, which was higher in all three groups of animals at eight weeks (experimental or animal variation, probably) was highest in group 3 animals, group 1 and 2 animals had similar activities. Group 3 animals had elevated malic enzyme activity for the duration of the experiment. Malic enzyme activity increased in group 1, becoming significantly elevated at 16 weeks and remaining significantly elevated until the end of the experiment.

There was no significant variation of hexokinase activity between the groups. All of the groups had high hexokinase, low glucokinase activity at twenty-four weeks probably due to dietary or other non-specific disturbances. Glucokinase activity in group 1 animals was reduced compared to groups 2 and 3, which had similar activities for this enzyme, until the thirty-two week time point.

The activities of the two gluconeogenic enzymes, glucose 6-phosphatase and phosphoenolpyruvate carboxykinase, were highest in group 2 animals and lowest in group 1 animals throughout the experiment. The largest differences in activity between these two groups was recorded at sixteen and twenty-four weeks. Intermediate activities of glucose 6-phosphatase and phosphoenolpyruvate carboxykinase were recorded in group 3 animals.

Similarly, the activities of glutamate dehydrogenase and aspartate aminotransferase were highest in group 2 animals and lowest in group 1 animals, with group 3 animals having intermediate activities, throughout the experiment. Both these enzymes were significantly reduced in the group 1 animals at thirty-two weeks.

In some of the animals in group 1, tumours, large enough to be assayed separately from the rest of the liver, had developed by thirty-two weeks. The results of the enzyme assays on these tumours and the apparently normal remainder of the liver 'host' liver are shown in Table 3.3.2. The tumour liver had significantly elevated thymidine kinase, glucose 6-phosphate dehydrogenase and malic enzyme activities,
TABLE 3.3.2 Comparison of Tumours Arising in Group 1 Animals After Thirty-two Weeks Treatment With Uninvolved 'Host' Liver

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>'HOST' LIVER (2)</th>
<th>TUMOUR (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymidine Kinase</td>
<td>1.94 ; 4.6 a) *</td>
<td>10.2 ± 0.6 a)***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b)*</td>
</tr>
<tr>
<td>Hexokinase</td>
<td>0.208 ; 0.28</td>
<td>0.316 ± 0.086</td>
</tr>
<tr>
<td>Glucose 6-Phosphate Dehydrogenase</td>
<td>1.35 ; 1.37</td>
<td>11.6 ± 1.3 a)**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b)**</td>
</tr>
<tr>
<td>Glutamate Dehydrogenase</td>
<td>31.4 ; 35.6</td>
<td>44.5 ± 2.7</td>
</tr>
<tr>
<td>Aspartate Aminotransferase</td>
<td>70.8 ; 88.4</td>
<td>57.9 ± 2.5 a)**</td>
</tr>
<tr>
<td>Glucose 6-phosphatase Dehydrogenase</td>
<td>10.9 ; 11.6 a)**</td>
<td>4.52 ± 0.74 a)***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b)**</td>
</tr>
<tr>
<td>Phosphoenolpyruvate Carboxykinase</td>
<td>1.31 ; 2.19</td>
<td>0.207 ± 0.022 a)**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b)*</td>
</tr>
<tr>
<td>Glucokinase</td>
<td>1.56 ; 1.7</td>
<td>1.22 ± 0.13 a)*</td>
</tr>
<tr>
<td>Malic Enzyme</td>
<td>1.06 ; 3.76</td>
<td>8.26 ± 1.15 a)**</td>
</tr>
</tbody>
</table>

Values are mean ± SEM U/g tissue. Figures in parenthesis indicate number of observations. Statistically significant differences from normal adult values, a); and 'host' liver, b) are: *, p<0.05; **, p<0.01; ***, p<0.005.
significantly depressed aspartate aminotransferase, glucose 6-phosphatase, phosphoenolpyruvate carboxykinase and glucokinase activities. The activities of hexokinase and glutamate dehydrogenase were not significantly different from controls. The enzymes of the host liver were mostly not significantly different from control values except for thymidine kinase which was significantly increased and glucose 6-phosphatase which was significantly decreased.

3.3.5. Correlation Analysis of the Enzyme Data

In order to compare the results obtained during a two-stage hepatocarcinogenesis with those obtained during chronic diethylnitrosamine treatment and during normal development Spearman's Rank Correlation analysis was again applied. The data from the two-stage experiment converted to normalised liver units by comparison with controls was compared with all stages of development and chronic diethylnitrosamine treatment and the analysis is shown in Tables 3.3.3, 3.3.4, and 3.3.5. No significant correlations were observed in either group 2 or group 3 with any stage of development or carcinogenesis except at the 95 - 90% probability level where there was some association of group 2 animals at twenty-four and thirty-two weeks with the post partum rat and an association of group 3 animals at twenty-four weeks with the liver of animals treated for eleven weeks with diethylnitrosamine. The enzyme profile of group 2 animals and group 3 animals were significantly correlated at eight weeks but thereafter there was no significant correlation between these two groups.

The animals of group 1 showed no significant correlation with those of group 2 but from 16 weeks onwards the correlation with animals from group 3 was significant. Both the tumours and the host liver at thirty-two weeks correlated significantly with the liver of the group 3 animals.

Group 1 animals did not, at any time during the experiment, correlate significantly with any stage of development. Significant similarities in the enzyme pattern of group 1 animals with the enzyme pattern during the later stage of chronic diethylnitrosamine-induced hepatocarcinogenesis were observed. This was especially pronounced
TABLE 3.3.3. Spearman's Rank Correlation Analysis of the Enzyme Patterns of Group 1 Animals (Normalised by Comparison With Control Animals) Compared With Enzyme Patterns During Development and During Chronic Diethylnitrosamine Induced Hepatocarcinogenesis

<table>
<thead>
<tr>
<th>TREATMENT TIME (WEEKS)</th>
<th>8</th>
<th>16</th>
<th>24</th>
<th>32</th>
<th>TUMOUR</th>
<th>HOST LIVER</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rs</td>
<td>t</td>
<td>Rs</td>
<td>t</td>
<td>Rs</td>
<td>t</td>
</tr>
<tr>
<td>Group 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.4</td>
<td>1.155</td>
</tr>
<tr>
<td>Group 3</td>
<td>.35</td>
<td>.989</td>
<td>.881</td>
<td>4.56**</td>
<td>.6333</td>
<td>2.165*</td>
</tr>
<tr>
<td>Group 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Foetal</td>
<td></td>
<td>3208</td>
<td>1.896</td>
<td>-.0417</td>
<td>-.046</td>
<td>.4958</td>
</tr>
<tr>
<td>Newborn</td>
<td>-.1292</td>
<td>-.396</td>
<td>-.6131</td>
<td>-.1901</td>
<td>1.625</td>
<td>.436</td>
</tr>
<tr>
<td>5 days</td>
<td>-.3</td>
<td>-.332</td>
<td>-.5752</td>
<td>-.1814</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10 days</td>
<td>-.3667</td>
<td>-.104</td>
<td>-.7857</td>
<td>-.3111*</td>
<td>-.0833</td>
<td>-.221</td>
</tr>
<tr>
<td>15 days</td>
<td>-.2167</td>
<td>-.587</td>
<td>-.6667</td>
<td>-.2191+</td>
<td>.1167</td>
<td>.311</td>
</tr>
<tr>
<td>Weanling</td>
<td>-.1333</td>
<td>.356</td>
<td>-.0952</td>
<td>-.234</td>
<td>.2167</td>
<td>.587</td>
</tr>
</tbody>
</table>

Diethylnitrosamine |

<table>
<thead>
<tr>
<th>Weeks</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.917</td>
<td>1.517</td>
<td>.4762</td>
<td>1.326</td>
<td>.5833</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>-.4167</td>
<td>1.213</td>
<td>1.687</td>
<td>.144</td>
<td>.45</td>
<td>1.333</td>
</tr>
<tr>
<td></td>
<td>.5942</td>
<td>1.761</td>
<td>.5290</td>
<td>1.53</td>
<td>.6292</td>
<td>2.142*</td>
</tr>
<tr>
<td></td>
<td>.6667</td>
<td>2.366+</td>
<td>.7857</td>
<td>3.111*</td>
<td>.7833</td>
<td>3.333*</td>
</tr>
<tr>
<td></td>
<td>.6333</td>
<td>2.165+</td>
<td>.6429</td>
<td>2.056*</td>
<td>.7833</td>
<td>3.333*</td>
</tr>
<tr>
<td></td>
<td>.65</td>
<td>2.623*</td>
<td>.7381</td>
<td>2.68*</td>
<td>.95</td>
<td>8.05***</td>
</tr>
</tbody>
</table>

Statistical significance is given by: *, p < 0.05; **, p < 0.01; ***, p < 0.005.
## TABLE 3.3.4 Spearman's Rank Correlation Analysis of Enzyme Patterns of Group 2 Animals Compared With Enzyme Patterns During Development and Chronic Diethylnitrosamine-induced Hepatocarcinogenesis

<table>
<thead>
<tr>
<th>Treatment Time (Weeks)</th>
<th>Rs 8</th>
<th>t</th>
<th>Rs 16</th>
<th>t</th>
<th>Rs 24</th>
<th>t</th>
<th>Rs 32</th>
<th>t</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 3</td>
<td>0.7</td>
<td>2.593*</td>
<td>0.4524</td>
<td>1.243</td>
<td>0.3625</td>
<td>1.029</td>
<td>0.5625</td>
<td>1.80</td>
</tr>
<tr>
<td>Foetal</td>
<td>-0.3125</td>
<td>-0.878</td>
<td>0.0417</td>
<td>0.102</td>
<td>0.2792</td>
<td>0.769</td>
<td>0.4292</td>
<td>1.257</td>
</tr>
<tr>
<td>Newborn</td>
<td>0.4625</td>
<td>-1.38</td>
<td>1.131</td>
<td>0.279</td>
<td>0.3958</td>
<td>1.14</td>
<td>0.4958</td>
<td>1.511</td>
</tr>
<tr>
<td>5</td>
<td>-0.4667</td>
<td>-1.396</td>
<td>0.238</td>
<td>0.60</td>
<td>0.6125</td>
<td>2.05 *</td>
<td>0.5208</td>
<td>1.614</td>
</tr>
<tr>
<td>10</td>
<td>0.44</td>
<td>-1.55</td>
<td>0.0238</td>
<td>0.058</td>
<td>0.5708</td>
<td>1.839</td>
<td>0.6458</td>
<td>2.238 *</td>
</tr>
<tr>
<td>15</td>
<td>-0.4157</td>
<td>-1.213</td>
<td>0.095</td>
<td>0.234</td>
<td>0.5292</td>
<td>1.65</td>
<td>0.6542</td>
<td>2.288 *</td>
</tr>
<tr>
<td>Weanling</td>
<td>-0.0875</td>
<td>-0.232</td>
<td>-0.238</td>
<td>-0.60</td>
<td>0.3042</td>
<td>0.8457</td>
<td>0.2792</td>
<td>0.769</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Diethylnitrosamine</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2 weeks</td>
<td>0.4583</td>
<td>-1.364</td>
<td>0.8333</td>
<td>3.693 *</td>
<td>0.2333</td>
<td>0.635</td>
<td>0.1667</td>
<td>0.447</td>
</tr>
<tr>
<td>4 weeks</td>
<td>-0.0333</td>
<td>-0.0888</td>
<td>-0.0952</td>
<td>0.234</td>
<td>-0.1292</td>
<td>-0.3945</td>
<td>-0.30</td>
<td>-0.832</td>
</tr>
<tr>
<td>6 weeks</td>
<td>-0.175</td>
<td>-0.47</td>
<td>0.2321</td>
<td>0.585</td>
<td>0.006</td>
<td>0.015</td>
<td>-0.2208</td>
<td>-0.599</td>
</tr>
<tr>
<td>8 weeks</td>
<td>-0.0292</td>
<td>-0.077</td>
<td>0.0952</td>
<td>0.234</td>
<td>-0.2708</td>
<td>-0.744</td>
<td>-0.3167</td>
<td>-0.883</td>
</tr>
<tr>
<td>10 weeks</td>
<td>-0.0542</td>
<td>-0.144</td>
<td>0.3095</td>
<td>0.797</td>
<td>0.0542</td>
<td>-0.277</td>
<td>0.0333</td>
<td>0.088</td>
</tr>
<tr>
<td>11 weeks</td>
<td>-0.2667</td>
<td>-0.737</td>
<td>0.4286</td>
<td>1.162</td>
<td>0.2708</td>
<td>0.744</td>
<td>0.3875</td>
<td>1.112</td>
</tr>
</tbody>
</table>

Statistical significance is given by: +, p<0.1; *, p<0.05; **, p<0.01; ***, p<0.005.
<table>
<thead>
<tr>
<th></th>
<th>8</th>
<th>16</th>
<th>24</th>
<th>32</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rs</td>
<td>t</td>
<td>Rs</td>
<td>t</td>
</tr>
<tr>
<td>Foetal</td>
<td>-0.2875</td>
<td>-0.794</td>
<td>-0.3869</td>
<td>-1.028</td>
</tr>
<tr>
<td>Newborn</td>
<td>-0.5708</td>
<td>-1.839</td>
<td>-0.5774</td>
<td>-1.732</td>
</tr>
<tr>
<td>5 days pp</td>
<td>-0.7833</td>
<td>-3.334*</td>
<td>-0.6429</td>
<td>-2.056†</td>
</tr>
<tr>
<td>10 days pp</td>
<td>-0.7</td>
<td>-2.593*</td>
<td>-0.7619</td>
<td>-2.881†</td>
</tr>
<tr>
<td>15 days pp</td>
<td>-0.5833</td>
<td>-1.9†</td>
<td>-0.619</td>
<td>-1.931†</td>
</tr>
<tr>
<td>Weanling</td>
<td>-0.4833</td>
<td>-1.461</td>
<td>-0.6667</td>
<td>-2.191†</td>
</tr>
</tbody>
</table>

Chronic DEN
2 weeks           | 0.35    | 0.989   | 0.2262   | 0.569    | 0.225    | 0.611    | 0.125    | 0.333    |
4 weeks           | -0.4524 | -1.243  | -0.0714  | -0.175   | -0.2042  | -0.552   | 0        | 0        |
6 weeks           | -0.0042 | -0.011  | 0.2321   | 0.585    | 0.0417   | 0.110    | 0.0958   | 0.255    |
8 weeks           | 0.2333  | 0.635   | 0.619    | 1.931    | 0.2542   | 0.695    | 0.2333   | 0.635    |
10 weeks          | 0.1833  | 0.493   | 0.381    | 1.009    | 0.1875   | 0.505    | 0.3833   | 1.098    |
11 weeks          | 0.0167  | 0.044   | 0.4762   | 1.326    | 0.5833   | 1.9†     | 0.3833   | 1.098    |

Statistical significance is given by: †, p < 0.1; *, p < 0.05; **, p < 0.01; ***, p < 0.005
at twenty-four weeks. At thirty-two weeks the association was only weak but in the animals with the large tumours both the host liver tissue and the tumours the association with the liver of rats treated with diethylnitrosamine was strong.

The livers of animals in group 1 was similar to those of group 3 so some of the changes observed could have been due to the phenobarbitone treatment. For this reason biochemical data from the group 1 animals was converted to liver units by comparison with group 3 animals. Spearman's rank correlation analysis was then applied again (Table 3.3.6). This time a significant correlation between the group 1 animals at eight weeks and the five day post partum rat and between group 1 animals at sixteen and twenty-four weeks and foetal animals was seen.

The livers of group 1 animals at eight weeks were not similar to those of rats treated with diethylnitrosamine but from sixteen weeks onwards the similarity between group 1 animals and rats during the later stage of diethylnitrosamine treatment was significant. The tumours arising at thirty-two weeks showed significant correlation with the later stages of hepatocarcinogenesis induced by chronic diethylnitrosamine treatment but the 'host' liver did not.

3.3.6. Aneuploidy in the Tumours Induced by Two-stage Hepato-carcinogenesis

Wax sections of liver tissue of group 2 and group 3 animals and host liver and tumour tissue from group 1 animals at thirty-two weeks were Feulgen stained and the DNA content estimated by scanning microdensitometry (Fig. 3.3.3).

A mode was observed at the same density value in the liver of group 2 and group 3 animals and the non-tumour liver of group 1 animals. There was a greater degree of scatter observed in the pre-neoplastic nodule and the tumour tissue.

Neither diethylnitrosamine nor phenobarbitone alone induced aneuploidy but aneuploidy arose as a consequence of tumour development.
**TABLE 3.3.6** Spearman's Rank Correlation Analysis of the Enzyme Patterns of Group 1 Animals (normalised by Comparison With Group 3 Animals) Compared With the Enzyme Patterns During Development and During Chronic Diethylnitrosamine-induced Hepatocarcinogenesis

<table>
<thead>
<tr>
<th></th>
<th>8</th>
<th>16</th>
<th>24</th>
<th>32</th>
<th>TUMOUR</th>
<th>HOST</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rs</td>
<td>t</td>
<td>Rs</td>
<td>t</td>
<td>Rs</td>
<td>t</td>
</tr>
<tr>
<td>Foetal</td>
<td>.4708</td>
<td>1.412</td>
<td>.7798</td>
<td>3.057*</td>
<td>.8958</td>
<td>5.333***</td>
</tr>
<tr>
<td>Newborn</td>
<td>.0375</td>
<td>.099</td>
<td>-.5595</td>
<td>-1.654</td>
<td>.6</td>
<td>1.984+</td>
</tr>
<tr>
<td>5 days</td>
<td>.7611</td>
<td>3.159*</td>
<td>-.7143</td>
<td>-2.5*</td>
<td>.55</td>
<td>1.742</td>
</tr>
<tr>
<td>10 days</td>
<td>.5333</td>
<td>1.668</td>
<td>-.8905</td>
<td>-3.378*</td>
<td>.3833</td>
<td>1.098</td>
</tr>
<tr>
<td>15 days</td>
<td>-.1429</td>
<td>-.354</td>
<td>-.8333</td>
<td>-3.693*</td>
<td>.4607</td>
<td>1.396</td>
</tr>
<tr>
<td>Weanling</td>
<td>-.0833</td>
<td>-.0221</td>
<td>-.1667</td>
<td>-.414</td>
<td>.3</td>
<td>.832</td>
</tr>
</tbody>
</table>

Diethylnitrosamine Weeks

| 2  | .4292 | 1.257 | .2381 | .6 | .7833 | 2.854* | .5458 | 1.724 | .6083 | 2.028+ | .1167 | .31 |
| 4  | .3292 | .922 | .5 | 1.414 | .4833 | 1.461 | .5125 | 1.579 | .3667 | 1.043 | -.3667 | -.16 |
| 6  | .2167 | .587 | .875 | 4.427** | .8 | 3.528* | .5875 | 1.921+ | .7792 | 3.289* | -.0708 | -.16 |
| 8  | .0708 | .188 | .7619 | 2.881* | .733 | 2.854* | .6958 | 2.563* | .8667 | 4.596** | -.3 | -.85 |
| 10  | .0625 | .166 | .8333 | 3.693* | .7667 | 3.159* | .5958 | 1.963+ | .8337 | 3.989** | -.1167 | -.36 |
| 11  | .1458 | .39 | .7619 | 2.881* | .833 | 3.989** | .6625 | 2.34+ | .8667 | 4.596** | -.15 | -.45 |

Statistical significance is given by: +, p < 0.1; *, p < 0.05; **, p < 0.01; ***, p < 0.005
Figure 3.3.3 DNA Content of Normal and Tumour Nuclei During 2-Stage Hepatocarcinogenesis, Diethylnitrosamine and Phenobarbitone Treatment

Abscissa: Density of Feulgen stain; arbitrary units

(i) and (ii) Uninvolved host liver of tumour-bearing group 1 animal at 32 weeks.

(iii) medium sized tumour in group 1 animal.

(iv) Preneoplastic nodule in liver of group 1 animal.

(v) large tumour in liver of group 1 animal.

(vi) small tumour in liver of group 1 animal.

(vii) Liver of group 2 animal.

(viii) Liver of group 3 animal.
3.3.7. Effect of Phenobarbitone on Developmental Enzyme Changes at Weaning

Neonatal animals were orally dosed daily from the tenth day after birth with phenobarbitone so that they received a daily dose approximately equivalent to that received by the rats in the two-stage carcinogenesis experiment. After weaning the rats received the phenobarbitone in the drinking water. Malic enzyme and glucokinase, two enzymes which do not appear until weaning, were assayed at three time intervals in these rats; three days before weaning, within the first day after weaning and on the third day after weaning. This experiment was performed to investigate the possibility that phenobarbitone exerts its carcinogenesis promoting activity by inhibiting differentiation. Hexokinase, an enzyme which remains relatively unchanged during the weaning period, was also assayed. The results are shown in Table 3.3.7. The liver of the treated animals was enlarged compared to control animals. Histologically this was due to centrilocular cell enlargement similar to that seen in the liver of adult animals treated with phenobarbitone.

Three days before weaning malic enzyme activity was not detectable in the livers of either treated or control rats. Glucokinase activity was very low in the control rats and not detectable in some of the control rats, however, in the treated rats the activity was significantly higher. Hexokinase activity was slightly higher in the control than the treated rats.

During the first post-weaning day malic enzyme activity was again not detectable in control animals but low activity was detectable in the treated animals. The activity of glucokinase was also higher in the treated rats than the control rats but the difference was not so marked as at the earlier time point. Hexokinase activity was similar in both the treated and the control animals.

Three days after weaning the situation was reversed, higher activities of all three enzymes were observed in the control rats compared with the treated rats. The reduced activities of malic enzyme and glucokinase in the treated animals, compared to controls, were significant, but the reduction in hexokinase was not significant.
<table>
<thead>
<tr>
<th></th>
<th>3 Days Prior to Weaning</th>
<th>Weaning</th>
<th>Within 24 hours of Weaning</th>
<th>3 days after Weaning</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (4)</td>
<td>Phenobarbitone Treated (4)</td>
<td>Control (4)</td>
<td>Phenobarbitone Treated (4)</td>
</tr>
<tr>
<td>Liver weight 100g body weight</td>
<td>3.23 ± 0.065</td>
<td>4.094 ± 0.058***</td>
<td>3.56 ± 0.16</td>
<td>4.4 ± 0.04+</td>
</tr>
<tr>
<td>Malic Enzyme</td>
<td>NOT DETECTABLE</td>
<td>NOT DETECTABLE</td>
<td>NOT DETECTABLE</td>
<td>0.295 ± 0.067</td>
</tr>
<tr>
<td></td>
<td>0.025 ± 0.025</td>
<td>0.231 ± 0.073*</td>
<td>0.573 ± 0.115</td>
<td>1.3 ± 0.29</td>
</tr>
<tr>
<td></td>
<td>0.081 ± 0.081</td>
<td>0.937 ± 0.292*</td>
<td>2.12 ± 0.43</td>
<td>3.47 ± 0.82</td>
</tr>
<tr>
<td>Glucokinase</td>
<td>0.251 ± 0.021</td>
<td>0.229 ± 0.017</td>
<td>0.277 ± 0.053</td>
<td>0.282 ± 0.011</td>
</tr>
<tr>
<td></td>
<td>0.814 ± 0.079</td>
<td>0.935 ± 0.064</td>
<td>1.0 ± 0.211</td>
<td>1.24 ± 0.04</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. The upper value is U/g tissue; the lower value is U/100g body weight.

Figures in parenthesis indicate the number of observations.

Statistically significant differences from control values are given by +, p<0.1; *, p<0.05; **, p<0.01; *** , p<0.005.
3.4. Effect of Chronic Diethylnitrosamine Administration on DNA Alkylation

Rat liver has a high capacity for the removal of $O^6$-alkylguanine from DNA, after a single dose of alkylating agents, compared to other tissues. In order to investigate the effect of chronic diethylnitrosamine treatment on the excision system rats receiving diethylnitrosamine in the drinking water for one, five and ten weeks were given a single dose of $[^{14}C]$ diethylnitrosamine or $[^{14}C]$ dimethylnitrosamine and the levels of alkylpurines in the liver DNA determined as described in the Methods section. The Sephadex G10 elution profile of 0.1N HCl hydrolysate of DNA from the liver of animals receiving diethylnitrosamine in the drinking water for five weeks and then given $[^{14}C]$ diethylnitrosamine (10 mg/kg: 16.0 mCi/mMol) twelve hours prior to death is shown in Fig. 3.4.1. The elution profile of liver DNA from rats treated with diethylnitrosamine for ten weeks then given $[^{14}C]$dimethylnitrosamine (0.9 mg/kg: 24.5 mCi/mMol) is shown in Fig. 3.4.2.

The levels of alkylated purines in the treated animals, compared with age-matched control animals, are given in Table 3.4.1. These results show that the 3-ethyladenine:7-ethylguanine ratio is virtually unchanged by chronic diethylnitrosamine treatment. Ethylation of guanine at the $O^6$ position was greatly reduced by chronic diethylnitrosamine treatment and this is clearly shown by the decrease in $O^6$-ethylguanine:7-ethylguanine ratio, which compensates for overall differences in the extent of alkylation. The reduction of $O^6$-ethylguanine became more marked with more prolonged diethylnitrosamine treatment.

Analysis of methylated purines after $[^{14}C]$ dimethylnitrosamine showed that there was a reduction in the 3-methyladenine:7-methylguanine ratio after one and five weeks but not after ten weeks diethylnitrosamine treatment and the reduction was not so marked as the reduction in the $O^6$-methylguanine:7-methylguanine ratio. Continued diethylnitrosamine treatment did not cause further reduction in the levels of $O^6$-methylguanine analogous to the effect on $O^6$-ethylguanine so that after ten weeks diethylnitrosamine treatment the $O^6$-methylguanine:7-methylguanine ratio was 57% of the control ratio whereas the $O^6$-ethylguanine:7-ethylguanine ratio was only 7% of the control ratio (Table 3.4.1).
Sephadex G-10 profile of 0.1N HCl hydrolysate of DNA from the liver of rats, treated for 5 weeks with diethylnitrosamine, killed 12 hours after a single dose of $[^{14}C]$diethylnitrosamine. Closed circles, solid line = radioactivity; Open circles, broken line $= E_{260}$; PyO, pyrimidine oligonucleotide; 3-EA, 3-ethyladenine; 7-EG, 7-ethylguanine; G, guanine; A, adenine; $O^6$-EG, $O^6$-ethylguanine.
Sephadex G-10 profile of 0.1N HCl hydrolysate of DNA from the liver of rats, treated for 5 weeks with diethylnitrosamine, killed 12 hours after a single dose of $[^{14}C]$ dimethylnitrosamine. Closed circles, solid lines = radioactivity; Open circles, broken lines = $E_{260}$; PyO, pyrimidine oligonucleotide; 3-MA, 3-methyladenine; 7-MG, 7-methylguanine, G, guanine; A, Adenine; $O_6$MG, $O_6$-methylguanine.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ethylpurine (μmol/mol parent base)</th>
<th>7-ethyladenine</th>
<th>7-ethylguanine</th>
<th>O^6^-ethylguanine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3-ethyladenine</td>
<td>7-ethylguanine</td>
<td>O^6^-ethylguanine</td>
<td></td>
</tr>
<tr>
<td>Diethylnitrosamine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 week</td>
<td>4.5</td>
<td>31.7</td>
<td>0.9</td>
<td>0.143 (105)</td>
</tr>
<tr>
<td>Control</td>
<td>4.3</td>
<td>31.6</td>
<td>2.0</td>
<td>0.136</td>
</tr>
<tr>
<td>5 weeks</td>
<td>7.5</td>
<td>50.4</td>
<td>3.2</td>
<td>0.149 (94)</td>
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<tr>
<td>Control</td>
<td>5.8</td>
<td>36.8</td>
<td>8.6</td>
<td>0.158</td>
</tr>
<tr>
<td>10 weeks</td>
<td>4.5</td>
<td>35.2</td>
<td>0.5</td>
<td>0.126 (106)</td>
</tr>
<tr>
<td>Control</td>
<td>5.1</td>
<td>42.6</td>
<td>8.9</td>
<td>0.119</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Methylpurines (μmol/mol parent base)</th>
<th>7-ethyladenine</th>
<th>7-ethylguanine</th>
<th>O^6^-ethylguanine</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>3-methyladenine</td>
<td>7-methylguanine</td>
<td>O^6^-methylguanine</td>
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</tr>
<tr>
<td>Diethylnitrosamine</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 week</td>
<td>7.8</td>
<td>433</td>
<td>8.5</td>
<td>0.018 (86)</td>
</tr>
<tr>
<td>Control</td>
<td>8.7</td>
<td>423</td>
<td>25.4</td>
<td>0.021</td>
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<tr>
<td>5 weeks</td>
<td>14.0</td>
<td>581</td>
<td>23.2</td>
<td>0.023 (85)</td>
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<tr>
<td>Control</td>
<td>11.5</td>
<td>426</td>
<td>32.7</td>
<td>0.027</td>
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<tr>
<td>10 weeks</td>
<td>3.4</td>
<td>160</td>
<td>3.8</td>
<td>0.021 (105)</td>
</tr>
<tr>
<td>Control</td>
<td>5.0</td>
<td>259</td>
<td>11</td>
<td>0.020</td>
</tr>
</tbody>
</table>

Figures in parenthesis are % of control ratios.
3.5. Changes After Partial Hepatectomy

3.5.1. Experimental Protocol

Partial hepatectomies were performed so that adult liver during regeneration could be compared to both liver during carcinogenesis and during development. Thus the extent to which not only tumorigenesis reflects tissue renewal but also dedifferentiated characteristics are acquired during tissue renewal could be investigated. Also, further testing of Greenstein's hypothesis (which has been substantiated by Knox) that on the one hand, foetal and tumour livers resemble each other and on the other hand, are different from regenerating and resting adult liver which resemble each other in turn could be performed.

Rats were partially hepatectomised and sham operated as described in the methods section and their livers were examined at the following intervals after operation: eighteen hours, twenty-four hours, forty-eight hours, three, five and seven days.

3.5.2. Changes in Liver Histology and Histochemistry After Partial Hepatectomy

At eighteen hours post operation no mitoses could be seen, as expected, but many of the nuclei of the hepatocytes appeared enlarged and hypochromatic with prominent nucleoli. Some cells had condensed cytoplasm and nuclei. A striking feature was the abundance of fat, principally periportally (Plate 5.1). This feature was not observed in control animals.

By twenty-four hours post operation a few mitoses could be seen principally in zones 1 and 2. Other nuclear changes were similar to those seen at eighteen hours (Plate 5.2). There appeared to be some slight sinusoidal dilation centriflobularly and in zone 2 the hepatocytes showed a 'cobblestone' arrangement (Plate 5.3). Lipid deposition, preferentially in zone 1 was greater than normal but reduced compared with eighteen hours after hepatectomy.

Glycogen deposition was reduced overall and particularly in zone 1, with similar changes being observed for glucose 6-phosphatase
PLATE 5.1  Liver of rat eighteen hours after partial hepatectomy. Fat deposition in zone 1. C.v., central vein; P.t., portal tract. Unfixed frozen tissue: ORO 100X mag.

PLATE 5.2  Mitotic figure and 'empty' looking nuclei in the liver 24 hours after partial hepatectomy. H & E 400X mag.
PLATE 5.3 Liver of rat twenty-four hours after partial hepatectomy. Hepatocytes show a 'cobblestone' arrangement. Fixed tissue: H & E 100X mag.
activity i.e. reduced and shifted from a predominance in zone 1 to zone 3 (Plates 5.4, 5.5). Glutamate dehydrogenase activity was reduced in zone 1 (Plate 5.6), so that hepatectomised animals had a less even distribution than sham and non-operated control animals. Both malic and glucose 6-phosphate dehydrogenase are located preferentially in zone 3 in normal animals and this distribution was unaltered by partial hepatectomy. The activity of these enzymes was reduced compared to sham-operated controls.

The 'cobblestone' arrangement of the hepatocytes was diminished by forty-eight hours post hepatectomy although in some areas, cords could not be distinguished. Mitoses were abundant and evenly spread throughout the tissue. Non mitotic nuclei were variable in size and appearance; some were large and hypochromatic with prominent nucleoli and nuclear margin, others were smaller and hyperchromatic. Many cells contained vacuoles but lipid deposition was reduced compared to earlier time points although higher than normal.

By three days post hepatectomy bile duct (Plate 5.7) proliferation was observed and the sinusoids were dilated periportal. The hepatocytes appeared enlarged with more eosinophilic cytoplasm than control animals. Fewer mitoses could be seen and fewer altered non-mitotic nuclei were observed. Lipid deposition was restricted to a small band of hepatocytes around the portal tracts. The deposition of glycogen was reduced as compared with the sham operated animals and located predominantly in zone 3. Glucose 6-phosphatase activity was restored in the periportal regions of the lobule by this time and in fact the activity of this enzyme was similar in all the zones of the functional liver acinus. Glutamate dehydrogenase remained reduced in the periportal regions. Malic enzyme and glucose 6-phosphate dehydrogenase were both reduced in activity compared to control animals but their lobular distribution remained unaltered.

Bile duct proliferation could still be seen at five days after the operation, though to a lesser extent than at three days. Mitoses were more infrequent, though still present, and few altered non-mitotic nuclei could be seen. Lipid deposition was no greater than normal. Glycogen deposition at this time was still reduced and located preferentially in zone 3 although glucose 6-phosphatase appeared to have
PLATE 5.4  Liver of rat twenty-four hours after partial hepatectomy. Glycogen deposition is reduced in zone 1. C.v., central vein; P.t., portal tract. Unfixed frozen tissue: PAS 40X mag.

PLATE 5.5  Same tissue as Plate 5.3. Glucose 6-phosphatase is shifted from predominantly zone 1 to zone 3. 40X mag.
PLATE 5.6 Same tissue as Plate 5.3. Glutamate dehydrogenase activity in zone 1 is less than normal. 40X mag.

PLATE 5.7 Liver of rat three days after partial hepatectomy. Bile duct proliferation. Fixed tissue: H & E 40X mag.
a slightly higher activity in zone 1 compared to zone 3. The activities and lobular distribution of glutamate dehydrogenase and glucose 6-phosphate dehydrogenase were similar in partially hepatectomised animals to sham operated animals. Malic enzyme activity appeared increased in the partially hepatectomised animals.

After seven days after the operation a few mitotic figures could still be seen but the hepatocytes appeared indistinguishable from those of control animals and there was no evidence of any bile duct proliferation. The glycogen deposits were restored to normal quantity although it still appeared that the heaviest deposits were in zones 2 and 3. No significant fat accumulation could be detected. Glucose 6-phosphatase had regained its normal lobular distribution, i.e. more active in zone 1 than zones 2 and 3, and the other enzymes appeared to have normal activities and distribution.

3.5.3. Liver Growth Following Partial Hepatectomy

The change in the relative liver weight of the partially hepatectomised rats compared to sham operated control rats during the course of the experiment is shown in Fig. 3.5.1. The relative liver weight of partially hepatectomised rats two and three weeks after operation are also shown. The liver increased rapidly in size between eighteen and seventy-two hours after the operation from less than 40% to more than 80% of the sham operated control. This finding agrees well with the observation that the greatest mitotic activity was between twenty-four and seventy-two hours. The growth of the liver levelled off after 3 days so that the relative liver weight of the partially hepatectomised animals was still slightly less than normal three weeks after the operation.

3.5.4. Enzyme Changes During Liver Regeneration

The changes in enzyme activity during liver regeneration are shown in Table 3.5.1. and in Fig. 3.5.2. i - ix, where the results are expressed as normalised liver units by comparison with sham operated animals.
Figure 3.5.1 Liver Regeneration After Partial Hepatectomy

Time After Partial Hepatectomy

Relative liver weight partially hepatectomised rats

Relative liver weight sham operated rats

18 24 48 72 120

Hours

1 2 3

Weeks
### TABLE 3.5.1. Changes in Enzyme Activities After Partial Hepatectomy

<table>
<thead>
<tr>
<th></th>
<th>TIME AFTER HEPATECTOMY OR SHAM OPERATION</th>
<th></th>
<th></th>
<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>16hrs (S)</td>
<td>24hrs (H)</td>
<td>48hrs (S)</td>
<td></td>
<td></td>
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<tr>
<td>Liver weight</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
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<tr>
<td>100g body weight</td>
<td>4.79 ± 0.25</td>
<td>1.79 ± 0.11***</td>
<td>4.91 ± 0.17</td>
<td>2.05 ± 0.09***</td>
<td>5.16 ± 0.20</td>
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<tr>
<td>Thymidine kinase</td>
<td>(2)</td>
<td>(2)</td>
<td>(2)</td>
<td>(3)</td>
<td>(4)</td>
</tr>
<tr>
<td></td>
<td>0.63 ± 0.95</td>
<td>19.5 ± 28.9**</td>
<td>3.16 ± 3.08</td>
<td>27.6 ± 8.3**</td>
<td>1.82 ± 0.52</td>
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<tr>
<td>Hexokinase</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
</tr>
<tr>
<td></td>
<td>0.208 ± 0.025</td>
<td>0.308 ± 0.032</td>
<td>0.237 ± 0.031</td>
<td>0.29 ± 0.03</td>
<td>0.253 ± 0.015</td>
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<td>Glucose 6-Phosphate dehydrogenase</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
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<tr>
<td></td>
<td>1.48 ± 0.09</td>
<td>1.55 ± 0.07</td>
<td>1.96 ± 0.36</td>
<td>1.55 ± 0.09</td>
<td>2.85 ± 0.79</td>
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<td>Glutamate dehydrogenase</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
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<tr>
<td></td>
<td>69.1 ± 6.2</td>
<td>57.1 ± 4.8</td>
<td>47.4 ± 4.2</td>
<td>43.4 ± 2.8</td>
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<td>Aspartate aminotransferase</td>
<td>(4)</td>
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<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
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<tr>
<td></td>
<td>125 ± 6</td>
<td>113 ± 3</td>
<td>79.8 ± 8.4</td>
<td>77.2 ± 9.8</td>
<td>112 ± 91</td>
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<tr>
<td>Glucose 6-Phosphatase</td>
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<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
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<tr>
<td></td>
<td>21.7 ± 2.3</td>
<td>18.3 ± 2.6</td>
<td>20.5 ± 1.5</td>
<td>15.9 ± 1.1*</td>
<td>20.9 ± 1.4</td>
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<td>Phosphoenolpyruvate</td>
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<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
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<tr>
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<td>1.78 ± 0.19</td>
<td>2.99 ± 0.27*</td>
<td>1.65 ± 0.25</td>
<td>2.12 ± 0.32</td>
<td>1.19 ± 0.23</td>
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<tr>
<td>Carboxykinase</td>
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<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
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<tr>
<td></td>
<td>2.03 ± 0.17</td>
<td>1.42 ± 0.16*</td>
<td>2.08 ± 0.09</td>
<td>1.67 ± 0.08*</td>
<td>2.16 ± 0.15</td>
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<td>Glucokinase</td>
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<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
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<tr>
<td></td>
<td>2.33 ± 0.23</td>
<td>2.7 ± 0.22</td>
<td>2.28 ± 0.22</td>
<td>1.51 ± 0.13*</td>
<td>3.56 ± 1.08</td>
</tr>
</tbody>
</table>

Values are mean ± SEM for H, partially hepatectomised animals and S, sham operated controls, assayed at the same time.

Figures in parenthesis indicate number of animals studied.

Significant differences are given by:

+ p =< 0.1, * p =< 0.05, ** p =< 0.01, *** p =< 0.005
### TABLE 3.5.1: Continued

<table>
<thead>
<tr>
<th></th>
<th>TIME AFTER HEPATECTOMY OR SHAM OPERATION</th>
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<th>7 Days</th>
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<td>H</td>
<td>S</td>
<td>H</td>
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<tr>
<td>Liver weight</td>
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<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
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<tr>
<td>100g body weight</td>
<td>4.41 ± 0.07</td>
<td>3.57 ± 0.05***</td>
<td>4.56 ± 0.14</td>
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<td>Thymidine kinase</td>
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<td>(4)</td>
<td>(3)</td>
<td>(3)</td>
</tr>
<tr>
<td></td>
<td>2.22 ± 0.79</td>
<td>11.6 ± 3.6**</td>
<td>1.88 ± 0.56</td>
<td>2.57 ± 0.51</td>
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<td>Hexokinase</td>
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<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
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<tr>
<td></td>
<td>0.287 ± 0.058</td>
<td>0.583 ± 0.081*</td>
<td>0.273 ± 0.015</td>
<td>0.687 ± 0.046***</td>
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<td>Glucose 6-Phosphatase</td>
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<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
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<td>Dehydrogenase</td>
<td>1.99 ± 0.14</td>
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<td>1.8 ± 0.44</td>
<td>1.94 ± 0.21</td>
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<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
</tr>
<tr>
<td>Dehydrogenase</td>
<td>43.2 ± 2.1</td>
<td>28.9 ± 2**</td>
<td>38.2 ± 2.7</td>
<td>27.3 ± 1.3*</td>
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<td>Aspartate</td>
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<td>(4)</td>
<td>(4)</td>
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<td>Aminotransferase</td>
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<td>(4)</td>
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<tr>
<td>Phosphoenolpyruvate</td>
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<td>10.4 ± 0.3**</td>
<td>17.8 ± 2.7</td>
<td>12.0 ± 1.7</td>
</tr>
<tr>
<td>Carboxykinase</td>
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<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
</tr>
<tr>
<td></td>
<td>1.25 ± 0.08</td>
<td>0.87 ± 0.09*</td>
<td>1.11 ± 0.06</td>
<td>1.44 ± 0.15</td>
</tr>
<tr>
<td>Glucokinase</td>
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<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
</tr>
<tr>
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<td>2.01 ± 0.14</td>
<td>0.796 ± 0.12***</td>
<td>1.82 ± 0.10</td>
<td>1.25 ± 0.01***</td>
</tr>
<tr>
<td>Malic enzyme</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
</tr>
<tr>
<td></td>
<td>2.88 ± 0.23</td>
<td>2.03 ± 0.16*</td>
<td>1.89 ± 0.35</td>
<td>2.28 ± 0.37*</td>
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</tbody>
</table>

Values are mean ± SEM for H, partially hepatectomised animals and S, sham operated controls, assayed at the same time.

Figures in parenthesis indicate number of animals studied.

Significant differences are given by:

+ p = 0.1, * p = 0.05, ** p = 0.01, *** p = 0.005
Figure 3.5.2. Changes in Enzyme Activities During Liver Regeneration

Error bars represent mean ± SEM of the values converted to normalised liver units (L.U.) by comparison with sham operated controls.

Figures on abscissa represent time after partial hepatectomy.

(i) TK; Thymidine kinase
(ii) HK; Hexokinase
(iii) G6PDH; Glucose 6-phosphate dehydrogenase
(iv) GDH; Glutamate dehydrogenase
(v) AAT; Aspartate aminotransferase
(vi) G6Pase; Glucose 6-phosphatase
(vii) PEPCK; phosphoenolpyruvate carboxykinase
(viii) GK; Glucokinase
(ix) Malic; Malic enzyme
As expected from the growth curve (Fig. 3.5.1.) and the observed mitotic rate, thymidine kinase was significantly elevated for the first three days after the operation and thereafter was not significantly different from controls. DNA synthesis starts between twelve and fifteen hours post-hepatectomy and reaches a peak between twenty and twenty-four hours (Leduc, 1964) however, thymidine kinase was still elevated at forty-eight hours post-hepatectomy.

Glucose 6-phosphate dehydrogenase was elevated at eighteen hours but this was not significantly different from controls. There was a reduction at both twenty-four and forty-eight hours but again this was not significant. There was an increase, not significant, observed at the five day post operation time point. Similar changes were observed for malic enzyme except that the reduction at twenty-four, forty-eight and seventy-two hours was significant as was the increase observed at five days.

Phosphoenolpyruvate carboxykinase activity was significantly increased at eighteen hours and glucokinase was significantly decreased at this time. This, together with the observation that glucose 6-phosphatase and hexokinase activities were not significantly different from control values, reflects the increased gluconeogenic, decreased glycolytic capacity of the liver remnant for the maintenance of constant blood sugar levels. Phosphoenolpyruvate carboxykinase remained elevated, though this was not significant, at all other time points except for the three day interval where it was significantly reduced. Glucose 6-phosphatase was also at its minimum value at three days and so was glucokinase but hexokinase was elevated significantly. Glucose 6-phosphatase decreased rapidly during the mitotic phase of liver regeneration but levels were restored to near normal between five and seven days after the operation.

There was a gradual increase in hexokinase activity for the first five days post hepatectomy which was significant from forty-eight hours until the maximum point at five days. By seven days the activity of hexokinase, like all the other enzymes studied was not significantly different from control animals.

The changes in activity, during regeneration, of aspartate aminotransferase and glutamate dehydrogenase were similar. An initial
decrease of activity at eighteen hours was partially restored at twenty-four hours this was followed by a further period of decreased activity between forty-eight hours and five days post operation, near normal values being achieved one week after the operation. This correlates well with the finding that urea output by the liver is diminished after partial hepatectomy (Leduc, 1964).

3.5.5. Statistical Analysis of the Enzyme Data

So that comparison of the regenerating liver with the liver during development and carcinogenesis could be made Spearman's Rank Correlation analysis was applied and the results are shown in Table 3.5.2.

Up to, and including forty-eight hours after hepatectomy the liver showed significant correlation with the liver of the newborn animal and the five day post partum animal. In addition, significant correlation with the foetal liver was shown by regenerating liver at forty-eight hours and three days after hepatectomy. At three days after operation the association with foetal liver was the only significant association with development. At twenty-four and forty-eight hours, the regenerating liver was significantly correlated with the liver at both ten and fifteen days post partum. No significant correlation with any stage of development was shown by the regenerating liver five days after the operation, but on the seventh post operative day the correlation with the liver of the five-day old rat was significant.

It seems that after hepatectomy during the early part of the phase of rapid growth the liver bears a distinct similarity with the liver of the perinatal animal and towards the end of this phase the similarity with foetal liver becomes more pronounced. Then, during the slow growing phase the pattern of enzyme activity resembles that of the liver of the five-day old animal again.

The liver three days after partial hepatectomy showed a significant correlation with the non-tumourous portions of liver of the animals with large liver tumours produced by phenobarbitone promotion of diethylnitrosamine-induction. Regenerating liver at eighteen hours, forty-eight hours, five and seven days after the operation showed significant correlation with the tumours of the two-stage carcinogenesis
**TABLE 3.5.2** Spearman's Rank Correlation Analysis of Enzyme Patterns During Liver Regeneration and During Development and Carcinogenesis

<table>
<thead>
<tr>
<th>Enzyme Pattern</th>
<th>Time After Hepatectomy</th>
<th>Statistic Significance</th>
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<tr>
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<td>Foetal</td>
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<td>5 days pp</td>
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<td>10 days pp</td>
<td>.6667</td>
<td>2.366*</td>
</tr>
<tr>
<td>15 days pp</td>
<td>.4833</td>
<td>1.461</td>
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<tr>
<td>Weanling</td>
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<td>1.396</td>
</tr>
<tr>
<td>Chronic DEN</td>
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<td></td>
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<tr>
<td>2 weeks</td>
<td>.6458</td>
<td>2.238*</td>
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<tr>
<td>4 weeks</td>
<td>.9167</td>
<td>6.068***</td>
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<td>6 weeks</td>
<td>.6375</td>
<td>2.189*</td>
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<tr>
<td>8 weeks</td>
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<td>1.396</td>
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<td>10 weeks</td>
<td>.5167</td>
<td>1.597</td>
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<tr>
<td>11 weeks</td>
<td>.5667</td>
<td>1.82</td>
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Statistical significance is given by: *, p<0.05; **, p<0.01; ***, p<0.005.
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<th>18 hrs</th>
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<th>48 hrs</th>
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<th>5 days</th>
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Statistical significance is given by: + p < 0.1; * p < 0.05; ** p < 0.01; *** p < 0.005.
experiment. Similarity in enzyme pattern between regenerating liver and during two-stage hepatocarcinogenesis was observed only occasionally viz: regenerating liver at five and seven days after the operation was similar to the liver of animals during the two-stage carcinogenesis experiment at twenty-four weeks and the correlation between the liver during two-stage carcinogenesis at thirty-two weeks and the liver eighteen hours after partial hepatectomy was significant.

No significant correlations were seen in the enzyme pattern of regenerating liver and the liver during chronic phenobarbitone feeding.

There was a significant correlation observed between the liver at twenty-four and forty-eight hours post hepatectomy and the group 2 animals (those which had received diethylnitrosamine for one week but no further treatment) at twenty-four and thirty-two weeks.
3.6. Transplantable Tumours and the Host Liver of Tumour-Bearing Animals

3.6.1 Experimental Protocol

In the chronic diethylnitrosamine feeding study there is the possibility that some of the changes observed may reflect a non-specific toxic effect of the carcinogenic changes alone. In order to investigate this problem and further investigate the foetal aspects of carcinogenesis, two transplantable tumours of different growth rates were studied. The host livers of tumour-bearing rats were also studied as there has been some suggestion that the tumour will induce dedifferentiation in the host liver of tumour-bearing rats (Introduction Section 1.3.4).

3.6.2. Histological Examination of the Tumours and the Host Livers

a) Rapidly-growing Tumour (UA)

The cells of this tumour were small and basophilic with a high nuclear/cytoplasmic ratio (Plate 6.1.). In some areas close to the blood vessels the cells appeared to be arranged in cords, in other areas the cells were arranged in islands and elsewhere the arrangement of the cells was amorphous with indistinct cellular junctions. Virtually no endothelial tissue could be seen even near the blood vessels. Further away from the blood vessels large necrotic areas were apparent. The majority of nuclei were very hypochromatic with very hyperchromatic margins and nucleoli giving them an 'empty' appearance as described earlier, this 'empty' look was very much exaggerated in some of the cells (Plate 6.2). Some of the nuclei were very large and numerous mitoses could be seen particularly around the periphery of the tumour. There was no glycogen detectable in the tissue and lipid deposition was mostly restricted to a few areas of necrosing cells.
PLATE 6.1 UA tumour, three weeks post-implantation. Small basophilic cells with high nuclear/cytoplasmic ratio. Fixed tissue: H & E 400X mag.

PLATE 6.2 UA tumour, three weeks post-implantation. 'Empty' looking nuclei. Fixed tissue: H & E/000X mag.
b) Slow-growing Tumour (WDA)

The cytoplasmic eosinophilia/basophilia was similar to normal hepatocytes and although the nuclear/cytoplasmic ratio was slightly greater than normal this was not so pronounced as in the UA tumour. Again the nuclei had a strikingly 'empty' appearance (Plate 6.3). There were many mitoses, some were abnormal. The cytoplasm of some cells was vacuolated varying from very fine vacuolation to large fatty vacuoles.

The cells mostly showed an amorphous arrangement (Plate 6.4) though in some areas a trabecular arrangement was apparent. Kupffer cells could not be seen.

Glycogen was not detectable in the tumour though some PAS positive material, probably mucopolysaccharides, was found near some blood vessels. Lipid droplets were apparent not only in necrotic areas but also in cells adjacent to the blood vessels and also some other areas (Plate 6.5).

c) Host Livers of Tumour-bearing Rats

There was no detectable difference in the histology of the host livers of rats, bearing either type of tumour, from control animals. There seemed to be a reduction in glycogen content in the livers of rats bearing the UA tumour with a focus of glycogen deficient cells seen in one animal (Plate 6.6). Lipid deposition was normal in both the host liver of the UA tumour-bearing rat and the host liver of the WDA tumour-bearing rat.

3.6.3. Enzyme Profiles of the Tumours and Host Livers

a) UA Tumour and the Host Liver of the UA Tumour-bearing Rat

The tumours were studied at the end of the third week after implantation, when the tumour is very large (about three times as large as the liver) and ready for transplantation. They were also studied two weeks after implantation when they are about 25% of their
PLATE 6.3  WDA tumour four weeks post-implantation. 'Empty' looking nuclei and fat vacuoles. Fixed tissue: H & E 400X mag.

PLATE 6.4  WDA tumour four weeks post-implantation. Hepatocytes in an amorphous arrangement. Fixed tissue: H & E 100X mag.
PLATE 6.5  WDA tumour four weeks post-implantation. Lipid deposition in non-necrotic area. Fixed tissue: ORO 40X mag.

PLATE 6.6  Focus of glycogen deficient hepatocytes in the liver of an animal bearing the UA tumour three weeks post-implantation. Fixed tissue: PAS 40X mag.
size at three weeks post-implantation. The host livers of the tumour-bearing rats were also studied at these two time points. The results of the enzymatic analysis are shown in Table 3.6.1.

The host liver of the animal bearing a tumour two weeks post-implantation shows no significant difference in enzyme activity, except for malic enzyme which is significantly reduced, compared to control livers. After the third week after implantation of the tumour the host liver shows a significant reduction in glucose 6-phosphate dehydrogenase and malic enzyme at the 95% level and an increase in thymidine kinase significant at the 90% level. No significant differences are seen with the other enzymes.

The relative liver weight is significantly reduced in the host animals compared to control animals at both two and three weeks post-implantation.

As expected, the tumour tissue showed significant alteration, in all the enzymes, compared with control liver, and the changes were of similar type both two and three weeks post-implantation. The magnitude of change was on the whole larger three weeks post-implantation than two weeks post-implantation.

Thymidine kinase, glucose 6-phosphate dehydrogenase and hexokinase were significantly elevated in the tumour tissue and all the other enzymes were significantly reduced compared with normal liver.

b) WDA Tumour and the Host Livers of the WDA Tumour-bearing Rat

The tumours and host livers were examined four weeks post-implantation when the tumour size was about 150% of the host liver. The results of the enzymatic analysis of the tissue are shown in Table 3.6.2.

In the host liver of the tumour-bearing rats there was a reduction in malic enzyme and in liver weight, all other changes were not significant. In the tumour tissue there were significant increases in thymidine kinase, hexokinase and glucose 6-phosphate dehydrogenase and reductions in the activities of the other enzymes measured except malic enzyme which was not significantly different from controls.
### TABLE 3.6.1. Enzyme Activities in UA Tumours and Host Livers at Two and Three Weeks Post Implantation

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<thead>
<tr>
<th>Enzyme Activities</th>
<th>HOST LIVER</th>
<th>TUMOUR TISSUE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Liver Values</td>
<td>2 weeks post-implantation (h)</td>
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<tr>
<td>Thymidine kinase U/g tissue</td>
<td>3.11 ± 0.9</td>
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<tr>
<td>Hexokinase U/g tissue</td>
<td>0.233 ± 0.012</td>
<td>0.253 ± 0.013</td>
</tr>
<tr>
<td>Glucose 6-Phosphate Dehydrogenase U/g tissue</td>
<td>1.31 ± 0.12</td>
<td>1.35 ± 0.2</td>
</tr>
<tr>
<td>Glutamate Dehydrogenase U/g tissue</td>
<td>36.2 ± 5.1</td>
<td>41.8 ± 1.5</td>
</tr>
<tr>
<td>Aspartate Aminotransaminase U/g tissue</td>
<td>103 ± 9</td>
<td>110 ± 2</td>
</tr>
<tr>
<td>Glucose 6-Phosphatase U/g tissue</td>
<td>17.3 ± 1.2</td>
<td>14.0 ± 0.9</td>
</tr>
<tr>
<td>Phosphoenolpyruvate Carboxykinase U/g tissue</td>
<td>1.83 ± 0.06</td>
<td>2.46 ± 0.58</td>
</tr>
<tr>
<td>Glucokinase U/g tissue</td>
<td>1.68 ± 0.05</td>
<td>1.68 ± 0.17</td>
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<tr>
<td>Malo Enzyme U/g tissue</td>
<td>1.78 ± 0.14</td>
<td>1.14 ± 0.2*</td>
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<tr>
<td>Liver weight 100g body weight</td>
<td>4.8 ± 0.09</td>
<td>4.34 ± 0.09*</td>
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Values are mean ± SEM. Figures in parenthesis indicate number of observations.
Statistical significance is given by: *, p < 0.1; **, p < 0.05; ***, p < 0.01; ****, p < 0.001
### TABLE 3.6.2. Enzymes in WDA Tumours and Host Livers

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<thead>
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<th>Enzyme</th>
<th>Control Liver (2)</th>
<th>Host Liver (4)</th>
<th>Tumour Tissue (4)</th>
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<td>Thymidine kinase</td>
<td>0.61; 3.17</td>
<td>0.934 ± 0.305</td>
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<td>Hexokinase</td>
<td>0.235; 0.265</td>
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<td>Glutamate Dehydrogenase</td>
<td>31.4; 45</td>
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<td>Aspartate Aminotransferase</td>
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<tr>
<td>Glucose 6-Phosphatase</td>
<td>18.2; 21.2</td>
<td>15.5 ± 2.2</td>
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<td>Phosphoenolpyruvate Carboxykinase</td>
<td>0.827; 0.989</td>
<td>0.919 ± 0.034</td>
<td>0.131 ± 0.016***</td>
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<td>Glucokinase</td>
<td>1.42; 1.86</td>
<td>1.53 ± 0.09</td>
<td>0.047 ± 0.015***</td>
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<td>Malic enzyme</td>
<td>2.51; 2.75</td>
<td>1.05 ± 0.13***</td>
<td>2.26 ± 0.28</td>
</tr>
<tr>
<td>Liver weight/100g body weight</td>
<td>5.16; 5.54</td>
<td>4.06 ± 0.10**</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SEM U/g liver. Figures in parenthesis indicate number of observations. Statistically significant differences from control animals are given by:

+ p < 0.1, * p < 0.05, ** p < 0.01, *** p < 0.005
3.6.4. Analysis of the Enzyme Data by Spearman's Rank Correlation

The enzyme activities were converted to normalised liver units by comparison with control animals and analysed by Spearman's Rank Correlation as described previously. The results of the analysis are shown in Table 3.6.3. The tumours show significant correlation with foetal tissue, but no other stage of development, with the livers of animals fed with diethylnitrosamine for eleven weeks and the tumours produced by phenobarbitone promotion of diethylnitrosamine carcinogenesis. Of the two transplantable tumours WDA, the slow growing tumour, showed the most significant correlation with the primary tumours induced by diethylnitrosamine. The transplantable tumours, especially UA, also showed significant correlation with regenerating liver after partial hepatectomy. The host liver of the WDA tumour-bearing rat was correlated with non-hepatoma areas of liver of rats bearing hepatomas induced by the two-stage carcinogenesis experiment but this was significant only at the 90% level. The host liver did not correlate with the other tissues studied. The host liver of the UA tumour-bearing rat correlated with the weanling animal liver at the 95% level, and foetal liver at the 90% level but did not correlate significantly with any of the other tissues, including the non-tumour liver of animals with primary hepatomas.

3.6.5. Changes in Enzyme Activity in the Liver During Pregnancy

Reports that transplantable tumours induce enzyme dedifferentiation in the host liver and the observation that some dedifferentiation was induced in the liver of the animal carrying the fast-growing tumour led to the speculation that an analogous situation might be the maternal liver during pregnancy. To investigate this possibility livers of 20-day pregnant females were assayed and the enzyme activities compared with non-pregnant females as shown in Table 3.6.4.

The pregnant females although showing differences in hepatic enzyme activity compared to controls do not exhibit a dedifferentiated enzyme pattern. This was confirmed by rank correlation analysis of the data (Table 3.6.5). No significant correlation was seen with the host
TABLE 3.6.3. Spearman's Rank Correlation Analysis of the Enzyme Patterns of Transplantable Hepatomas and Host Liver Compared with the Enzyme Patterns of Liver During Development, Regeneration and Primary Hepatocarcinogenesis

<table>
<thead>
<tr>
<th>Compared Tissue</th>
<th>UA Host Liver</th>
<th>WDA Host Liver</th>
<th>UA Tumour</th>
<th>WDA Tumour</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rs t</td>
<td>Rs t</td>
<td>Rs t</td>
<td>Rs t</td>
</tr>
<tr>
<td>Foetal</td>
<td>0.6375 *</td>
<td>2.189 *</td>
<td>0.2342</td>
<td>0.695</td>
</tr>
<tr>
<td></td>
<td>0.7042</td>
<td>2.624 *</td>
<td>0.7292</td>
<td>2.815 *</td>
</tr>
<tr>
<td>Newborn</td>
<td>0.4542</td>
<td>1.349</td>
<td>0.1542</td>
<td>0.413</td>
</tr>
<tr>
<td></td>
<td>0.5272</td>
<td>1.65</td>
<td>0.3125</td>
<td>0.870</td>
</tr>
<tr>
<td>5 Day old</td>
<td>0.5667</td>
<td>1.82</td>
<td>0.1633</td>
<td>0.493</td>
</tr>
<tr>
<td></td>
<td>0.458</td>
<td>1.724</td>
<td>0.3167</td>
<td>0.883</td>
</tr>
<tr>
<td>10 Day old</td>
<td>0.5667</td>
<td>1.82</td>
<td>0.3667</td>
<td>1.056</td>
</tr>
<tr>
<td></td>
<td>0.0667</td>
<td>0.177</td>
<td>0.167</td>
<td>0.311</td>
</tr>
<tr>
<td>15 Day old</td>
<td>0.55</td>
<td>1.72</td>
<td>1.333</td>
<td>0.356</td>
</tr>
<tr>
<td></td>
<td>0.392</td>
<td>1.002</td>
<td>0.2</td>
<td>0.54</td>
</tr>
<tr>
<td>Weanling</td>
<td>0.7</td>
<td>2.59 *</td>
<td>0.2</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td>0.592</td>
<td>1.761</td>
<td>0.167</td>
<td>0.311</td>
</tr>
<tr>
<td>E1W 11 Weeks</td>
<td>0.0833</td>
<td>0.221</td>
<td>-0.2</td>
<td>-0.54</td>
</tr>
<tr>
<td></td>
<td>0.7875</td>
<td>3.381 **</td>
<td>0.9833</td>
<td>14.31 ***</td>
</tr>
<tr>
<td>Host Liver in Group 1 rats</td>
<td>-0.2</td>
<td>-0.54</td>
<td>0.55</td>
<td>1.742</td>
</tr>
<tr>
<td></td>
<td>-0.0208</td>
<td>-0.055</td>
<td>-0.1833</td>
<td>-0.493</td>
</tr>
<tr>
<td>Tumour in Group 1 rats</td>
<td>-0.0167</td>
<td>-0.054</td>
<td>-0.2667</td>
<td>-0.732</td>
</tr>
<tr>
<td></td>
<td>0.7542</td>
<td>3.033 *</td>
<td>0.8167</td>
<td>3.794 **</td>
</tr>
</tbody>
</table>

Regenerating Liver

<table>
<thead>
<tr>
<th></th>
<th>UA Host Liver</th>
<th>WDA Host Liver</th>
<th>UA Tumour</th>
<th>WDA Tumour</th>
</tr>
</thead>
<tbody>
<tr>
<td>15h p.o.</td>
<td>0.15</td>
<td>0.401</td>
<td>0.0333</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>0.8292</td>
<td>3.624 **</td>
<td>0.6167</td>
<td>2.073 +</td>
</tr>
<tr>
<td>24h p.o.</td>
<td>0.5667</td>
<td>1.82</td>
<td>0.3501</td>
<td>0.989</td>
</tr>
<tr>
<td></td>
<td>0.6625</td>
<td>2.74 +</td>
<td>0.2833</td>
<td>0.782</td>
</tr>
<tr>
<td>48h p.o.</td>
<td>0.2893</td>
<td>0.782</td>
<td>0.2333</td>
<td>0.635</td>
</tr>
<tr>
<td></td>
<td>0.8958</td>
<td>5.333 ***</td>
<td>0.6893</td>
<td>2.476 *</td>
</tr>
<tr>
<td>3 Day p.o.</td>
<td>0.333</td>
<td>0.935</td>
<td>-0.0873</td>
<td>-0.221</td>
</tr>
<tr>
<td></td>
<td>0.9208</td>
<td>6.288 ***</td>
<td>0.95</td>
<td>8.05 ***</td>
</tr>
<tr>
<td>5 Day p.o.</td>
<td>0.0625</td>
<td>0.166</td>
<td>0.0125</td>
<td>0.033</td>
</tr>
<tr>
<td></td>
<td>0.8458</td>
<td>4.195 **</td>
<td>0.7125</td>
<td>2.687 *</td>
</tr>
<tr>
<td>7 Day p.o.</td>
<td>0.1333</td>
<td>0.356</td>
<td>0.2333</td>
<td>0.653</td>
</tr>
<tr>
<td></td>
<td>0.7875</td>
<td>3.381 *</td>
<td>0.5833</td>
<td>1.9 +</td>
</tr>
</tbody>
</table>

Statistical significance is given by: + p < 0.1, * p < 0.05, ** p < 0.01, *** p < 0.005
<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>Control</th>
<th>Pregnant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexokinase</td>
<td>0.25 ± 0.04</td>
<td>0.33 ± 0.04</td>
</tr>
<tr>
<td>Glucose 6-Phosphate Dehydrogenase</td>
<td>4.23 ± 0.53</td>
<td>2.98 ± 0.44+</td>
</tr>
<tr>
<td>Glutamate Dehydrogenase</td>
<td>49.4 ± 1.6</td>
<td>37.8 ± 5.3</td>
</tr>
<tr>
<td>Aspartate Aminotransferase</td>
<td>93.4 ± 2.0</td>
<td>122 ± 6**</td>
</tr>
<tr>
<td>Glucose 6-Phosphatase</td>
<td>13.3 ± 0.5</td>
<td>8.1 ± 0.3***</td>
</tr>
<tr>
<td>Glucokinase</td>
<td>1.78 ± 0.21</td>
<td>1.90 ± 0.34</td>
</tr>
<tr>
<td>Malic enzyme</td>
<td>2.56 ± 0.21</td>
<td>2.52 ± 0.72</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM of 4 observations in U/g tissue

Statistically significant differences are given by:

+ $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$
TABLE 3.6.5. Spearman's Rank Correlation Analysis of the Enzyme Pattern of Pregnant Female Liver with Other Tissues

\[ df = 5 \]

<table>
<thead>
<tr>
<th>Compared Tissue</th>
<th>Liver of Pregnant Female</th>
<th>( R_s )</th>
<th>( t )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foetal</td>
<td>0.2054</td>
<td>0.697</td>
<td></td>
</tr>
<tr>
<td>Neonatal</td>
<td>-0.2232</td>
<td>-0.512</td>
<td></td>
</tr>
<tr>
<td>5 days post partum</td>
<td>0.1429</td>
<td>0.323</td>
<td></td>
</tr>
<tr>
<td>10 &quot; &quot; &quot;</td>
<td>0.1071</td>
<td>0.241</td>
<td></td>
</tr>
<tr>
<td>15 &quot; &quot; &quot;</td>
<td>-0.0714</td>
<td>-0.160</td>
<td></td>
</tr>
<tr>
<td>Weanling</td>
<td>0.4911</td>
<td>1.261</td>
<td></td>
</tr>
<tr>
<td>Host liver of UA tumour-bearing rat</td>
<td>0.5</td>
<td></td>
<td>1.291</td>
</tr>
<tr>
<td>&quot; &quot; &quot; WDA &quot; &quot; &quot; &quot;</td>
<td>0.5357</td>
<td>1.419</td>
<td></td>
</tr>
<tr>
<td>Regenerating liver</td>
<td>18 hours</td>
<td>0.3929</td>
<td>0.955</td>
</tr>
<tr>
<td>24 &quot;</td>
<td>0.7143</td>
<td>2.282+</td>
<td></td>
</tr>
<tr>
<td>48 &quot;</td>
<td>0.3571</td>
<td>0.855</td>
<td></td>
</tr>
<tr>
<td>3 days</td>
<td>0.3929</td>
<td>0.955</td>
<td></td>
</tr>
<tr>
<td>5 &quot;</td>
<td>0.3661</td>
<td>0.880</td>
<td></td>
</tr>
<tr>
<td>7 &quot;</td>
<td>0.5357</td>
<td>1.419</td>
<td></td>
</tr>
</tbody>
</table>

Statistical significance is given by: \(+ p < 0.1\), \(-* p < 0.05\)
liver of the tumour-bearing rats, nor regenerating liver after partial hepatectomy beyond the 90% level.

The correlation with regenerating liver twenty-four hours after operation may be due to the fact that the liver of the pregnant female is a growing tissue.
CHAPTER 4

DISCUSSION
DISCUSSION

4.1. Comparison of Developing and Regenerating Rat Liver, Rat Liver During Chronic Diethylnitrosamine-induced Hepatocarcinogenesis and Transplantable Rat Hepatocellular Carcinomas

4.1.1. Histology and Histochemistry

The histological appearance of adult rat liver as described in this thesis (Section 3.1.1. a) agrees closely with what is already well-documented (Elies, 1963; Bloom and Fawcett, 1975) and will not be discussed further. The lobular localisation of lipids, glycogen and the enzymes assayed histochemically (Section 3.1.1. a) also agrees well with the observations of others (Rappaport, 1963; Wachstein, 1963; Jungermann and Sasse, 1978; Rieder et al., 1978; Teutsch, 1978; Wilson, 1978).

Most of the changes observed during foetal and postnatal development of rat liver reported here (Section 3.1.1b,c) have also been recorded by other workers for various mammalian species. These changes include: the gradual decrease in haematopoietic tissue during late foetal and early neonatal periods (Jézéquel et al., 1965; Greengard et al., 1972); the gradual development of lobular architecture (McKellar, 1949; Wilson et al., 1963); changes in cell-size, nuclear/cytoplasmic ratios and ploidy (McKellar, 1949; Doljanski, 1960; Jézéquel et al., 1965) changes in liver weight relative to body weight and the mitotic activity of the hepatocytes (McKellar, 1949; Rohr et al., 1971; Leeson and Cutts, 1972) the build-up of glycogen stores in late foetal liver, their rapid decline immediately after birth and subsequent reaccumulation at weaning (Deane, 1944; Shelley, 1961; DuBois, 1963; Jézéquel et al., 1965; Phillips et al., 1967; Chiu and Phillips, 1974), this sequence agrees closely with biochemical measurements of hepatic glycogen (Snell and Walker, 1973).

a) Architectural Arrangement of Hepatocytes

In regenerating liver after partial hepatectomy the earliest changes
observed were glycogen depletion and pronounced lipid accumulation in the periportal region (Section 3.5.2.) as reported by others as a result of post-operative stress (for refs. see Brinkmann et al., 1978). Mitoses first appeared periportal then spread throughout the lobule (Section 3.5.2.; Harkness, 1952; Rabes et al., 1975). The normal liver architecture of the adult was lost and the hepatocytes assumed a 'cobblestone' appearance (Plate 5.2). In this respect the architectural arrangement became more 'immature'.

The architectural arrangement of the hepatocytes in the pre-neoplastic nodules often appeared different from that observed in the resulting tumour. In the preneoplastic nodule the cells were arranged amorphously (Plates 2.11, 2.18) as seen also in the embryonic liver, whereas frequently the tumours exhibited a two-cell thick trabecular arrangement (Plate 2.24) reminiscent of the late foetal or neonatal liver. The hepatoma cells themselves were similar to the late foetal or neonatal hepatocyte in terms of acidophilia in most cases (Plates 2.18, 2.22, 2.26, 1.15, 1.17) but in others they were more basophilic and similar to the early foetal hepatocyte (Plate 2.24, 1.12).

The two transplantable hepatomas studied also exhibited abnormal architecture. The slower growing transplantable tumour (WDA) appeared similar to the primary diethylnitrosamine-induced tumour in terms of acidophilia, cell-size and differentiated appearance (Plates 2.26, 6.3, 6.4). The rapidly growing tumour (UA) was characterised by smaller basophilic cells and resembled the early foetal hepatocytes to a greater extent (Plates 6.1, 1.12).

b) Biliary Hyperplasia

Little mention is made in the literature of bile duct proliferation or increasing periportal vasculature during development as observed in the present work (Section 3.1.1. c; Plates 1.24, 1.25). Jézéquel et al. (1965) observed that bile ducts were seen only rarely in the first twenty-four hours of post-natal life of the mouse. McKellar (1949) observed an increase in the number as well as size of the lobules in the postnatal period and suggested that this was brought about by the branching of the central veins and portal tracts. Similarly, Wilson
et al. (1963) suggested that the development of venous channels and the sinusoidal network by the expansion of vascular elements was associated with an increase in the number of lobules postnatally. Since the number of lobules increases postnatally, the demand for increased vasculature to these new lobules is the most likely explanation for the increase in vascular elements periportally. This would seem to be supported by the observed bile duct proliferation in regenerating liver after partial hepatectomy (Plate 5.6). In this situation, there is an increase in the number as well as size of the lobules, as the liver mass is restored, and this presumably stimulates the proliferation of vascular tissue. In the case of the bile ducts, the proliferative response seems greater than might be expected, resulting in the multiplication of bile ducts beyond that required for new lobules. However, it appears that hepatocytes can differentiate into bile duct epithelium in the foetal mouse liver (Wilson et al., 1963) and that 'oval' cells, which are derived from bile duct cells, may proliferate (in some carcinogenesis regimes) and differentiate into hepatocytes (Williams, 1980). Thus, it may be that the two cell-types may be interconvertible under appropriate conditions and bile duct proliferation may be one aspect of the growth response to specific stimuli. Biliary hyperplasia was also observed during tumour induction (Plate 2.24). The role of biliary hyperplasia in conditions of normal and abnormal liver growth warrants further investigation before conclusions regarding the significance of this feature can be made.

Certain architectural features (viz. lack of normal hepatocyte arrangement; biliary hyperplasia) are common to immature, regenerating and neoplastic rat liver. Whereas in regenerating liver there seems to be a recapitulation of developmental growth i.e. that first there is the lack of arrangement of the hepatocytes into cords, which is typical of the foetal and early neonatal periods. This sequence of events does not seem to take place during chronic diethylnitrosamine-induced hepatocarcinogenesis.

c) Nuclear Changes

The nuclei of the hepatocytes of immature, regenerating and
(pre-)neoplastic (including transplantable hepatomas) rat liver also appear similar in that there is a peripheralisation of the chromatin and the nucleoli are prominent (Sections 3.1.1, b, 3.5.2, 3.2.2, 3.6.2. a,b). This is most marked in the (pre-)neoplastic hepatocytes. The nuclear changes which result in such 'empty' looking or vesicular nuclei are characteristic of well-differentiated hepatocellular carcinomas and have been observed by many authors (Jones and Butler, 1978; Ward et al., 1979; Emmelot and Scherer, 1980). Similar observations have been made by Farber (1976) who suggests that these nuclear changes precede the reactivation of quiescent cells and that preneoplastic cells, like foetal hepatocytes, have a greater number of options for different biological behaviour than are open to the mature hepatocyte.

Other histological and ultrastructural similarities between foetal, neonatal, regenerating and (pre-)neoplastic hepatocytes have been reported (Bruni, 1973; Ogawa et al., 1979a; 1979b).

d) Lipid Accumulation

Lipid accumulation is another feature common to the developing hepatocytes, regenerating hepatocytes and preneoplastic hepatocytes during diethylnitrosamine hepatocarcinogenesis (Plates 1.13, 1.19, 5.1, 2.14, 2.19). In developing rat liver the lipid is thought to be an energy store whereas in regenerating liver its presence is a result of stress, the fat having been released by adipose tissue. The observation of lipid accumulation in the preneoplastic hepatocytes and early hepatocellular carcinomas but not in the more advanced tumours induced by diethylnitrosamine agrees well with the observation of transient fat accumulation during nitrosomorpholine-induced hepatocarcinogenesis (Bannasch, 1968). Lipid accumulation was observed in some areas of the WDA tumour (Plate 6.5) but there was an absence of fat in the rapidly growing UA tumour. It has been suggested that whereas the more dedifferentiated hepatocellular carcinomas have lost the capacity for both synthesis and export of fat the more differentiated tumours have only lost the export capability and thus may accumulate fat (Peters, 1976). Increased neutral lipid content (usually in association with decreased phospholipid content) has been observed in
some hepatomas (Hruban et al., 1972; Araki and Okazaki, 1976; Reuber, 1977; Mizejewski et al., 1979). Decreased lipid content has been observed in other hepatomas (Ruggieri and Fallani, 1979) and the suggestion has been made that lipid accumulation is of no direct relevance (Steele and Jenkin, 1974). However, defective control of cholesterol and fatty acid metabolism is a common feature in hepatomas (Majerus et al., 1968; Sabine et al., 1968; Bricker and Levey, 1972) and dietary fat influences both the acute toxicity and carcinogenic potential of aflatoxin B₁ (Newberne et al., 1979). Human hepatocellular carcinoma is also associated with elevated liver lipid levels (Araki and Okazaki, 1976; Newberne et al., 1979). Choline deficiency, which induces fatty liver, also enhances hepatocarcinogenesis (Rogers and Newberne, 1969; Sells et al., 1979) but the preneoplastic foci have reduced fat content compared to the surrounding tissue. Clofibrate and nafenopin, hypolipidemic drugs, also enhance carcinogenesis in various tissues, including the liver, but this may not be via an effect on the lipid content of the tissues (Svoboda and Azarnoff, 1979). Lavletes and Coleman (1980) have proposed that the loss of control of cholesterol and lipid synthesis constitutes a drain from the Krebs cycle thereby activating glycolysis thus accounting for the higher glycolytic rate of tumours. There also appears to be a correlation between the ability of tumours to resist attack by the immune system and the ability to synthesise lipid (Schlager et al., 1978), although the reasons for this are far from clear.

In the work presented in this thesis, where neutral fat is seen in developing, regenerating and (pre-)neoplastic hepatocytes there seems to be some suggestion that the presence of fat in these cells is linked to their mitotic capacity especially as the fat accumulation in regenerating liver is periportally located, i.e. where cell multiplication is initiated.

There is also some suggestion from the literature that lipid accumulation after partial hepatectomy is related to cell division. An infusion of glucose and insulin which inhibits the release of fatty acids from adipose tissue and prevents their accumulation in the liver, also inhibits the regeneration of hepatic tissue after partial hepatectomy (Simek et al., 1967). A mixture of amino acids and hormones
which increases the supply of fatty acids to the liver in the intact animal also induces a similar cellular response, including DNA synthesis, to that induced by 70% partial hepatectomy (Short et al., 1972). Furthermore, in cross-circulation experiments between partially hepatectomised and intact animals, there is a correlation between circulating free fatty acid concentrations and the effectiveness of the blood from the operated animals to stimulate DNA synthesis (Short et al., 1972).

On the other hand, Leiberman (1969) found that lipid accumulation and glycogen loss, but not DNA synthesis, in regenerating liver after partial hepatectomy, were inhibited by intravenous glucose infusion suggesting the stress-related nature of lipid accumulation. Nevertheless this does not rule out the possibility that some changes in lipid or carbohydrate metabolism are obligatory for the passage of the liver cell through the pre-replicative phase, but that these changes are not essential for DNA synthesis. Furthermore, lipidic microvacuoles are often found in basophilic liver cells during viral hepatitis, and are considered a characteristic sign of regeneration (Desmet, 1978).

It would be interesting and worthwhile to pursue further the observation of lipid accumulation in developing, regenerating and (pre-)neoplastic hepatocytes with a view to delineating the precise role of fat accumulation in these hyperplastic conditions and the role of lipid metabolism in normal and malignant hyperplasia. It is noteworthy in this connection that diaminopropanol ('a specific inhibitor of liver polyamine biosynthesis) blocks regenerative growth and fatty acid biosynthesis after partial hepatectomy in rats (K. Snell, personal communication of unpublished work of M. Brosnan). Polyamines may therefore provide the common metabolic link between these metabolic processes.

It appears therefore that there are a number of histological features common to immature, regenerating and (pre-)neoplastic hepatocytes as summarised in Table 4.1.

e) Differences Between Centrilobular and Periportal Hepatocytes

During liver regeneration there is a loss of glycogen and glucose 6-phosphatase activity from portal areas of the lobule, where they are
<table>
<thead>
<tr>
<th>Feature</th>
<th>Foetal liver</th>
<th>Neonatal liver</th>
<th>Regenerating liver</th>
<th>Pre-neoplastic liver</th>
<th>Primary Hepatocellular Carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amorphous arrangement of hepatocytes</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Multicell trabeculae</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Biliary hyperplasia</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glycogen loss</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lipid accumulation</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+ and -</td>
</tr>
<tr>
<td>'Empty' looking nuclei</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Increased mitoses</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Feature observed, +; Not observed, -. 

**TABLE 4.1** Histological Features Common to Developing, Regenerating and (Pre-)Neoplastic Hepatocytes
normally concentrated (Plates 5.3, 5.4, 1.4, 1.7). Brinkmann et al., (1978) observed a similar shift in glucose 6-phosphatase activity but, although observing a reduction in glycogen, this still retained its predominance periportally. The other enzymes assayed histochemically i.e. glutamate dehydrogenase, glucose 6-phosphate dehydrogenase and malic enzyme, are normally located centrilobularly and this zonation was not affected by partial hepatectomy (Section 3.5.2). Other enzymes also show differing activities in different parts of the liver lobule (Wachstein, 1963; Jungermann and Sasse, 1978). No such metabolic zonation is seen in the foetal or neonatal liver (Brinkmann et al., 1978).

Not only are periportal hepatocytes the first to replicate after partial hepatectomy (Harkness, 1952) but also cross-circulation experiments between partially hepatectomised and intact animals results in greater DNA synthesis in the periportal hepatocytes in the liver of the intact animals (Bücher et al., 1969). Poisoning with CCl₄ results in centrilobular necrosis but the compensatory hyperplasia arises periportally (Dinman et al., 1968). Mitoses in resting adult liver is also restricted to the periportal regions (Emmelot and Scherer, 1980).

The centrilobular hepatocytes represent the more differentiated type in that they have a more developed drug metabolising and detoxifying system and the 'hyperdifferentiation' induced by phenobarbitone is more pronounced centrilobularly.

The finding that there are two populations of hepatocytes: the differentiated centrilobular cells; and the periportal cells capable of cell division may have some bearing on Bannasch's (1968) results. He observed that nitrosomorpholine induced toxic changes centrilobularly but that the changes leading to hyperplasia and preneoplasia occurred periportally (see also Section 4.6).

f) Lobe Differences

In the chronic diethylnitrosamine administration experiments nodularity was most marked in the posterior right lateral lobe (Section 3.2.2; Plate 2.21). Presumably the tumours that developed in the initial carcinogenesis experiment arose principally from this lobe (though the lobe of origin was not determined in this experiment partly
because the large size of the tumours made it difficult to say exactly where it came from). Opie (1944) found that primary hepatocellular carcinomas arising after butter yellow administration also occurred most frequently in this lobe. This fact may have some relevance to experiments with regenerating liver as this is one of the lobes remaining after 2/3 partial hepatectomy. If this lobe is more susceptible to carcinogenesis then the increased incidence of hepatomas after partial hepatectomy may not only be due to increased carcinogenicity in dividing cells but also in a particular species of susceptible dividing cells.

Alternatively, blood may perfuse this lobe at different rates, which might give them an altered (enhanced) response to the carcinogen. Possibly this lobe receives a higher dose of carcinogen than the others. Although generally there is not much variation between the blood supply to the individual lobes the right lobe of the liver is the only one to receive both greater cardiac and portal blood as a percentage of its weight (Vide infera, Solt et al., 1977a). Tracer studies with radioactively-labelled carcinogens could be used to determine the distribution of the carcinogen throughout the liver and might reveal whether the posterior right lateral lobe receives a higher dose. Also the possibility of metabolic differences between the posterior right lateral lobe and the other lobes requires further investigation. Preliminary experiments to determine whether the lobes removed at partial hepatectomy differ from those remaining showed that there was no difference in the enzyme activities measured in the present work between the right and caudate lobes assayed together and the median and left lobes assayed together (N. J. Curtin, unpublished data).

4.1.2 Similar Histological Aberrations Observed in Other Conditions

The absence of typical adult liver architecture is not found only during development, regeneration after partial hepatectomy and carcinogenesis. Similar changes are found after experimental injury particularly in chronic toxicity studies. Bile duct proliferation accompanies almost all sub-acute or chronic experimental or human injury (Rubin and Popper, 1967). There is a loss of one-cell thick
plates after CCl₄ intoxication (McLean et al., 1969) and in rats fed a choline deficient diet, multicellular plates of hepatocytes, similar to those found in the embryo or neonate, are seen (Hartroft, 1964). In human liver too, hyperplastic nodules with plates of hepatocytes two or more cells thick are seen in otherwise healthy livers. These lesions are not associated with fibrosis, give no symptoms and are without clinical significance (Rubin and Popper, 1967). In viral hepatitis various degenerative changes occur, including the arrangement of hepatocytes into two-cell thick plates or tubules (Desmet, 1978). Ascites are also found in rats after chronic CCl₄ intoxication (McLean et al., 1969).

Lipid accumulation is a frequent accompaniment of toxic damage but as a rule, even when it is severe, this feature interferes little with hepatic function (Popper et al., 1978). Various nuclear changes also take place in liver injury, viz: hypertrophy of the nucleoli (Rees, 1978; Desmet, 1978); nuclear hyperchromasia giving them a 'malignant' appearance (Desmet, 1978). Nucleolar hypertrophy is a feature common to regenerating liver and many pathological states and, as the nucleolus is the site of ribosomal RNA synthesis, this is thought to reflect altered RNA synthesis (Rees, 1978).

4.1.4 Changes in Enzyme Activities During Rat Liver Development, Regeneration and Carcinogenesis

a) Comparison of Foetal Liver and Liver During Chronic Diethylnitrosamine Treatment

The changes in enzyme activities during development are well documented (Section 1.4) and the work presented here (Section 3.1.3) confirms the data in the literature. During chronic diethylnitrosamine administration enzyme changes occurred before histological changes could be observed (Section 3.2.5). At first these changes were not significant presumably because of dilution by normal tissue. It is unlikely that necrosis and restorative hyperplasia were responsible for these changes because not only is the low cytotoxicity of diethyl-
nitrosamine well-established but also very little evidence of cell
damage could be seen. Such evidence of cytotoxicity that was seen in
these early stages was reduction in the glycogen content of centri-
lobular cells and a low level of isolated cell necrosis. Of the early
enzyme changes, the reduction in glucose 6-phosphatase has been
reported in foci of altered cells arising after carcinogen treatment
by many authors (Kitagawa, 1971; Scherer et al., 1972; Eltz et al., 1977;
Pugh and Goldfarb, 1978; Du et al., 1979; Bannasch et al., 1980; Emmelot
and Scherer, 1980; Ogawa et al., 1980). In the histochemical exami-
nation of the tissue after six weeks of diethylnitrosamine treatment I
was unable to detect any foci of reduced glucose 6-phosphatase, though
it was reduced centrilobularly (Plate 2.2, 2.4) and this has been
noted previously with diethylnitrosamine hepatocarcinogenesis (Rabes
et al., 1970). It should be noted here that for the histochemical
study of the tissue at six weeks only small pieces of tissue were
examined and foci of altered cells could inadvertently have been missed.

With the exception of thymidine kinase and hexokinase, all the
liver enzymes measured were reduced during the early period of carcino-
genesis when histological changes were minimal i.e. up to eight weeks.
Hexokinase and thymidine kinase are both foetal enzymes, the others
(with the exception of glucose 6-phosphate dehydrogenase which shows
a biphasic pattern of expression during development) increase in activity
after birth. Therefore the decrease in adult enzymes and gain in foetal
enzymes is detectable very early on, long before histological changes
develop.

At eight weeks when more marked histological changes occur (viz:
variable fibrosis; cellular atypia; hyperplasia; fat accumulation) malic
enzyme and glucose 6-phosphate dehydrogenase were elevated. Whereas
this represents an increase in a foetal enzyme in the case of glucose
6-phosphate dehydrogenase, malic enzyme is an adult enzyme and the
adaptation is therefore developmentally anomalous. The enzyme profile
of the liver at eight weeks is qualitatively but not quantitatively
identical to that of the final tumour and probably represents a truly
preneoplastic change even though histological investigation of the
tissue at this time does not give an unequivocal diagnosis of preneoplasia.
Further experiments using different carcinogens and non-carcinogenic
isomers, if available, should be carried out to determine if these changes are characteristic solely of diethylnitrosamine-induced hepatocarcinogenesis or hepatocarcinogenesis in general. If similar changes occurred with a variety of different carcinogens then hepatocarcinogenicity trials could be shortened using the acquisition of characteristic enzyme patterns as an end-point rather than the development of tumours.

Since 70 - 90% of human cancers may be attributed to an environmental origin, the continuing production of potentially carcinogenic chemical substances and drugs requires urgent attention if we are not to add inadvertently to this environmental burden. The magnitude of the task requires more rapid assessment than can be provided by full-scale carcinogenicity tests in vivo and by more appropriate tests than those in vitro systems relying on mutational reversions in prokaryotic organisms.

In addition, the assay of these phase-specific enzymes that alter during carcinogenesis in biopsy material might be a useful diagnostic probe.

In a series of stop experiments (i.e. carcinogen administration for varying times followed by no treatment, during which the liver is studied) Barbason et al. (1979a,b) treated rats with a similar daily dose to that used in the experiments described in this thesis (and also found that neoplasia developed after eleven weeks of continuous administration). After one months treatment persistent foci developed but tumours failed to arise, treatment for two months resulted in an increase in size, but not number, of the foci and a loss of homeostatic control such that tumours developed. Thus the hepatocytes do not become programmed for malignancy until the second month of treatment, this is compatible with my findings that the enzyme profile of the final tumour is not expressed until the eighth week of treatment.

The enzyme changes in the chronic diethylnitrosamine-induced tumours (Section 3.2.5) have been reported by other authors using different carcinogens and in transplantable tumours, viz: reduction in glucose 6-phosphatase (Rabes et al., 1970; Kitagawa, 1971; Scherer et al., 1972; Goldfarb and Pitot, 1976; Knox, 1976; Weber, 1977; Bannasch, 1978; Sato et al., 1978; Du et al., 1979; Eigenbrodt and Glossman, 1980; Emmelot and Scherer, 1980; Ogawa et al., 1980); reduction in phosphoenolpyruvate
carboxykinase (Knox, 1976; Weber, 1976); reduction in glutamate dehydrogenase (Ono, 1966; Knox, 1976; Weber, 1977); reduction in aspartate aminotransferase (Knox, 1976); reduction in glucokinase and increase in hexokinase (Goldfarb and Pitot, 1976; Knox, 1976; Weber, 1977; Sato et al., 1978); increase in glucose 6-phosphate dehydrogenase (Criss, 1974; Goldfarb and Pitot, 1976; Knox, 1976; Weber, 1976; Bannasch, 1978; Sato et al., 1978; Du et al., 1979); increase in thymidine kinase (Knox, 1976; Weber, 1977). Malic enzyme was found to be increased in slow growing hepatomas (Knox, 1976) but Weber (1966) believes that the variation in malic enzyme activity between transplantable hepatomas with no correlation with growth rate means that changes in malic enzyme activity are coincidental to neoplasia.

b) Transplantable Hepatomas

Similar enzymic deviations from the normal liver were observed in the two transplantable hepatomas with the exception of malic enzyme which, in both tumours, was decreased in activity. The changes in the rapidly growing tumour were qualitatively, but not quantitatively, similar to those observed in the primary tumour induced by diethylnitrosamine. The slower growing tumour was not only qualitatively similar to the primary tumour but the enzyme changes observed also showed more quantitative similarities to the primary tumour. This slower growing tumour was also more similar to the primary tumour on histological grounds.

c) The Mechanistic Significance of the Enzyme Adaptations During Carcinogenesis

The enzyme alterations in tumours (as described in the present work and in the literature) have been attributed to an expression of 'foetalism' (Knox, 1976) or, in contrast, to bear no similarity to the immature liver (Weber, 1973; 1975; 1977). Knox uses a vast amount of data collected from many different laboratories to illustrate the similarity between tumours and their foetal counterpart, but makes no suggestion as to how such a foetal situation arises - whether neoplasia
is accompanied by, or is the result or cause of a return to a more immature state. Weber has concentrated principally on the metabolic imbalance of tumours, as observed in his own studies, rather than a comparison of neoplastic and foetal tissues. This leads him to conclude that cancer cells have a biochemical commitment to proliferation and that this confers on them a selective growth advantage. However, foetal cells are also biochemically committed to proliferate and therefore display a similar biochemical phenotype.

The enzyme changes, with the exception of malic enzyme, observed during chronic diethylnitrosamine-induced hepatocarcinogenesis (Section 3.2.5) are compatible with both the views of Knox and Weber. There is an increase in foetal enzyme activities and a decrease in adult enzyme activities and the enzyme profile during the latter stages of carcinogenesis correlates significantly with the foetal enzyme profile (Table 3.2.2). This is in agreement with the hypothesis of foetalism in tumours. The results presented in this thesis are also compatible with the findings of Weber (i.e. that the neoplastic cell has a biochemical pattern which confers on it a selective growth advantage) in that enzymes of DNA synthesis are increased (elevation of thymidine kinase and glucose 6-phosphate dehydrogenase) gluconeogenesis is decreased (reduction in glucose 6-phosphatase and phosphoenolpyruvate carboxykinase) amino acid catabolism is decreased (reduction in glutamate dehydrogenase and aspartate aminotransferase) and there is a loss of regulatory control over glycolysis (the low Km enzyme, hexokinase, is increased and the high Km enzyme, glucokinase, is decreased). This means that the cells programmed for growth and division, rather than the maintenance of blood glucose levels and urea synthesis, will proliferate at the expense of other 'disadvantageous' phenotypes.

Weber (1974; 1980) however, usually takes the liver of the six-day old rat to be representative of the immature liver, as prior to this time there are substantial amounts of haematopoietic tissue, which would interfere with the interpretation of hepatic measurements. However, the sudden changes in enzymes in the liver during late foetal and early postnatal life, during which time there is a gradual decrease in the haematopoietic tissue, is thought to indicate that these changes represent predominantly the changing biochemistry of the hepatocytes.
In addition the biochemistry of the liver of the suckling rat at six days post partum is very different from that of the foetal rat (Section 3.2.5; Vernon and Walker, 1968; Greengard, 1971) and it is not truly immature. From the results presented in this thesis it can be seen that both the primary (Table 3.2.2) and the transplantable hepatomas (Table 3.6.3) bear little resemblance to the postnatal rat but they show striking similarity to the foetal livers. Even when studying foetal liver, however, Weber (1975; 1977) fails to detect a similarity in the metabolic pattern with that of neoplastic liver. Although he observes a reduction of gluconeogenic enzymes (glucose 6-phosphatase, fructose 1.6-diphosphatase, phosphoenolpyruvate carboxykinase and pyruvate carboxylase) in both foetal and tumour liver he does not find an increase in glycolysis in the foetal tissue as seen in the tumours. In fact he reports reduced glycolytic enzyme (hexokinase, phosphofructokinase and pyruvate kinase) activities in the foetal liver. However, hexokinase and phosphofructokinase are both elevated in the foetal liver (Table 3.1.1; Burch et al., 1963; Jamdar and Greengard, 1970; Knox, 1976) and pyruvate kinase is only slightly less active in the foetus than in the adult (Vernon and Walker, 1968). Weber also reports reduced glucose 6-phosphate dehydrogenase in foetal liver, whereas it is elevated in the tumours. However, other measurements of this enzyme have shown it to be elevated in the foetus compared to the adult (Burch et al., 1963; Table 3.1.1). Weber expresses his results as activities per average cell for comparison with normal adult livers whereas most other authors express enzyme activities in terms of activities per g tissue or per mg protein. The foetal cell is about half the size of the adult cell and so this might account for some of the discrepancy in results. Weber has not measured thymidine kinase in foetal liver, although this enzyme is reported to be elevated in both foetal liver (Table 3.1.1; Machovich and Greengard, 1972) and tumour tissue (Table 3.2.1; Weber, 1972).

The results presented in this thesis are in general agreement with the views of Knox and give no support to the statement of Weber that the metabolic pattern of the cancer cell is specific to neoplasia and a similar pattern cannot be found in immature liver.
d) The Anomalous Behaviour of Malic Enzyme

In Weber's notion of molecular correlation malic enzyme is assigned to a category of 'coincidental changes' as the activity of this enzyme shows no correlation with growth rate (Weber and Lea, 1967; Weber, 1972). The increase in activity of malic enzyme in the primary tumours is anomalous to a foetal concept of neoplasia as one would expect a reduction in activity of this adult enzyme if neoplasia is characterised by a foetal-like metabolic pattern. There must be some biological reason for the increase in malic enzyme in the preneoplastic nodules and primary tumours induced by diethylnitrosamine as its increase in activity coincides with the transformation to the preneoplastic state.

Malic enzyme is generally thought to be associated with lipogenesis (Ramos and Leveille, 1974). Its increase at eight weeks coincides with significant fat deposition at this time and the two events may well be connected. Lipid accumulation seems to be associated with cell division and/or DNA synthesis as mentioned above (Section 4.1.1. d). Since hyperplasia increases from the eighth week of diethylnitrosamine treatment onwards this might be partly responsible for the increase in fat deposition and malic enzyme activity.

Alternatively, others have suggested that malic enzyme activity is not associated with fatty acid synthesis. That malic enzyme fails to correlate with another NADPH generating pathway, the pentose phosphate pathway, and with the utilization of NADPH for fatty acid synthesis during development (Madvig and Abraharn, 1980) and under different dietary conditions (Stark et al., 1975) has been demonstrated. The NADPH generated by malic enzyme may instead serve as a source of reducing equivalents for DNA synthesis. Malic enzyme was elevated after partial heptectomy during the first DNA synthesising phase at eighteen hours (Table 3.5.1), but then declined in activity even though active DNA synthesis continued. On the other hand reducing equivalents generated by malic enzyme activity might be involved in glutathione reduction (Stark et al., 1975; Rognstad, 1980). Sauer et al. (1980) have suggested that increases in malic enzyme are progression-linked in a series of transplantable hepatomas (in contrast to Weber's observation that malic
enzyme does not correlate with growth rate). These workers suggest that malic enzyme is involved in energy production i.e. by the conversion of excess malate to pyruvate which is then available for oxidation to $CO_2$ via pyruvate dehydrogenase. Thus, flux through the Krebs cycle may be maintained with malate as the sole respiratory substrate. These functions of malic enzyme are summarised in Fig. 4.1.

**Fig. 4.1 Involvement of Malic Enzyme With DNA Synthesis, Lipogenesis, Glutathione Reduction and Energy Production**

[Adapted from Sauer et al., (1980)]

Production of $CO_2$ from malate was found to be increased in some transplantable tumours whereas fatty acid oxidation was reduced (Cederbaum and Rubin, 1976).

The possible association of malic enzyme with glutathione reduction is interesting as alterations in glutathione metabolism are observed in hepatocellular carcinomas and preneoplastic lesions. One early change in hepatocytes is the elevation of $\delta$-glutamyl transpeptidase (Harada et al., 1976; Ida, 1977; Cameron et al., 1978; Boelsterli, 1979; Demi and Oesterle, 1980; Fiala et al., 1980). $\delta$-glutamyl transpeptidase is a glutathione degrading enzyme involved in the transport of amino acids (Tateishi et al., 1980) and present in foetal hepatocytes (Kalengayi et al., 1975). Other enzymes involved in glutathione metabolism are altered: glutathione oxidation in hepatocellular carcinomas is reduced.
because of a decrease in glutathione peroxidase and increase in glutathione reductase (Pinto et al., 1980a,b). The glutathione content of preneoplastic foci is increased (Fiala et al., 1976) and it has been suggested that high glutathione levels could be responsible, at least in part, for the resistance of preneoplastic foci to carcinogen cytotoxicity by enhancing the capacity of the cell for detoxification of electrophiles by conjugation (Fiala et al., 1976; Demi and Oesterle, 1980).

e) Enzyme Activities in Regenerating Liver

Weber (1966; 1975; 1980) and Knox (1976) are both agreed that the metabolic pattern of the regenerating liver after partial heptectomy is different from that of hepatocellular carcinomas. These authors have studied the regenerating liver only at twenty-four hours after partial heptectomy and suggested that the growth rate at this time is similar to that in the tumour. However, at twenty-four hours the cells are only just becoming mitotically active and only a few cells are dividing, the majority of the cells being either quiescent or in a pre-replicative phase. Much of the growth at this time-point is due to cellular hypertrophy which may partly be accounted for by lipid accumulation.

Care was taken in the experiments with partially heptectomised rats to assay the liver at the same time each morning as there is a circadian rhythm in DNA synthesis and subsequent cell division, the peak of DNA synthesis occurring at the end of the dark phase (Schulte-Herman, 1975). Variation in the enzyme profile in these experiments therefore was not due to diurnal rhythm.

Weber (1975) observed no change in the activity of glucose 6-phosphatase or phosphoenolpyruvate carboxykinase. In the work presented here (Table 3.5.1) at twenty-four hours, and at subsequent times, an elevation of the latter enzyme and reduction of the former was seen. An increase in the activity of phosphoenolpyruvate carboxykinase has been noted by others (Brinkmann et al., 1978; Katz, 1979). However, these authors failed to observe decreased glucose 6-phosphatase activity although Brinkmann et al. (1978) do observe a periportal loss of this enzyme. Knox (1976) also reports near normal activity of this enzyme.
in the regenerating liver twenty-four hours after partial hepatectomy. Brinkmann et al. (1978) and Katz (1979) report a reduction in glucokinase activity and this, in the absence of any differences in hexokinase and other glycolytic enzymes, coupled with the increase in phosphoenolpyruvate carboxykinase means that there is an enhanced capacity for gluconeogenesis in the regenerating liver. Enhanced gluconeogenesis is necessary to prevent the animal developing severe hypoglycaemia. The maintenance of blood glucose levels after partial hepatectomy has been described previously (Leduc, 1964; Daniel et al., 1980).

In the work presented here (Table 3.5.1) for the first three days after partial hepatectomy the loss of glucokinase was accompanied by an increase in hexokinase in contrast to the findings of unchanged hexokinase by Weber (1975), Brinkmann et al. (1978) and Katz (1979). Other authors report an increase in hexokinase (Sato et al., 1969; Knox, 1976) although in some cases the elevation was very slight (Walker and Potter, 1972). The increase in hexokinase was not as marked as the decrease in glucokinase (Table 3.5.1) and the results probably indicate a reduced glycolytic capacity overall. The reduction in glucose 6-phosphatase activity during liver regeneration seen in the experiments presented here is at variance with the need for enhanced gluconeogenesis. Possibly the production of this enzyme is incompatible with cell division which would explain its loss periportally. Three days after the operation not only is glucose 6-phosphatase activity at a minimum but also phosphoenolpyruvate carboxykinase activity is lower than control levels. This loss of gluconeogenic capacity might be somewhat offset by the fact that glucokinase is also greatly reduced (although hexokinase remains elevated) at three days after the operation.

Glucose 6-phosphate dehydrogenase, whilst being elevated in the pre-replicative phase (eighteen hours after the operation) was reduced during the period of most rapid growth (Table 3.5.1). Knox (1976) also observed a reduction of this enzyme during liver regeneration. This finding was unexpected as the pentose phosphate pathway, which generates NADPH and ribose, is thought to be integral to DNA synthesis. Malic enzyme follows a similar course after partial hepatectomy i.e. that it is elevated at eighteen hours is reduced up to three days after the operation and is elevated at five days (Table 3.5.1). Thus there
is a general lowering of NADPH production between one and three days after partial heptectomy.

Longenecker and Williams (1979) also found a decrease in pentose phosphate pathway activity in cells isolated from regenerating liver twenty-four hours after partial hepatectomy. In normal liver 30% of glucose is metabolised through this pathway but in regenerating liver this was reduced to 12%. They suggest two explanations for this: 1) that there is a large increase in total glucose metabolism during regeneration which enables sufficient carbon flux through the pentose phosphate pathway to support the synthesis of required growth intermediates; or 2) that there is no increase in carbon flux and new questions about the role of the pentose phosphate pathway must be investigated.

Thymidine kinase, as expected was greatly elevated during the first two days after partial hepatectomy correlating with the requirement for DNA synthesis during the rapid growth phase (Table 3.5.1). As the proliferative activity of the liver decreased after three days so thymidine kinase activity declined to reach near normal levels at five and seven days.

There was a reduction in glutamate dehydrogenase and aspartate aminotransferase during regeneration (Table 3.5.1) and this finding agrees well with the observation that urea output by the regenerating liver is reduced (Leduc, 1964). Reduced protein breakdown and unaltered protein synthesis seen in regenerating liver may be responsible for net protein gain and growth (Scornik, 1975).

The pattern of enzyme activity at three days showed similar features to that seen in the later stages of carcinogenesis, hepatic tumours and the foetal liver. At this stage too, bile duct proliferation was evident, a feature which is also found in the developing tumours and in the neonatal (but not foetal) liver (Section 4.1.1 b). Scholla et al. (1980) have performed DNA/RNA hybridizing experiments with normal and regenerating liver and found that whereas at twelve and twenty-four hours after the operation the sequences in the regenerating liver were similar to normal at three days they were different. This difference was mostly due to changes in the frequency of existing sequences rather than the appearance of new species. It would be interesting to determine
if there was an increased similarity in RNA sequences between foetal liver and regenerating liver three days after partial hepatectomy.

Some similarities between foetal and regenerating liver (Walker and Potter, 1972; Bonney et al., 1973) and between transplantable hepatomas, especially slow growing ones, and regenerating liver (Walker and Potter, 1972; Schapira et al., 1973) have been observed.

Portal blood flow is important in liver regeneration, it is increased after partial hepatectomy (Weinbren, 1973) and only those areas supplied with portal blood grow (Starzl and Putnam, 1975). Portal blood will stimulate growth of liver explants but peripheral blood will not (Schindler et al., 1975). Pancreatic hormones, particularly insulin, are thought to be the important factors (Bücher, 1975). There may be an altered chemical environment in the liver after partial hepatectomy due to increased portal blood flow which may be partly responsible for some of the changes.

The elevation of phosphoenolpyruvate carboxykinase in regenerating liver is similar to the situation in neonatal liver but different from foetal and neoplastic liver. It may therefore possibly be useful as a diagnostic discriminant between regenerating liver after injury and neoplastic or preneoplastic conditions of the liver. The reduction in glucose 6-phosphate dehydrogenase may also help distinguish between regenerating and cancerous liver.

4.1.4. Oncofoetal Changes Found in Non-Neoplastic State

Two commonly used markers for hepatocarcinogenesis, i.e. elevations of δ-glutamytranspeptidase and α-fetoprotein, may be found in regenerating liver. Hyperplastic nodules that arise after portacaval anastomosis not only have a similar architectural appearance to preneoplastic nodules (Weinbren and Washington, 1976) but also contain hepatocytes with δ-glutamyl transpeptidase activity (Muller et al., 1974; Colombo and Gigon, 1979). Liver regeneration after poisoning with CCl₄ is accompanied by an elevation of δ-glutamyl transpeptidase (Harada et al., 1976). Similarly ethanol treatment causes an elevation of δ-glutamyl transpeptidase (Ishii et al., 1978; Idéo et al., 1980).
The increase in α-fetoprotein is also not entirely specific to hepatocarcinogenesis. Rises, albeit transitory, in α-fetoprotein are seen after partial hepatectomy and to a greater extent, after poisoning with CCl₄ (Stillman and Sell, 1979). The increase is not correlated necessarily with mitotic activity, as when an animal undergoes partial hepatectomy and CCl₄ treatment there is low mitotic activity but α-fetoprotein levels are elevated (Mohanty et al., 1978). Other hepatotoxins also cause an increase in α-fetoprotein (Sell and Becker, 1978). Phenobarbitone induces an elevation of α-fetoprotein within six hours of administration, i.e. before cell division, and in this case the elevation is thought to be associated with hypertrophy (Sell and Becker, 1978).

The increase in α-fetoprotein during carcinogenesis may be associated with oval cell proliferation as during acetamidofluorene carcinogenesis, which induces necrosis and oval cell proliferation, there is an increase in α-fetoprotein during the early stages whereas with diethylnitrosamine, which has low toxicity and does not induce oval cell proliferation, there is no increase in α-fetoprotein levels until tumours form (Stillman and Sell, 1979). Similarly, when high, necrogenic doses of nitrosonomorpholine are given there is a rise in α-fetoprotein accompanied by oval cell proliferation in which α-fetoprotein can be demonstrated histologically. With low doses of nitrosonomorpholine α-fetoprotein was only detected in the serum and in the neoplastic hepatocytes once hepatomas had developed (Kuhlman, 1978). Non α-fetoprotein secreting transplantable hepatomas are also known to occur (Sell and Morris, 1974). Ethionine, on the other hand, causes an increase in α-fetoprotein in the absence of injury (Stillman and Sell, 1979).

The decrease in glucose 6-phosphatase activity, which is also used as a preneoplastic marker, is seen with CCl₄ poisoning (Idéo et al., 1971) and in degenerating hepatocytes during hepatitis infection (Takemoto, 1979). Similarly, the increase in glucose 6-phosphate dehydrogenase is also seen with CCl₄ poisoning (Idéo et al., 1971). Furthermore in experimental injury by a variety of toxins an increase in foetal enzymes and isoenzymes and a decrease in adult enzymes and isoenzymes has been observed (Taketa et al., 1976).
During chronic diethylnitrosamine treatment most of the enzymic alterations are consistent with an approach to foetalism, i.e. thymidine kinase and hexokinase increase and the other enzymes decrease in activity. The deviation from normal activities becomes more marked with continued diethylnitrosamine administration as expected if more cells are becoming affected by the carcinogen. Exceptions to the gradual change are glucose 6-phosphate dehydrogenase and malic enzyme which are at first depressed but become elevated later (Table 3.2.1). The pattern of glucose 6-phosphate dehydrogenase expression is therefore similar to a reversal of the pattern of its expression during normal differentiation (Fig. 3.2.1.(iii)). Malic enzyme, as referred to above (Section 4.1.3d) is anomalous in this respect. The gradual nature of the changes of activity for the other enzymes on the whole means that the nature of the change is similar to a reversal of normal differentiation. However, glucose 6-phosphatase and phosphoenolpyruvate carboxykinase show postnatal rises. This is not reflected by an increase in their activities, prior to a decrease, during diethylnitrosamine-induced hepatocarcinogenesis. Glucose 6-phosphatase and phosphoenolpyruvate carboxykinase respond perinatally to an increased demand for gluconeogenesis because of perinatal hypoglycaemia and because of the low carbohydrate diet during suckling. Since the animals during the carcinogenesis experiment were not on a low carbohydrate diet and not expected to become hypoglycaemic until large tumours arise, this lack of elevation of these two enzymes is not unexpected.

Statistical analysis however, reveals that during the early stages of chronic diethylnitrosamine-induced hepatocarcinogenesis the liver showed a significant correlation with both the weanling and the five-day old rat liver. After six weeks however, the only significant correlation was seen with the foetal liver (Table 3.2.2). This would seem to suggest that prior to adopting a foetal pattern of enzyme activity, the liver during carcinogenesis passes through less immature patterns i.e. it undergoes a stepwise retrodifferentiation.
The enzyme pattern of the transplantable tumours also showed statistically significant correlation with foetal liver (Table 3.6.3) reinforcing the idea that neoplasia is associated with foetalism. Both tumours, particularly WDA also correlated significantly with the enzyme pattern of the primary hepatoma induced by diethylnitrosamine (Table 3.6.3). This suggests that the enzyme changes in the primary tumour were characteristically neoplastic.

This retrodifferentiation was also seen in the regenerating liver after partial heptectomy. Up to, and including forty-eight hours after the operation the greatest correlation was observed with the neonatal rat between five and ten days post partum (Table 3.5.2). During this period the regenerating liver correlated significantly with the early stages of hepatocarcinogenesis too (Table 3.5.2).

At three days, however, when most of the rapid phase of cell division is over, in contradiction of Greenstein's (1954), Knox's (1976) and Weber's (1975) statements, the regenerating liver correlated significantly with foetal liver. It did not correlate with the other developmental stages. At this point a significant correlation was also seen with the later stages of carcinogenesis. Curiously, all stages of regeneration correlated significantly with the UA tumour (Table 3.6.3).

These data suggest that both the regenerating liver and the liver during carcinogenesis undergo a similar set of changes to arrive at a foetal metabolic pattern. The preneoplastic liver remains trapped in this foetal state (possibly because of continued presence of the carcinogen) whereas the regenerating liver retains the capacity to redifferentiate again passing through metabolic patterns similar to that of the ten-day old rat liver and the liver during early carcinogen treatment. The proposed sequence of events taking place during differentiation, regeneration and hepatocarcinogenesis are given in Figure 4.2.

These results are consistent with the view expressed earlier (Walker and Potter, 1972) that a fully mature hepatocyte cannot undergo division without some degree of dedifferentiation and that similar stages of dedifferentiation occur in both regenerating and precancerous liver to give rise to a population of cells capable of cell division. After division the cells undergo maturation or reoentogeny, and whereas
Figure 4.2 Differentiation and Dedifferentiation During Development, Regeneration and Carcinogenesis

DIFFERENTIATION

(i) Foetal Liver \(\rightarrow\) neonatal liver \(\rightarrow\) adult liver

(ii) 3 days p.h. \(\leftarrow\) 1 - 2 days p.h. \(\rightarrow\) 7 days p.h. \(\rightarrow\) adult liver

(iii) Preneoplastic/neoplastic liver \(\leftarrow\) early stages of hepatocarcinogenesis \(\rightarrow\) adult liver

DEDIFFERENTIATION

(i) normal differentiation (ii) liver regeneration: p.h., time after partial hepatectomy. (iii) hepatocarcinogenesis
this is complete in the regenerated liver after partial hepatectomy the process is blocked at various stages in the precancerous liver. As support for their view that the precancerous hepatocytes undergo a certain degree of maturation they show that the precancerous lesions induced by 3'-methyl-4-dimethylaminoazobenzene have more foetal characteristics than do some of the 'minimal deviation' transplantable hepatomas. It is possible though that their carcinogenesis regime induces more dedifferentiated tumours than those used to produce the 'minimal deviation' hepatomas. Dimethylaminoazobenzene is known to produce poorly differentiated hepatocellular carcinomas and cholangiosarcomas (Sato et al., 1978). It is therefore not valid to say that the pre-neoplastic lesions will give rise to less dedifferentiated tumours. However, the majority of altered foci are capable of maturation to normal hepatocytes and only a small proportion of them give rise to tumours (Kitagawa and Sugano, 1973). In the work presented here statistical analysis of the enzyme data shows that the liver of the rat treated for six weeks with diethylnitrosamine shows greater similarity with the foetal liver, and the regenerating liver at three days after the operation, than it does subsequently (Tables 3.2.2, 3.5.2). This is presumably due to the anomalous increase in malic enzyme activity after this time, though it may reflect some degree of maturation. Furthermore, the amorphous mass of hepatocytes in the preneoplastic nodule (Plates 2.11, 2.18) is representative of more immature architectural forms than the trabecular arrangement of many of the ultimate tumours (Plates 2.24, 3.32). Further investigation is required to determine if maturation is taking place in preneoplastic hepatocytes destined to develop into tumours.

Contrary to the supposition that the preneoplastic hepatocytes may undergo a certain degree of maturation before the ultimate tumours arise is the findings of Sato et al. (1978). These workers found an increase in enzymic immaturity during the progression from preneoplastic nodules to well differentiated hepatomas during acetylaminofluorene carcinogenesis.

Uriel (1976; 1979) maintains that tissue regeneration and neoplastic change are accompanied by stepwise retrodifferentiation, i.e. a recapitulation of ontogeny in reverse sequence. In the case of regeneration, as renewal is accomplished, there is a redifferentiation along the
the same pathway to give rise to cell types characteristic of the original cell, as in liver regeneration. The results obtained in the present study, as described above (summarised in Fig. 4.2) agree with this hypothesis. The dedifferentiated cells may alternatively redifferentiate along a different pathway to give rise to other cell types - metaplastic shift (Uriel, 1976; 1979). Such a metaplastic shift is seen in Wolffian lens regeneration i.e. removal of the lens from certain urodel amphibians results in fully differentiated iris cells undergoing a series of steps of dedifferentiation and then lens regeneration begins with the synthesis of lens-specific proteins. An analogous metaplastic shift in liver regeneration and carcinogenesis might be the observed bile duct proliferation with oval cells differentiating into hepatocytes as described in section 4.1.1. b. Sugimura et al. (1972) have observed properties in hepatomas which do not correspond to their normal foetal counterpart, this may also be due to differentiation to a cell-type different from the original cell-type.

Other workers (Malkin et al., 1978) have measured a battery of seven different enzymes, which show developmental phase-specific patterns of expression, in transplantable hepatomas and primary hepatomas induced by the Solt and Farber (1976) regime i.e. diethylnitrosamine induction, acetaminofluorene selection. Contrary to the results expressed in this thesis they found that the enzymic profile exhibited by the tumours was not characteristic of any particular phase of ontogeny and therefore was inconsistent with the view of synchronous, stepwise retrodifferentiation of mature hepatocytes or a systematic phase-specific arrest in normal development. However, they took the peak activity of the enzymes during foetal or neonatal growth to be their standard immature enzyme values for comparison. Thus for some of the enzymes e.g. glucose 6-phosphatase high activity was taken to represent the immature state but in the foetus these enzymes are low in activity. Also they examined the tumours i.e. the end-point of carcinogenesis and not preneoplastic stages so they cannot unequivocally say that a process of retrodifferentiation did not take place. Potter (1973), in an attempt to explain the apparent diversity of enzyme activities in a spectrum of tumours suggested that the various enzymes were locked in at different developmental stages so that any specific
hepatoma would not have an enzyme pattern that corresponds to any particular developmental age in normal liver. In the work presented here the enzymes seem to vary in their rate of dedifferentiation (Fig. 2.2 (i - ix)). Nevertheless, statistical analysis reveals that overall there is an underlying stepwise retrodifferentiation suggesting a degree of synchrony of enzymic dedifferentiation.

It seems likely from the experimental data presented here and in the literature (Section 4.1.1 a - c; 4.1.2; 4.1.3 a,b,e; 4.1.4) that many of the changes observed during hepatocarcinogenesis are not specific to neoplastic change but are also seen in regenerating liver after partial hepatectomy and following toxic injury. This is presumably because of the underlying mechanism of dedifferentiation in hyperplasia whether it be normal, regenerative cell division or abnormal, malignant growth. Further work is needed before such changes can be said to be unequivocally related to cancer. Studies should be carried out to determine the changes during acute and chronic liver injury to enable biological and biochemical discriminants between restorative and malignant growth to be identified. Once such discriminants are known they could be used as more specific markers for neoplasia. One possible biochemical discriminant may be the elevation of phosphoenolpyruvate carboxykinase in regenerating liver after partial hepatectomy. Although it remains to be determined if this enzyme is also elevated during chronic injury.
4.2 Dedifferentiation in Host Livers of Tumour-Bearing Rats

Contrary to expectation the host liver of the tumour-bearing rats showed little evidence of dedifferentiation (Section 3.6.3; Table 3.6.3) as described by others (Section 1.3.4). There was a significant elevation in thymidine kinase activity in the liver of the UA tumour-bearing rats (Table 3.6.1) but this was not the case in the liver of the WDA tumour-bearing rat (Table 3.6.2). There was no significant increase in hexokinase, glucose 6-phosphate dehydrogenase or decrease in glucokinase as reported previously (Suda et al., 1966; Herzfeld and Greengard, 1972; Greengard, 1979; Herzfeld et al., 1980). There was however, a significant decrease in malic enzyme activity in the host liver of both tumour-bearing rats (Tables 3.6.1, 3.6.2) as described by Herzfeld and Greengard (1972). The host liver of the UA tumour-bearing rat did show slightly significant correlation (0.1 > p > 0.05, Table 3.6.3) to the foetal liver, probably by virtue of the elevation of thymidine kinase and depression of malic enzyme and glucose 6-phosphatase activities (Table 3.6.1).

With the exception of the human study (Herzfeld et al., 1980) the tumours used in the above mentioned studies were not hepatocellular carcinomas. However, dedifferentiation has been demonstrated in the host livers of rats bearing transplantable hepatomas (de Rosa and Pitot, 1978; Matthei, 1979). Because of the diversity of tumour biochemistry these tumours may secrete different chemical messengers or cause different metabolic disturbances from the hepatocellular carcinomas used in the present study. Hepatomas and other tumours may produce toxic metabolites or hormones, some of which have been extracted and shown to affect host liver enzymes (Goldfarb and Pitot, 1976). A common feature in cancer is cachexia, and hypoglycaemia may develop because of the large glucose uptake by the tumour (Cochrane and Williams, 1976). This may explain the glycogen depletion seen in the host liver of the UA tumour-bearing rat (Plate 6.6) since the rate of glycogen depletion in tumour-bearing rats was found to surpass that of starved rats in one study (Hori et al., 1958). Hyperlipidaemia is also often seen in advanced cancer because of the energy demand of the tumour (Pitot, 1978) and the tumour may release a lipid-mobilising factor (Kitada et al., 1980).
An increase in liver weight is often seen in tumour-bearing animals (Yeakel, 1948), this is partly due to increased water content (Rechcigl et al., 1961) and partly due to increased DNA synthesis (Morgan and Cameron, 1973), especially during most active tumour growth (Hori et al., 1958), and mitotic activity of the liver (Annau et al., 1951; Baserga and Kisieleski, 1961; Trotter, 1961). If transplantable tumours are inducing host liver growth then this might explain the dedifferentiated pattern observed by some authors as experiments with regenerating liver (described above: Section 4.11 a - c, 4.1.3 e) indicate that growth is accompanied by morphological and enzymic dedifferentiation. The increase in liver size in these cases is partly due to increased functional demand by the growing tumour i.e. work hypertrophy (Naora and Naora, 1964; Theologides and Pegelow, 1970).

In the experiments with the UA and WDA tumours host liver growth was not induced which may account for the lack of enzymic dedifferentiation. Work hypertrophy also occurs during pregnancy (Poo et al., 1940) and a similar, increased, response to partial hepatectomy is seen in both tumour-bearing and pregnant animals (Theologides and Zaki, 1969). It might be expected therefore that changes in the enzymic activity in the liver of tumour-bearing rats would be comparable to changes in enzymic activity in maternal liver during pregnancy. However, preliminary experiments indicate that there is no significant correlation between the liver of pregnant rats and tumour-bearing rats (Table 3.6.5), The liver of pregnant rats did not exhibit a dedifferentiated pattern of enzymes although an increase in hexokinase activity (not significant) and decrease in glucose 6-phosphatase (p < 0.005) was observed (Table 3.6.4). A slightly significant correlation (0.1 ≥ p ≥ 0.05) between the liver during pregnancy and the liver twenty-four hours after partial hepatectomy was observed, which may have been due to the hypertrophic and hyperplastic response of the liver during pregnancy. This requires further investigation before the biological significance of this correlation can be ascertained.
4.3 Two-Stage Hepatocarcinogenesis

Diethylnitrosamine appears to act as a multistage carcinogen, and in a similar dietary regime to the chronic diethylnitrosamine treatment reported in this thesis Barbason et al. (1979a, b) divided the action of the carcinogen into three stages. The first stage, induction, corresponding to one month of treatment, causes the appearance of preneoplastic nodules which are under homeostatic control and which fail to give rise to tumours. A second month of treatment causes an increase in size of the nodules, but not an increase in their number, and a 100% tumour incidence. This corresponds to the second stage, promotion, during which no new neoplastic foci are induced. If diethylnitrosamine is administered for a further two weeks, or continuously until death the tumours arise and kill the animal sooner than if administration had been discontinued at eight weeks. This is the progression stage.

In a chronic carcinogenesis regime biochemical and histological investigations into the changes occurring in a two-stage carcinogenesis model cannot be accurately made as the continued presence of the carcinogen might induce changes other than promotion. For this reason in the experiments reported in this thesis carcinogenesis initiated by diethylnitrosamine was promoted by phenobarbitone using a method described by Kitagawa and Sugano (1978). This method was chosen because it did not involve the use of partial hepatectomy which, as already described, induces dedifferentiation in the liver.

4.3.1 Pathological Changes

a) Changes Due to the Action of Phenobarbitone

Many of the pathological changes observed in the animals treated with both diethylnitrosamine and phenobarbitone were due to the action of phenobarbitone as they were, on the whole, shared by the control animals receiving phenobarbitone alone. These changes were: liver growth; centrilocular cell enlargement; decreased glycogen and increased fat deposition.
Liver growth in animals treated with phenobarbitone is due mainly to cytoplasmic hypertrophy and partly due to hyperplasia (Henderson et al., 1964; Kunz et al., 1966; Emmelot and Scherer, 1980). In the work presented here no increased thymidine kinase activity (Table 3.3.1) or mitotic activity (Section 3.2) could be seen in the livers of the rats treated with phenobarbitone alone (until the thirty-two week time point when cell necrosis and compensatory regeneration was seen). This is because phenobarbitone induces mitosis only transiently in normal hepatocytes, though initiated hepatocytes continue to respond to the mitogenic action of phenobarbitone (Emmelot and Scherer, 1980). Ruttiman (1972) also failed to observe increased thymidine kinase incorporation into DNA and mitotic index in phenobarbitone treated rats.

The cytoplasmic hypertrophy occurring centrilobularly (Plate 3.3) is due to an increase in smooth endoplasmic reticulum which accompanies the increased drug metabolising activity seen in these areas (Henderson et al., 1964; Orrenius et al., 1965; Jones and Fawcett, 1966; Staubli et al., 1969).

Phenobarbitone-induced centrilobular glycogen depletion has been observed in the hamster (Jones and Fawcett, 1966) and in the rat (Kast and Nishikawa, 1979). Lipid deposition in the liver of rats treated with phenobarbitone has also been reported (Sorrell et al., 1973).

Focal hydropic degeneration, occasionally accompanied by necrosis was seen in the liver of rats treated with phenobarbitone, particularly in those rats pretreated with diethylnitrosamine (Plate 3.14). Focal hydropic degeneration leading to necrosis is a common feature of ageing rat liver (Knook and Hollander, 1978). It seems therefore that phenobarbitone not only induces hyperdifferentiation but also accelerates the ageing process of the liver especially after diethylnitrosamine pretreatment. It has been postulated that ageing and carcinogenesis are two related phenomena (Pitot, 1977a) so an acceleration of the ageing process by phenobarbitone might contribute to its action as a promoter.
b) Changes Due to the Action of Diethylnitrosamine

In animals which had received diethylnitrosamine but no further treatment, no histological abnormalities could be detected. This is presumably because if initiated hepatocytes are different from their non-initiated counterparts they are so few in number that they cannot be seen unless promoting stimulus induces their multiplication.

c) Changes Due to DiethylNitrosamine Followed by Phenobarbitone

Until the development of tumours the livers of rats treated with diethylnitrosamine and phenobarbitone were histologically very similar to the livers of rats receiving phenobarbitone alone. Focal accumulations of fat and focal accumulation or depletion of glycogen were seen, however. There was no evidence of cirrhosis or biliary hyperplasia, as seen in the chronic diethylnitrosamine study, prior to tumour formation but tumours arising in association with bile duct proliferation were seen (Plate 3.17). The tumours induced by this two-stage carcinogenesis experiment appeared histologically similar to those induced by chronic diethylnitrosamine administration.

Aneuploidy was observed in the tumours (Figure 3.3.3), being most marked in the large tumours and least obvious in the preneoplastic nodules. Such aneuploidy was absent in the non-tumourous areas of the liver of these animals and in the livers of animals treated with either diethylnitrosamine or phenobarbitone alone. Aneuploidy, therefore, is not induced by diethylnitrosamine and phenobarbitone either separately or in conjunction but develops during tumour formation as suggested in the Introduction (Section 1.5.2. a).

4.3.2 Histochemical Alterations Induced by DiethylNitrosamine and/or Phenobarbitone

Diethylnitrosamine treatment, when not followed by prolonged phenobarbitone promotion, failed to induce histochemically demonstrable enzymic change in the liver (Section 3.3.2). The reason for this is that the overwhelming majority of hepatocytes are not initiated by the
carcinogen treatment so the biochemical alterations (if indeed any occur) in the initiated hepatocytes are masked by the normal hepatocytes.

When, however, diethylnitrosamine initiation was promoted by phenobarbitone foci of altered enzymic activity, which were frequently associated with histochemical changes (increased fat deposition, increased or decreased glycogen deposition), were detected. Some of the changes observed in these foci have been observed in two-stage hepatocarcinogenesis regimes by other authors. The loss of glucose 6-phosphatase and the gain of γ-glutamyl transpeptidase are the most commonly reported findings (Solit and Farber, 1976; Tatematsu et al., 1977; Pitot et al., 1978; Sirica et al., 1978; Sells et al., 1979). Elevated glucose 6-phosphate dehydrogenase has also been reported (Bannasch et al., 1980).

In the work presented here an additional focal alteration was observed, viz: increased malic enzyme activity (Plate 3.21) glutamate dehydrogenase was also decreased focally but this was not very marked. These enzyme changes correspond to those seen in the final tumours and could be used as additional markers for preneoplasia in carcinogenicity tests. It was uncommon to find all the marker enzymes altered in a single focus and the heterogeneity of the foci is a common feature in experimental hepatocarcinogenesis (Kitagawa, 1971; Becker, 1978; Sirica et al., 1978; Ogawa et al., 1980). It has been suggested that some of the biochemical changes observed in preneoplastic nodules may be as a result of an altered microenvironment due to changes in blood flow to these areas. Such alterations in blood flow could be responsible for some of the phenotypic heterogeneity (Farber, 1980). Reduced blood flow to hyperplastic nodules and liver tumours, compared to the surrounding tissue has been reported (Solit et al., 1977a). Butler (1978) has also remarked that when artificially perfusing rat liver, hyperplastic nodules fail to perfuse. Restricted blood flow might be an important factor in some of the enzymic and histochemical changes, such as glycogen depletion. It has also been suggested (Ogawa et al., 1980) that elevated γ-glutamyl transpeptidase and decreased glucose 6-phosphatase and ATPase activities may not necessarily be essential components of carcinogenesis but might be indicative of altered blood flow through the nodules. Arterial blood is increased and portal blood
is decreased in the nodules, and in portacaval anastomosis, where arterial blood is also increased and portal blood is decreased there is also an increase in γ-glutamyl transpeptidase. The hypertrophic liver has a poorer blood supply (Kunz et al., 1966) and this may be responsible for increased γ-glutamyl transpeptidase activity in the liver of phenobarbitone-treated animals (Ratanasavanh et al., 1979; Goldberg et al., 1981). In the work described in this thesis phenobarbitone administration alone failed to induce γ-glutamyl transpeptidase activity in hepatocytes consistently (Plate 3.12) though some hepatocytes staining for this enzyme were detected at the eight week time-point (Plate 3.7).

In view of the fact that there is an altered blood flow, similar to that in hepatomas, in the liver after portacaval anastomosis and that this is often accompanied by the development of nodular liver, (Weinbren, 1978) this experimental manipulation would provide a suitable control tissue for study. Investigation of such tissue might help to distinguish changes occurring in (pre-)neoplastic liver nodules that are specific to neoplasia from those which are due to altered blood flow and/or non-neoplastic nodular hyperplasia in the liver.

4.3.3 Enzyme Biochemistry During Two-Stage Hepatocarcinogenesis

a) Enzyme Changes in Phenobarbitone-Treated Rats

Phenobarbitone alone induced changes in the activities of some of the enzymes measured. The elevation of glucose 6-phosphate dehydrogenase and malic enzyme activity observed in the present study (Table 3.3.1) have been reported previously (Kunz et al., 1966). These workers report that drugs which cause liver enlargement stimulate the pentose phosphate pathway.

Other changes were observed in the phenobarbitone-treated group of animals, many of which were qualitatively, but not quantitatively, similar to those observed in rats treated with both diethylnitrosamine and phenobarbitone. Compared to the animals which had received diethylnitrosamine alone (and were essentially histologically and
biochemically normal) animals which received phenobarbitone had reduced glucose 6-phosphatase, phosphoenolpyruvate carboxykinase, aspartate aminotransaminase and glutamate dehydrogenase. There was an increase, also, in thymidine kinase at thirty-two weeks which probably represents hyperplasia compensating for the cell mass lost due to the hydropic/necrotic foci. The decreased glucose 6-phosphatase activity was an unexpected finding as it was expected that this microsomal enzyme would increase with the proliferation of endoplasmic reticulum induced by phenobarbitone. However, a decrease in glucose 6-phosphatase in the liver of animals treated with phenobarbitone has been reported elsewhere (Orrenius et al., 1965).

Whether the similarity in enzymic change induced by prolonged phenobarbitone treatment to those induced by carcinogen treatment means that phenobarbitone is a carcinogen in its own right, as suggested previously (Rossi et al., 1977), or that it is acting as a promoter of other unidentified carcinogens in the environment (Pitot and Sirica, 1980) or that phenobarbitone acts as a promoter by inducing such enzyme changes requires further investigation.

b) Enzyme Changes After Short-Term DiethylNitrosamine Treatment

The biochemical measurement of enzyme activities in the livers of animals treated with diethylnitrosamine alone (Table 3.3.1) like the histological and histochemical investigation of these tissues, revealed that the livers were essentially normal.

c) Enzyme Changes After DiethylNitrosamine and Phenobarbitone Administration

In general, the enzyme changes observed in the livers of rats subjected to both initiation and promotion (Table 3.3.1) were expected from the chronic carcinogenesis experiments (i.e. increased thymidine kinase, glucose 6-phosphate dehydrogenase and malic enzyme, decreased glucose 6-phosphatase, phosphoenolpyruvate carboxykinase, glutamate dehydrogenase, aspartate aminotransferase and glucokinase). However, there was no elevation in hexokinase activity until tumours developed.
The elevation of malic enzyme in the two-stage hepatocarcinogenesis regime was greater than in the chronic carcinogenesis experiments, presumably because of the effect of phenobarbitone on normal liver (i.e. induction of increased malic enzyme activity). The other enzyme changes were similar or less marked in the two-stage regime. The reduced response of thymidine kinase activity in this regime probably reflects the slower rate of tumour production, the reduced amount of liver affected, the lower tumour incidence and the lower toxicity of two-stage hepatocarcinogenesis compared with chronic diethylnitrosamine administration. This may also explain the fact that the other enzyme activities deviated less from normal in the two-stage regime.

4.3.4 Rank Correlation Analysis of the Data

Animals which had received diethylnitrosamine alone failed to show any significant correlation with any of the developmental stages or with the later stages of carcinogenesis (Table 3.3.4). This was in keeping with their normal histological appearance and was expected from the enzyme data. However, at sixteen weeks there was a significant correlation with two weeks of chronic diethylnitrosamine treatment. This may reflect a metabolic similarity of the liver initiated by one week of diethylnitrosamine treatment without further manipulation and two weeks of continuous diethylnitrosamine treatment. However, this seems unlikely as neither before nor after sixteen weeks was there any significant correlation.

The observation that phenobarbitone induces hyperdifferentiation, as judged by cytoplasmic structure (Staubli et al, 1969) is borne out by the fact that the enzyme pattern of the liver of rats treated with phenobarbitone correlated negatively with all the immature developmental stages of the liver (Table 3.3.5).

Because of this enzymic hyperdifferentiation induced by phenobarbitone, livers of rats which received both diethylnitrosamine and phenobarbitone (when normalised with control animals) failed to exhibit any significant correlation with any of the stages of differentiation (Table 3.3.3). They did however, correlate with the later stages of chronic diethylnitrosamine-induced hepatocarcinogenesis. When the hyperdifferentiation
effect was negated (by normalisation comparison with phenobarbitone treated rats) however, there was a significant correlation with foetal liver as well as the later stages of hepatocarcinogenesis.

At thirty-two weeks the loss of correlation between diethylnitrosamine plus phenobarbitone-treated rats which had not developed tumours with early developmental stages and the later stages of carcinogenesis probably reflects the maturation of the majority of altered hepatocytes into phenotypically normal hepatocytes as described previously (Farber, 1980; Ogawa et al., 1980). Curiously the tumours failed to exhibit a significant correlation with foetal livers, although they did correlate significantly with the later stages of chronic carcinogenesis and the transplantable hepatomas. This outcome of the correlation analysis was unexpected as, with the exception of malic enzyme, all post-natal enzymes were decreased and all foetal enzymes increased. Therefore, despite the rank correlation analysis, I believe the tumours to be exhibiting a dedifferentiated pattern of enzymic activity.
4.4. Mechanisms of Promotion

Most work concerning the mechanisms of action of promoters has concerned the well-established skin model using phorbol esters and has been reviewed recently (Diamond et al., 1980). The evidence to date points to no single factor concerning the mechanism of their action conclusively. They may inhibit cell differentiation in some systems (Diamond et al., 1978; Fibach et al., 1979); or they may induce dedifferentiation, as indicated by the appearance of foetal proteins (Colburn, 1980); in other systems they may induce differentiation (Lotem and Sacks, 1979; Nakayasu et al., 1979); they may have transient inhibitory effect on differentiation (Muñoz et al., 1979); or they may elicit a heterogeneous differentiation response. It has also been suggested that phorbol ester may increase the stem cell population via a transient block in their differentiation (Diamond et al., 1978b).

Phorbol esters have been shown to induce sister chromatid exchange (Kinsella and Radman, 1978; Weinstein et al., 1979) and tumour promotion might therefore be the result of aberrant mitotic activity causing the expression of carcinogen-induced recessive mutations. Boutwell (1974) suggests that promoters may act as gene activators. However, phorbol esters have been shown to act on the cytoplasm and induce in enucleated cells similar morphological changes to those seen in intact cells (Nagle and Blumberg, 1980). Phorbol esters are also hyperplastic agents but the dose response for hyperplasia is different from that for promoting activity (Boutwell, 1974).

Multistage carcinogenesis has been demonstrated in many systems and some speculative ideas as to the mechanisms involved have been reviewed recently (Berenblum, 1979; Boyland, 1980a).

4.4.1 Promotion of Liver Carcinogenesis

An excellent review of this subject has recently become available (Farber, 1980). Two principal experimental approaches have been made. There is the model of differential inhibition as demonstrated by the Solt and Farber (1976) model. This involves the single (or short-term administration of an initiating carcinogen followed by continuous feeding with a different carcinogen during which the animals undergo partial hepatectomy. The basis for this is that initiated hepatocytes
(like preneoplastic nodules and hepatocellular carcinomas) are resistant to the cytotoxicity and mitoinhibitory action of the second carcinogen and are therefore able to proliferate in response to partial hepatectomy, while the normal hepatocytes are prevented from proliferating by the presence of the second carcinogen. Alternatively there is the model of differential stimulation such as prolonged phenobarbitone feeding as performed in this thesis. The mechanism of this stimulation is unclear but it may be that the transient mitotic response induced in normal hepatocytes by phenobarbitone is prolonged in initiated hepatocytes (Emmelot and Scherer, 1980). This method also selects many more histochemically altered foci than does the differential inhibition model. More than 90% of the induced foci undergo remodelling and only a very small proportion are destined to become tumours. At present it is not possible to distinguish between the stable and transient foci (Pitot and Sirica, 1980).

a) Action of Phenobarbitone as a Promoter

Phenobarbitone is a mitogen and this may be an important factor in its mechanism of action. Peraino et al. (1975) have observed that phenobarbitone and DDT (dichlorodiphenyltrichloroethane), which enhance liver carcinogenesis, increase DNA synthesis and liver weight whereas other microsomal enzyme inducers, amobarbital and diphenylhydantoin, neither stimulate DNA synthesis or liver weight increase, nor enhance tumour formation. Other xenobiotic inducers which stimulate adaptive liver growth, butylated hydroxytoluene and α-hexachlorocyclohexane, also promote liver tumours, and the mitotic index is higher in the preneoplastic nodule than in the surrounding tissues (Ohde et al., 1979).

Phenobarbitone also enhances the mitotic response to partial hepatectomy (Japundžić et al., 1967; Ruttiman, 1972; Tuczek et al., 1975) if administered prior to the operation. If phenobarbitone is administered at the time of partial hepatectomy, however, there is a delay of and decrease in DNA synthesis and mitotic activity (Becker and Lane, 1968; Burki et al., 1971; Schindler et al., 1975). This two-directional effect of phenobarbitone is analogous to the effect of phorbol esters on differentiation and is also reflected in the results obtained from
the study of phenobarbitone on enzymic differentiation presented in this thesis (Section 3.3.6). When neonatal rats were given phenobarbitone daily from the tenth day after birth until weaning and continuously thereafter there was a two-directional response in the appearance of the adult enzymes, glucokinase and malic enzyme. Prior to weaning phenobarbitone induced differentiation in that glucokinase activity was significantly elevated three days before weaning and malic enzyme was induced to appear on the day of weaning (Table 3.3.7). This is in keeping with the idea that phenobarbitone induces hyperdifferentiation as mentioned earlier (Section 4.3.1.a). However, three days after weaning both these enzymes were significantly reduced in phenobarbitone treated groups indicating an inhibition of differentiation. Thus it seems that phenobarbitone, like the phorbol esters, both induces and inhibits differentiation. The role of differentiation/dedifferentiation in tumour promotion is still therefore unclear.

In the present work other effects of phenobarbitone which might play a role in tumour promotion were observed. These were the acceleration of ageing, centrilobular hypertrophy (Section 4.3.1.a) and the induction of enzyme changes which were qualitatively similar to those occurring during carcinogenesis (Section 4.3.3.a). The relationship between ageing and cancer has previously been discussed (Pitot, 1977a). Centrilobular hypertrophy, by causing a reduction in blood flow, may create a favourable environment for tumour growth and might contribute to the enzymic alterations which, in turn may enhance tumorigenesis by creating a biochemical imbalance in cellular metabolism that facilitates tumour growth.

The observation that phenobarbitone, when given simultaneously with, or soon after, partial hepatectomy delays the time of onset of DNA synthesis and mitosis is similar to that of Craddock (1976) with dimethylnitrosamine as described in the Introduction (Section 1.5.2.c). She found that when dimethylnitrosamine was given six hours after partial hepatectomy there was a delayed time of onset of DNA synthesis and cell division but dimethylnitrosamine administration given twenty-four hours after partial hepatectomy did not inhibit DNA synthesis and cell division. The former experimental regime gave a low incidence of tumours
and the latter resulted in a high tumour incidence. She concluded that the delay in DNA synthesis was due to repair replication of DNA damaged by dimethylnitrosamine, when DNA synthesis was not delayed there was replication of damaged DNA which resulted in the high tumour incidence. However, phenobarbitone does not covalently bind to DNA, so that the delay in DNA synthesis need not necessarily be the result of DNA repair. Administration of CCl₄ can also cause a delay in DNA synthesis after partial hepatectomy (Schulte-Herman, 1974). Repair of damaged DNA cannot be said unequivocally to be the key event in cell-cycle susceptibility to carcinogenesis: DNA synthesis starts between fourteen and sixteen hours after partial hepatectomy and peaks at twenty-four hours (Leduc, 1964); foreign compound metabolism is reduced in partially hepatectomised animals (Gram et al, 1968; Hilton and Sartorelli, 1970) and the metabolism of the carcinogen would take some time. Thus, DNA damage may occur after the bulk of DNA replication has taken place in animals given carcinogen twenty-four hours after partial hepatectomy. Subsequent work (Ishikawa et al, 1980) in which diethylnitrosamine was administered before partial hepatectomy and phenobarbitone was given after the operation, does however indicate that DNA repair is related to the induction of enzyme altered islands considered to be precursors of tumours.

b) Cell Division and Tumorigenesis

That cell division enhances tumorigenesis is demonstrated by experiments with regenerating liver after partial hepatectomy or CCl₄ poisoning (Pound and McGuire, 1978a, b) and with young growing animals (reviewed by Craddock, 1978). However, the degree of stimulation of cell division by partial hepatectomy is not directly proportional to the degree of enhancement of carcinogenesis (Becker, 1979). My own speculations on this problem are that the dedifferentiated nature of dividing cells might be a contributory factor in the enhancement of tumorigenesis by cell division. The induction of immature metabolic patterns by partial hepatectomy or the presence of a pre-existing immature metabolism, such as in the foetal or young animal might provide a favourable situation for the growth of preneoplastic hepatocytes.
If dedifferentiation is a feature of carcinogenesis, it may be that carcinogens act primarily on dedifferentiated hepatocytes or the induction of cell division (and hence dedifferentiation) in cells altered by carcinogen interaction may allow the expression of the cancer phenotype. Dedifferentiated hepatocytes would be present at high frequencies in the young animal and in the adult liver after partial hepatectomy, but at low frequency in the resting adult liver unless the cytotoxicity of the carcinogen induces compensatory hyperplasia. The carcinogen may then inhibit further differentiation of the cell. The carcinogenic potential of a given compound may therefore depend partly on its cytotoxicity and partly on its ability to inhibit cell differentiation. The stimulatory effect of enhanced cell division on carcinogenesis need not necessarily depend on enhanced replication of DNA damaged by the carcinogen, it could also be due to the creation of a population of cells on which the carcinogen may act and/or the creation of a suitable biochemical milieu for the growth of abnormal cells as postulated (Fig. 4.3). The possibility that carcinogens themselves might inhibit differentiation could be investigated by administering a hepatocarcinogen to suckling rats and studying the behaviour of the late suckling cluster of enzymes at weaning as described for phenobarbitone (Section 3.3.6).
Figure 4.3 Postulated Mechanism of Involvement of Phenobarbitone and Cell Division in Liver Carcinogenesis

INITIATED CELL  

PHENOBARBITONE OR CELL DIVISION  

ALTED HISTOLOGY.  
ENZYME CHANGES.  
DEDifferentiation.  

TUMOURS

NORMAL CELL  

PHENOBARBITONE OR CELL DIVISION  

ALTED HISTOLOGY.  
ENZYME CHANGES SIMILAR TO TUMOUR OR DEDifferentiation.  

NO TUMOURS

INITIATED CELL  

NO PHENOBARBITONE NO CELL DIVISION  

NO ALTERED HISTOLOGY.  
NO ENZYMIC ALTERATION.  
NO DEDifferentiation.  

TUMOURS VERY RARELY
4.5 Summary of Biochemical Data on Foetal and Regenerating Rat Liver, Primary and Transplantable Hepatocellular Carcinomas and Host Livers of Tumour-Bearing Rats

The accumulated enzyme data in the present work compared with the enzyme data of Knox (1976) and Greengard (1979) are shown in Fig. 4.4 (i-ix). In general foetal livers, regenerating liver, transplantable hepatomas and primary tumours induced by chronic or two-stage hepatocarcinogenesis are similar (qualitatively, if not quantitatively) in their enzymic deviation from normal. The histological similarity of immature, regenerating and neoplastic liver has been summarised above (Table 4.1).

The acquisition of foetal characteristics during neoplastic transformation does not appear to be specifically linked to malignancy since similar foetal characteristics are expressed by non-malignant, regenerating liver. Other authors report similar histological and biochemical aberrations after toxic damage (c.f. Sections 4.1.2, 4.1.4). Ibsen and Fishman (1979) have reviewed the subject and cite numerous examples of foetal enzymes not only in hepatocarcinogenesis but also in regenerating liver after partial hepatectomy or toxic damage. Although they say it is not possible to clearly define the relation of oncodevelopmental gene expression to cancer they suggest that retrodifferentiation-redifferentiation is linked to the promotion phase.

The results reported in this thesis are consistent with the view held by Uriel (1979) that: a) resemblance between cancerous and foetal cells is highly significant since it stems from the convergence of biological properties; b) this resemblance is nevertheless not intrinsically specific to neoplasia since it can also be shown by non-cancerous cells and tissues growing under the influence of certain deleterious agents or in response to aggressions of varied aetiology; and c) retrodifferentiation seems to be a unique mechanism of cell rejuvenation underlying both neoplastic development and regenerative processes.

The understanding of the mechanisms involved in dedifferentiation is contingent upon advances in knowledge about the control of gene expression during normal differentiation. Parallels between cell
Figure 4.4 Enzyme Activities in Foetal, Regenerating Hepatomas and Host Liver: Combined Experimental and Previously Reported Data

F; foetus 14 - 16 days gestation
LF; foetus 19 - 20 days gestation
24; 24 hours after hepatectomy
72; 3 days after hepatectomy
CD; tumours arising after chronic diethylnitrosamine treatment
2S; tumours arising after 2-stage carcinogenesis
WD; WDA transplantable hepatomas
UA; UA transplantable hepatomas
HW; host liver of WDA tumour-bearing rats
HU; host liver of UA tumour-bearing rats
KF; foetal liver reported by Knox (1976)
KR; liver 24 hours hepatectomy reported by Knox
KI°; primary hepatoma reported by Knox
KS; slow-growing transplantable hepatoma reported by Knox
KU; undifferentiated rapidly-growing transplantable hepatoma reported by Knox
GH; host liver reported by Greengard (Herzfeld et al, 1980)

(i) TK; Thymidine kinase
(ii) HK; Hexokinase
(iii) G6PDH; Glucose 6-phosphate dehydrogenase
(iv) GDH; Glutamate dehydrogenase
(v) AAT; Aspartate aminotransferase
(vi) G6Pase; Glucose 6-phosphatase
(vii) PEPCK; Phosphoenolpyruvate carboxykinase
(viii) GK; Glucokinase
(ix) Malic; Malic enzyme
differentiation and carcinogenesis have been the subject of a review article (Dustin, 1972) and a book (Saunders, 1978). The mechanisms of the regulation of histone-coding gene expression in normal and neoplastic cells have been studied (Stein et al., 1978). The role of the various nuclear and cytoplasmic nucleic acids and proteins in gene regulation and the expression of the cancer phenotype have been reviewed by Busch (1979). The possibility that carcinogenesis may result from alterations in non-histone proteins leading to the inactivation of genes specifying adult functions and genes regulating cell division has been investigated (Gronow, 1980).

Furthermore investigations are currently in progress to elucidate the mechanisms of gene misregulation by carcinogens using a regulatory test system in Drosophila involving specific structural genes under the control of mobile DNA insertions, or transposable elements (Fahmy, 1980).

The genetic reprogramming in regenerating liver is unlikely to be the direct result of a mutation, but whether the underlying mechanism for the reprogramming of genetic information leading to cancer is mutational is as yet undetermined. Because of the possible involvement of mutational events in carcinogenesis, a study of the chronic effect of diethylnitrosamine on DNA alkylation and subsequent excision was carried out.
4.6 Enhancement of $\text{O}^6$-Alkylguanine Excision by Chronic Diethylnitrosamine Administration

If $\text{O}^6$-alkylguanine is the promutagenic lesion induced by alkylation agents, one would expect that chronic administration of diethylnitrosamine would inhibit the excision of $\text{O}^6$-alkylguanine, causing an increased chance of mis-coding, thereby leading to a high tumour incidence. However, this was not found to be the case - in fact there was a considerable reduction in the levels of $\text{O}^6$-ethylguanine in treated rats. This was evident after only one week pretreatment with diethylnitrosamine with further reductions in $\text{O}^6/N^7$ ratios after five and ten weeks pretreatment. Pretreatment did not affect $3'$-ethyladenine/$N^7$-ethylguanine ratios. The reduction of $\text{O}^6$-ethylguanine persistence in DNA suggests that chronic diethylnitrosamine administration enhances the repair capacity for this lesion. The repair process is not specific for $\text{O}^6$ ethylation as there is also a reduction of $\text{O}^6$ methylguanine, induced by dimethylnitrosamine, in the diethylnitrosamine pretreated rats.

This induction of a repair system is similar to the 'adaptive' response of bacteria i.e. enhanced DNA repair induced by pretreatment with low levels of an alkylating agent reduces the agent's toxicity and mutagenicity (Karran et al., 1979). The induction of $\text{O}^6$ alkylguanine repair is also seen after chronic dimethylnitrosamine treatment (Montesano et al., 1979) and again the effect was specific for $\text{O}^6$-methylguanine ($3'$-methyladenine levels were unaffected by pretreatment). The repair is probably due to enhanced enzyme activity, rather than redistribution of the $\text{O}^6$-alkylation to other regions where removal is more rapid, as cell-free liver preparations from rats pretreated with dimethylnitrosamine have an enhanced capacity for $\text{O}^6$ repair of methylated DNA in vitro (Montesano et al., 1980). Renard and Verly (1980a, b) have isolated a factor from rat liver chromatin which induces the disappearance of $\text{O}^6$-ethylguanine from DNA after ethylnitrosourea alkylation. This factor appears to be an enzyme rather than a stoichiometric reagent in that it is inactive at $0^\circ C$, remains active after two hours at $37^\circ C$ in the presence of excess substrate and is competitively inhibited by the reaction product.

Not only does pretreatment with alkylating agents enhance the
capacity of rat liver for the excision of $O^6$-alkylguanine but pre-
treatment with other carcinogens also has this effect. The two
carcinogens tested, acetaminofluorene and aflatoxin B$_1$ both induce
$O^6$-methylguanine excision after a single dose of $[^{14}C] \text{dimethylnitrosamine}$ (Buckley et al., 1979; O'Connor and Margison, 1981).

Metabolic activation of the carcinogen seems to be necessary
for the induction of $O^6$ excision, pretreatment with the direct acting
alkylating agent $N$-methyl-$N$-nitrosourea has no effect, or an inhibitory
(or overloaded) effect on the repair of $O^6$-methylguanine after a
single dose of $N[^{14}C]\text{-methyl-}N\text{-nitrosourea}$ (Margison, 1981) or
$[^{14}C] \text{dimethylnitrosamine}$ (O'Connor and Margison, 1981) whereas pre-
treatment with 1,2-dimethylhydrazine, an agent metabolised to an
alkylating species via a different route from that of dimethylnitro-
samine does enhance the repair of $O^6$-methylguanine after a single dose
of $[^{14}C] \text{dimethylnitrosamine}$ (Margison, 1981). Coupled with the
observation that dimethylnitrosamine pretreatment fails to enhance
$O^6$-methylguanine disappearance after a single dose of $N[^{14}C]\text{-methyl-}
N\text{-nitrosourea}$ (Margison et al., 1976b) these results suggest that the
direct acting alkylating agents alkylate a different cell population
from that alkylated by those requiring metabolic activation. The
enzymes necessary for the metabolic activation of dimethylnitrosamine
to the toxic alkylating species are located preferentially in the
centrilobular region and therefore the toxic changes are seen in this
region (Pegg, 1977). $N$-methyl-$N$-nitrosourea has a very short half-
life \textit{in vivo} and might therefore be expected to alkylate periportal
cells preferentially.

Induction of repair of alkylation at the $O^6$ position of guanine
should result in a reduced tumour incidence, however the pre-treatment
regime clearly induces a large number of tumours. One explanation for
this paradox is that the induced repair system might be error-prone
(Kleihues et al., 1979). Also, most of the agents which induce $O^6$-alkyl
guanine repair also damage the DNA and are hepatotoxic as well as
hepatocarcinogenic and thus cause restorative hyperplasia and DNA
replication. This might be the most important factor as far as hepato-
carcinogenesis is concerned as alkylated DNA can be replicated (Craddock
and Henderson, 1978). Hence the protective effect of $O^6$ repair against
tumour induction might be more than offset by the induced proliferation of cells and replication of faulty DNA.
4.7. Relevance of the Histological and Biochemical Data to Human Cancer

As described in the Introduction (Section 1.2.) there are many pathological and etiological similarities between human and rat liver tumours. The human liver also exhibits phase-specific profiles of enzyme activity which show sequential similarities to those observed in the rat. Thus, during the second trimester enzymes belonging to the late foetal cluster in rats are present at about half the adult level but those belonging to the neonatal and late suckling cluster are present at very low levels or undetectable (Greengard, 1977). It is a major conclusion from the present work that hepatocarcinogenesis is accompanied by dedifferentiation. The dedifferentiation is detectable by measuring enzymes that exhibit phase-specific profiles of activity. If a similar process is operating in human cancer then similar changes in the activity of phase-specific enzymes might be expected to occur.

In general the enzyme pattern of human hepatocellular carcinomas differs from normal human liver with respect to the same enzymes showing differences between rat hepatoma and normal rat liver (Boxer and Shonk, 1966). Reduced phosphoenolpyruvate carboxykinase and glucose 6-phosphatase and other gluconeogenic enzyme activities are observed in human liver tumours, like their rodent counterparts (Yeung et al., 1973; Cyanis et al., 1975; Hammond and Balinsky, 1978a). The increase in hexokinase and other glycolytic enzymes was not as consistent as in rat hepatomas, however (Balinsky et al., 1973; Cyanis et al., 1975) though there is overall a loss of adult enzymes and gain of foetal enzymes (Cyanis et al., 1975; Goto et al., 1977; Hammond and Balinsky, 1978b). Enzymes of pyrimidine and DNA synthesis are elevated in both rat and human liver tumours with the exception of thymidine kinase which was not elevated in the human hepatomas (Cummins and Balinsky, 1980). The elevation of glucose 6-phosphate dehydrogenase in rat liver carcinogenesis as described in this thesis is also observed in human liver tumours (Balinsky et al., 1973; Yeung et al., 1973). There is also a low hepatoma frequency in glucose 6-phosphate dehydrogenase deficient males in a Bantu population. The high incidence of glucose 6-phosphate dehydrogenase deficiency in this population may not only be due to its
protective effect against malaria but also because of a reduced mortality from liver tumours (Mbessa et al., 1978). The similarity between human and rodent liver tumours means that the short-term in vivo carcinogenicity tests proposed (page 220) are likely to be particularly relevant to the detection of agents potentially hepatocarcinogenic in humans.

Human liver tumours, when assayed for adenosine triphosphatase and glucose 6-phosphatase deficiency and γ-glutamyl transpeptidase elevation exhibit phenotypic heterogeneity with respect to these markers, like rat tumours (Gerber and Thung, 1980). Cell-lines derived from various human tumors, including liver tumours, showed no predictable coexpression of markers of carcinogenesis - in fact there was a negative correlation between markers such that when one was found it was unlikely that another marker would be exhibited by that tissue (Neuwald et al., 1980). For this reason and because of the lack of specificity of any single biochemical or pathological marker to carcinogenesis, the use of a battery of biochemical and pathological characteristic should be employed for diagnostic purposes. At present the diagnosis is often made by histological and cytological investigation of liver biopsy material (taken with or without the direction of liver scans). About 70% of patients with liver cancer being detected in this way (Tao et al., 1979). False positives may arise because of the cytological similarity of malignant hepatocytes with regenerating hepatocytes in inflammatory lesions (Atterbury et al., 1979). Histochemical enzyme analysis of these biopsy specimens should produce greater diagnostic success. However, in view of the heterogeneity of both preneoplastic nodules and hepatocellular carcinomas a battery of histological, cytological and biochemical criteria should be investigated to determine the overall pattern of the change. Many currently accepted markers, such as α-fetoprotein, γ-glutamyl transpeptidase and glucose 6-phosphatase, are found in non-neoplastic lesions (see above, Sections 4.1.2, 4.1.4) and the use of a battery of markers together with a further study of non-neoplastic lesions to identify discriminants (possible phosphoenolpyruvate carboxykinase elevation in non-malignant hyperplasia) may help distinguish neoplastic from non-neoplastic lesions. It should be emphasised that there is a large degree of histological and biochemical
overlapping between tumours and non-malignant lesions and therefore knowledge of the clinical history of the patient is of undoubted value. I believe also that this overlap will render the search for a single neoplastic marker futile for the reason that retrodifferentiation is the underlying mechanism involved in both regenerative processes and neoplastic development. Study of a battery of enzymes may reveal metabolic imbalances more likely to be indicative of cancer than of non-malignant hyperplasia. Future studies designed to aid tumour diagnosis should therefore bear in mind the similarities between malignant and non-malignant hyperplasia and concentrate on measurements of multiple histological and biochemical criteria. Studies aimed at the control of neoplasia could be directed at investigation of regenerating liver in order to elucidate those factors which enable the tissue to redifferentiate into normal tissue in this situation.
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