Br. J. exp. Path. (1975) **56**, 103

DIVERSE MECHANISMS OF HEPATOCELLULAR INJURIES DUE TO CHEMICALS: EVIDENCE IN RATS ADMINISTERED CARBON TETRACHLORIDE OR DIMETHYLNITROSAMINE

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Received for publication October 2, 1974

Summary.—Differences in acute hepatotoxicity of carbon tetrachloride (CCl_4) and dimethylnitrosamine (DMN) have been tested in normal foetal, newborn and adult rats, foetal, newborn and adult rats pretreated with phenobarbitone and partially hepatectomized adult rats. While CCl_4 is non-toxic to the foetal and newborn liver, DMN induces identical necrosis at all ages. Prior dosing with phenobarbitone augments CCl_4 toxicity only in the adult and the newborn but the foetus continues to be resistant. Such pretreatment, on the other hand, significantly reduces the effects of DMN on liver in all animals. Partial hepatectomy makes the liver less susceptible to CCl_4 and more so to DMN. Such diversities of hepatic response to the two toxins can be accounted for by the levels of the respective toxifying enzymes in the liver cell in different situations.

AN IMPRESSIVE number of chemicals, including several commonly used drugs, possess hepatotoxic properties (Sherlock, 1968; Rouiller, 1964) manifested as one or more of a wide range of alterations with varying degrees of severity. These occur in spite of the liver cell being endowed with rich detoxifying enzyme systems (Gillette, 1963). It is in fact these very drug metabolizing enzymes which paradoxically convert certain chemicals to potent hepatotoxic agents (Slater, 1966; Recknagel, 1967). Physiological detoxification is carried out mainly by an electron transferring enzyme chain located in the hepatocytic microsomes. It is now known that the most extensively studied hepatotoxic chemical, carbon tetrachloride (CCl_4) is noxious not by itself but only through a metabolite liberated either at the cytochrome P450 or the TPNH cytochrome-C-reductase terminal of this microsomal enzyme chain (Chopra *et al.*, 1972; Castro *et al.*, 1968). Procedures which modify these enzymes one way or the other, not only correspondingly alter physiological conjugations (Catz and Yoffe, 1962; Trolle, 1968) but augment or reduce injury induced by CCl_4 (Chopra *et al.*, 1972; McLean and McLean, 1966; McLean, 1967; Nayak, Chopra and Ramalingaswami, 1970; Garner and McLean, 1969; Stenger, Miller and Williams, 1970). The various morphological, biochemical and topographic alterations in the liver induced by CCl_4 seem to be directly related to the presence and quantity of these enzymes (Chopra *et al.*, 1972; Nayak *et al.*, 1970). Recent work carried out by us and also by others has shown that by certain prior manipulations a state of insensitivity or tolerance to this chemical can be achieved (Das, Chopra and Nayak, 1974; Dambrauskas and Cornis, 1970; Ugazio, Koch and Recknagel, 1972, 1973).

Dimethylnitrosamine (DMN), another potent hepatic toxin, has also to be metabolized by the liver cell before exerting its injurious effect (McLean and Verschuren, 1969; Murvish and Sidransky, 1971; Venkateshan, Argus and Acces, 1970; Pound, Horn and Lawson, 1973*a*). These two chemicals, CCl_4 and DMN,

not only produce variable centrizonal liver cell necrosis in several species of animals but are believed to induce hepatic lesions in the human almost identical to those in rats (IARC Monograph, 1972). However, unlike carbon tetrachloride, DMN is strongly oncogenic to the rat liver either in a single large or repeated small doses (IARC monograph, 1972; Magee and Barnes, 1959). While enough information is not yet available on the subject, there is evidence to indicate that the pathway of DMN metabolism in the liver may be distinct from that of CCl_4 (McLean and Verschuren, 1969; Pound, Lawson and Horn, 1973*b*). During some of our studies on nitrosamine induced oncogenesis, we observed certain distinct differences between the toxic effects of this chemical and those of CCl_4 in different situations. The present communication is a report on the findings of a study designed to define, in clearer terms, the differences in hepatotoxic potential of the two chemicals and to explore the possible reasons for these.

MATERIALS AND METHODS

Animals.—Groups of normal foetal, newborn and adult rats, foetal, newborn and adult rats pretreated with phenobarbitone and partially hepatectomized young adult rats were administered CCl_4 or DMN as shown in Table I. For studies on foetuses, the chemicals were injected subcutaneously in the back of 17–20 day pregnant animals in dosages appropriate to provide enough concentrations to the foetus. Intraperitoneal injection was avoided because of the risk of direct puncture of a foetus or amniotic sac. Direct injection of toxins into the foetus after laparotomy was also considered unnecessary. Foetuses pretreated with phenobarbitone *in utero* were administered the toxic chemicals after birth. All newborns were injected with the various chemical solutions subcutaneously in the shoulder region within 1–4 h of birth. They were then allowed to be nursed until they were killed.

Pretreatment with phenobarbitone consisted of 3 daily injections of the chemical. In adult and newborn rats the toxic chemical was administered 24 h after the last injection while in the case of foetuses, since the exact time of birth could not be predicted, the time gap between the last injection of phenobarbitone and intoxication varied between 8 and 24 h. Partially hepatectomized rats were given the toxins 24 h after surgery. Adult rats were given DMN and phenobarbitone by the intraperitoneal route and CCl_4 by intragastric intubation.

Chemicals.—Dimethylnitrosamine was prepared synthetically and its purity tested (courtesy of the Department of Biochemistry, Patel Chest Institute, Delhi University). Solutions in physiological saline were injected in dosages varying between 5 and 50 mg/kg body weight. Carbon tetrachloride (BDH, analytical reagent) was administered as solution in paraffin oil at dosages of 100–500 μl/100 g body weight. Aqueous solution of sodium phenobarbitone was injected in a dose of 80 mg/kg (Table I). Along with every experimental group control animals were injected physiological saline or liquid paraffin in volumes comparable with those of the toxic chemicals (Table I).

Surgery and mode of death.—Partial hepatectomy was performed on young adult rats

TABLE I.—*Outline of Experimental Set-up**

Animal	Pretreatment	Controls	Hepatotoxin dose: CCl_4 (u. l/100 g)	Hepatotoxin dose: DMN (mg/kg)	Sacrifice (h after intoxication)
Foetus (14)	None	(4)	200 to mother (4)	50 to mother (6)	24
Foetus (26)	Phenobarbitone	(6)	200 (12)	30 (8)	24
Newborn (16)	None	(4)	200 (6)	30 (6)	24
Newborn (12)	Phenobarbitone	(4)	200 (4)	30 (4)	24–48
Adult (18)	None	(4)	100–500 (8)	15–50 (6)	24–48
Adult (14)	Partial hepatectomy	(2)	35, 170† (6)	5† (6)	24–96
Adult (10)	Phenobarbitone	(2)	100 (4)	30 (4)	24

* Figures within parentheses indicate number of animals used.

† Calculated in terms of equivalent dose in animals with intact liver: 100 and 500 μl/kg for CCl_4 and 15 mg/kg for DMN.

weighing 80–120 g according to the method of Higgins and Anderson (1931), resulting in ablation of approximately two-thirds of the total liver. For the first 24 h after surgery, the animals were denied food and allowed free access to a 20% glucose solution in water.

All animals were killed 24–96 h after intoxication. Foetuses and newborns were decapitated while older animals were quickly exsanguinated by cutting the heart following light ether anaesthesia.

Structural and histochemical examinations.—After removal of the liver, some representative slices were transferred immediately to the cryostat maintained at −20° and others were fixed in neutral buffered formalin. Fresh cryostat sections were stained for lipid by oil red O and for glucose-6-phosphatase (G-6-Pase) (Chopra *et al.*, 1972). Paraffin sections of formalin fixed tissue were stained with haematoxylin and eosin. Ultrastructural examination of the liver was carried out in foetuses and newborns treated with 3 doses of phenobarbitone. After the usual fixation of tiny bits of fresh tissue in 1% osmium tetroxide in veronal buffer, dehydration in alcohol and Epon embedding, sections were stained with uranyl acetate and lead hydroxide and examined under a Hitachi HU-11A electron microscope.

Biochemical estimation.—Hepatic triglycerides were estimated in some groups of foetuses and newborns according to the method of van Handel and Zilversmit (1957) using livers removed at the time of killing.

RESULTS

Carbon tetrachloride toxicity

In the adult animal centrizonal loss of G-6-Pase increased accumulation of lipid and necrosis of centrizonal hepatocytes occurred consistently, the extent and severity of lesion being directly related to the dose of chemical administered (Chopra *et al.*, 1972; Das *et al.*, 1974). Not infrequently a row of balloon cells surrounded the area of necrosis.

The liver of untreated foetuses, newborns and foetuses administered phenobarbitone showed no significant alterations following exposure to CCl_4 both at 24 and 48 h. There was no loss of G-6-Pase, no excess of lipid and no necrosis when compared with corresponding controls (Fig. 1a, 1c). Some of the control untreated newborn rats had demonstrable droplets of lipid in the liver, distributed diffusely, while pretreatment with phenobarbitone considerably increased stainable hepatic lipid (Fig. 1e). Thus, in all animals previously exposed to this chemical, excess of hepatic lipid was found irrespective of whether or not they were subsequently treated with CCl_4.

Newborns pretreated with phenobarbitone when exposed to CCl_4, on the other hand, showed distinct changes in the liver. Characteristic balloon cells, either diffusely distributed or arranged in a row some distance away from central vein (Fig. 2), focal or centrizonal areas of necrosis, degenerating cells with clumped eosinophilic cytoplasm and hazy outlines and necrobiotic cells resembling Councilman bodies (Fig. 2), were seen in varying combinations in every animal. The zonal pattern of necrosis as seen consistently in adult animals, was however, not so clearly apparent. Loss of G-6-Pase was evident in the centrilobular area. Presence of excess lipid, on the other hand, could not be appreciated since even the controls treated either with saline or liquid paraffin showed relatively heavy steatosis induced by phenobarbitone.

In adult rats pretreated with phenobarbitone, CCl_4 induced explosive lesions far in excess of those in normal rats given identical doses of the toxin. Detailed descriptions of these lesions have been reported by us earlier (Chopra *et al.*, 1972; Nayak *et al.*, 1970).

Partially hepatectomized animals treated with the non-lethal dose of 35 μl/kg or lethal dose of 170 μl/kg showed alterations considerably less severe than those induced in normal animals given comparable dosages (100 and 500 μl/kg respectively) of the toxin observed in our earlier studies (Chopra *et al.*, 1972, Nayak *et al.*, 1970; Das *et al.*, 1974). All parameters, loss of G-6-Pase, lipid accumulation and extent and severity of necrosis were less. Also, unlike in

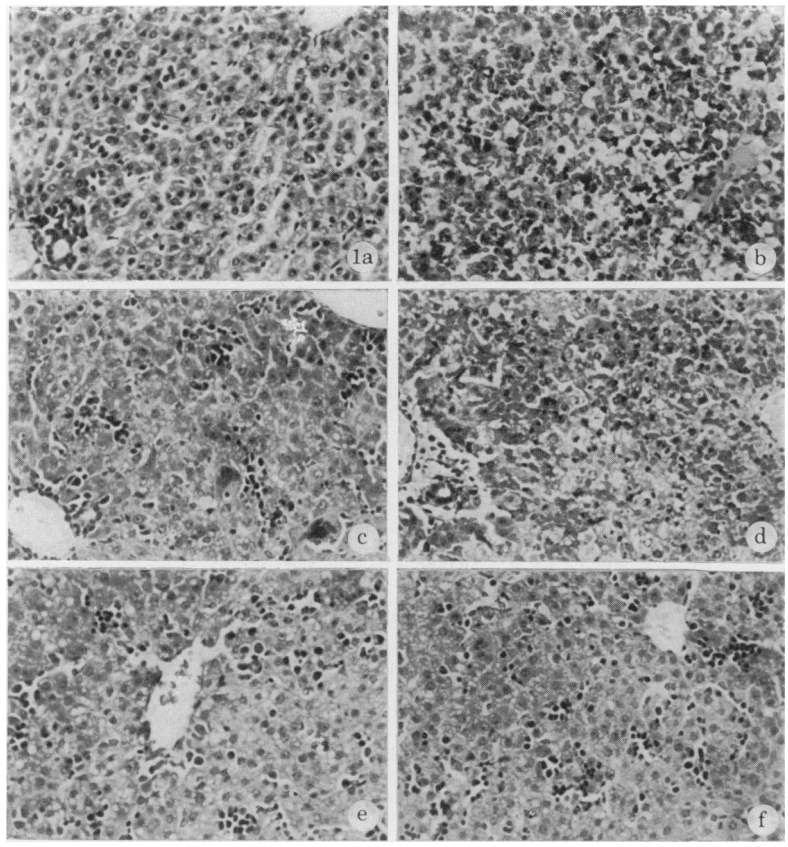

FIG. 1.—Effects of CCl_4 and DMN on livers of normal untreated rat foetus (a, b), untreated newborn rat (c, d) and of rat foetus pretreated with phenobarbitone (e, f). CCl_4 shows no necrosis in any of the three. DMN on the other hand produces haemorrhagic necrosis in normal foetus and newborn livers (b, d) which is almost completely prevented by prior treatment with phenobarbitone (f). Phenobarbitone pretreated livers show a significant number of fatty vacuolations. Foci of haemopoiesis, including megakaryocytes, so prominently seen are normal for this age. H. and E. ×145.

the normal rat, the larger dose was not fatal.

Dimethylnitrosamine toxicity

In the adult animal a 15 mg dose induced only mild centrizonal congestion of the sinusoids but no necrosis or steatosis (Fig. 3a) while with the 30 and 50 mg dose, in addition to intense congestion and haemorrhage, half to two-thirds of the lobule showed necrosis of the haemorrhagic or sometimes of the coagulative type (Fig. 3b). No significant fatty change or balloon cells were seen. There was loss of G-6-Pase in the central necrosed area.

Normal foetuses and newborns given DMN in doses of 30–50 mg/kg showed intense congestion and haemorrhagic necrosis of the centrizonal area identical to those in adults (Fig. 1b, 1d). In foetuses, newborns and adults previously exposed to phenobarbitone on the other hand, identical doses of DMN produced only mild congestion without necrosis, almost like what was seen in the adult given the 15 mg dose (Fig. 1f, 3c).

In control animals no hepatocellular damage or other alterations were seen.

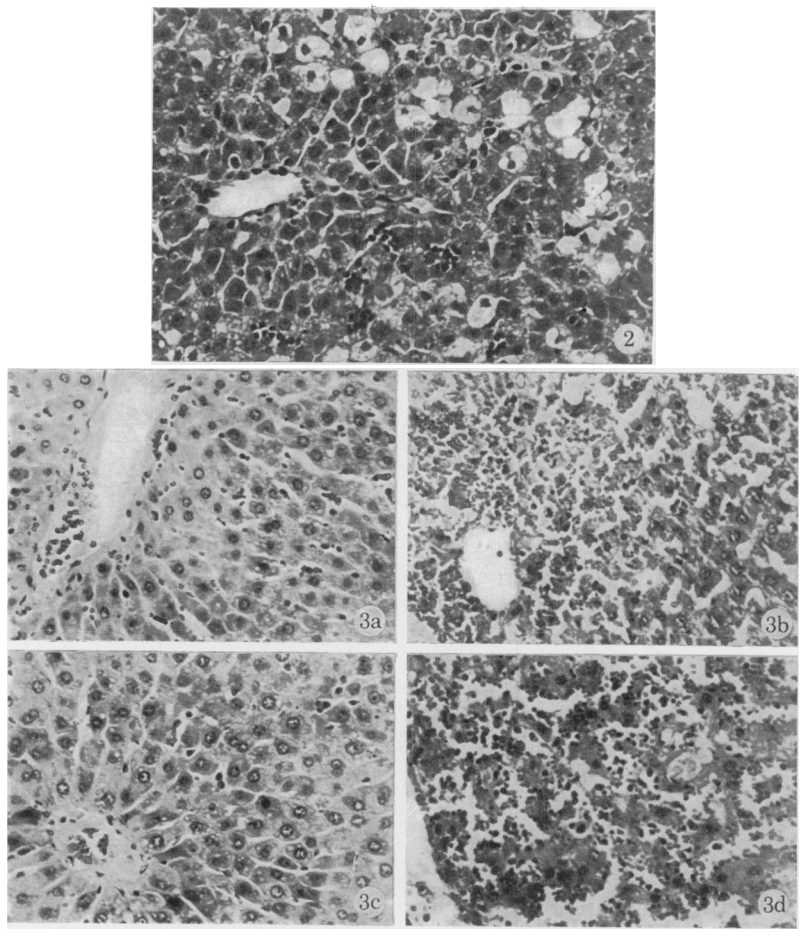

FIG. 2.—Liver of newborn rat first treated with phenobarbitone and subsequently given CCl_4. Balloon cells, necrobiotic hepatocytes with clumped, swollen cytoplasm and Councilman-like bodies are clearly seen. H. and E. ×280.

FIG. 3.—DMN hepatotoxicity in normal, partially hepatectomized and phenobarbitone pretreated adult rats. In the normal animals, the 15 mg dose (a) produces only congestion but almost no necrosis, whereas with the 30 mg dose (b) significant haemorrhagic necrosis of the central area of the lobule occurs. In the phenobarbitone pretreated animal, on the other hand, (c) the same 30 mg dose appears to have very little toxic effect. In the partially hepatectomized rat (d) 5 mg of toxin/kg equivalent to the 15 mg dose in the intact animal produces marked necrosis. H. and E. ×145.

Partially hepatectomized animals treated with 5 mg DMN/kg equivalent to the non-lethal, non-necrogenic 15 mg dose for the normal rat with intact liver, showed centrizonal necrosis, most prominently developed 48–96 h after intoxication (Fig. 3d). Several animals in fact died during this phase with lesions identical to those induced by a 30–50 mg dose in the normal animal.

Control animals receiving physiological saline showed only features of hepatocytic regeneration.

Electron microscopy

In foetuses pretreated with phenobarbitone *in utero*, hepatocytes were somewhat larger compared with those of adults and their nuclei appeared relatively prominent. A number of lipid

droplets, some of them of considerable size, were distributed throughout the cytoplasm. Often these were seen to be coalescing. The endoplasmic reticulum was not proliferated and in fact the smooth components were few and far between. Other cytoplasmic and nuclear components were qualitatively and quantitatively normal. In the newborn rats treatment with phenobarbitone induced almost identical steatosis but in addition a mild proliferation of the smooth endoplasmic reticulum was seen. The latter was quantitatively less than that observed by us earlier in adult rats administered phenobarbitone (Chopra *et al.*, 1972).

The values for hepatic triglycerides in groups of animals that were studied are presented in Table II. Carbon tetrachloride does not appear to induce any steatosis in the foetal or newborn liver. Phenobarbitone administration, on the other hand, does increase liver triglycerides though in phenobarbitone pretreated foetuses CCl_4 does not increase the lipids any further.

TABLE II.—*Hepatic Triglyceride Values in some of the Experimental Groups*

Group	Hepatic triglyceride* (mg/g of wet weight)
Foetus, untreated	2·1
Foetus, CCl_4 treated	2·0
Foetus, phenobarbitone treated	3·55
Foetus, phenobarbitone and CCl_4 treated	3·70
Newborn, untreated	2·65
Newborn, CCl_4 treated	2·8

* Mean of 3 estimations.

DISCUSSION

Carbon tetrachloride and dimethylnitrosamine are both well known for their hepatotoxic property in the adult animal, the nature of two injuries being only slightly different. While acute necrosis of centrizonal hepatocytes is common to both, CCl_4 produces a significant steatosis and in injury induced by DMN marked sinusoidal congestion and haemorrhage occur. The present study has shown other distinct differences between the acute hepatotoxic potential of the two chemicals in a variety of situations (Table III). Carbon tetrachloride, even in twice the average adult toxic dose, shows no effect on the foetal or newborn liver. This phenomenon is well documented by earlier workers (Dawkins, 1963; Bhattacharyya, 1965). In fact, these observations provided some of the initial clues to the concept of enzymatic mediation of CCl_4 toxicity and it is now believed that livers of newborn and foetal rats lack certain microsomal enzyme systems which convert CCl_4 into its toxic metabolite (Slater, 1966; Recknagel, 1967).

TABLE III.—*Comparison of Hepatotoxicity due to Carbon Tetrachloride and Dimethylnitrosamine in Various Experimental Groups*

Group	Hepatotoxicity*	
	CCl_4	DMN
Foetus, untreated	0	++
Foetus, phenobarbitone treated	0	±
Newborn, untreated	0	++
Newborn, phenobarbitone treated	+	±
Adult, untreated	++	++
Adult, phenobarbitone treated	+++	± to 0
Adult, partially hepatectomized	+	+++

* ++ = necrosis with or without other regressive changes comparable to adult, +++ = more than ++, + = slightly less than ++, ± = significantly less than +, 0 = no significant lesion.

Augmentation of microsomal enzymes, particularly of those involved in drug metabolism, including cytochrome P450, by phenobarbitone or DDT considerably increases the subsequent toxic manifestation of CCl_4 in the adult animal (Chopra *et al.*, 1972; McLean and McLean, 1966; Nayak *et al.*, 1970; Garner and McLean 1969; Stenger *et al.*, 1970). Peculiarly enough, in foetuses pretreated with phenobarbitone *in utero*, the normal insusceptibility of the liver at birth to

CCl_4 could not be altered. Phenobarbitone, nevertheless, appears to be acting on these livers as excess lipid demonstrable by histochemical and ultrastructural alterations was consistently seen even though the explanation for this steatosis is not entirely clear. On the other hand, administration of phenobarbitone after birth quickly made the liver prone to injury by CCl_4 though not to the same extent as in the adult untreated animal (Chopra *et al.*, 1972, Nayak *et al.*, 1970; Reynolds and Yee; 1968). Dawkins (1963) and Bhattacharyya (1965) have shown that susceptibility to the type of hepatic injury induced by CCl_4 in adult rats is attained in the newborns not earlier than 6 or 7 days of life. Phenobarbitone treatment of our newborn rats has brought forward this susceptibility age to the 4th postnatal day. These findings indicate that while hepatic microsomal enzymes responsible for toxic manifestations of CCl_4 are lacking both in the livers of foetal as well as newborn rats, they seem to appear only when phenobarbitone is administered after birth (Fig. 4). In the adult animals, phenobarbitone induces microsomal enzymes responsible for toxifying CCl_4 (Chopra *et al.*, 1972; Nayak *et al.*, 1970; Garner and McLean, 1969; Stenger *et al.*, 1970), and also increases the organelle component housing them, namely smooth endoplasmic reticulum (Herdson, Garvin and Jennings, 1964; Orrenius, Ericsson and Ernster, 1965). In our experiments, following phenobarbitone treatment this component showed no proliferation in foetuses and only mild increase in newborns, directly paralleling their respective responses to CCl_4 toxicity (Table III). It appears, therefore, that along with the rapid alterations in enzymatic and structural makeup of the rat hepatocyte occurring at birth (Dallner, Siekevitz and Palade, 1966) the adult type response to phenobarbitone stimulation also appears only at this phase, possibly as a result of certain changes in regulating or operative gene. Unlike CCl_4, DMN produces centrilobular necrosis and sinusoidal congestion in foetal and newborn livers comparable with those in the adult. Thus, the enzyme system responsible for the toxic manifestation of DMN is present equally at all ages, pre- and postnatally. While centrizonal haemorrhagic necrosis due to DMN is well documented in adult rats (Butler and Hard, 1971), Bhattacharyya (1965) reported negative results in livers of suckling rats exposed to DMN administered through the mother's diet. This may be because he used a very small non-necrogenic dose of approximately 10 mg/kg. Recent studies seem to suggest that it is not DMN itself but a metabolite of this chemical that induces necrosis and tumours of the liver cell (McLean and Verschuren, 1969; Murvish and Sidransky, 1971; Venkatesan *et al.*, 1970; Pound *et al.*, 1973*a*, *b*). Microsomal demethylases convert DMN to alkylating derivatives, which possibly exert the oncogenic effect through incorporation in DNA and RNA while formaldehyde liberated as a byproduct may well be

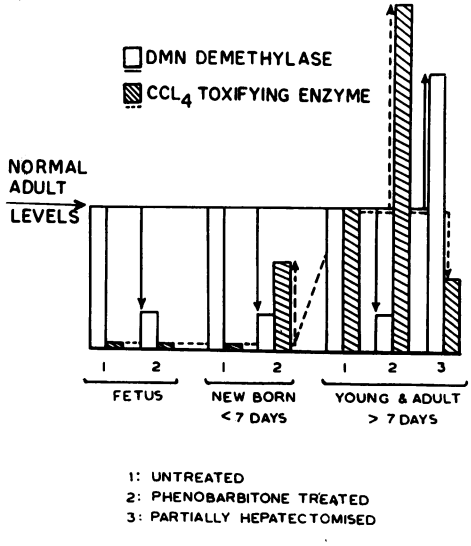

FIG. 4.—Hypothetical levels of hepatocytic enzymes which convert DMN and CCl_4 to their active toxic metabolites at various ages in the normal rat and their responses to treatment by phenobarbitone and partial hepatectomy.

responsible for necrosis. That the foetal and newborn livers contain adequate concentrations of the DMN metabolizing enzyme is apparent from the fact that liver tumours can be easily induced by DMN administered during these early periods of life (Toth, Magee and Subik, 1964; Toth, 1968). Also, procedures leading to a quick drop in the level of this enzyme significantly reduce hepatotoxicity following DMN administration and breakdown of this chemical in the liver (Pound *et al.*, 1973*a*, *b*).

In our experiments, prior treatment of foetuses, newborns and adults with phenobarbitone reduced the toxic effect of DMN inasmuch as no significant necrosis was apparent. This again is the converse of what is seen in the case of CCl_4 under identical conditions (Table III) and may be due to reduction of demethylation of DMN. There is evidence to indicate that this enzyme can be induced not by phenobarbitone but by benzo-α-pyrene (McLean and Verschuren, 1969). A reduction in metabolism of DMN and DEN (diethylnitrosamine) following the administration of phenobarbitone has recently been reported (Murvish and Sidransky, 1971; Venkatesan *et al.*, 1970; Magour and Nievel, 1971).

Since amelioration of DMN toxicity due to this chemical was also observed by us in the foetal as well as post-natal period, it may be assumed that in the rat, the enzyme toxifying DMN is identically controlled and expressed in all developmental phases (Fig. 4).

Partial hepatectomy offers yet another situation to highlight the difference in CCl_4 and DMN induced liver injury. In this model, toxic effects of the two chemicals were respectively decreased and heightened as compared with those in the adult animal possessing intact liver (Table III). A regenerating liver has been shown to be somewhat resistant to CCl_4 but we believe that its heightened susceptibility to the necrogenic effect of DMN, as observed in our study, has not been described earlier. Since the dose of DMN administered to our partially hepatectomized animals was corrected for the reduction of total liver tissue available, the increased toxicity is not due to excessive level of chemical. Craddock (1971) showed a remarkably high yield of tumours in regenerating rat livers following only a single exposure to a relatively small dose of DMN. He did not however study the acute effects of the toxin. We have ourselves observed a marked polyploidy of the liver 3–6 weeks after a single small dose of DMN administered 24 h after two-thirds hepatectomy (unpublished observation).

The entire spectrum of enzyme alterations occurring in regeneration following ablation of two-thirds of the liver is not precisely identified. However, it has been recognized that in this situation the hepatocytes partly acquire some properties of the foetal phase, namely reappearance of alpha foetoprotein synthesis (Gilli and Masseyeff, 1974; Nayak *et al.*, 1975). It may be that other enzymatic environments also shift towards what is prevalent in the foetal period, at least partly explaining the pattern of altered response to the toxins.

It is possible that different toxins are metabolized in the liver through a variety of enzymatic pathways. Thus, their effects and alterations would not only be mediated through different channels but would be entirely dependent on the concentrations of such enzymes.

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