Biochem. J. (1966) **100**, 564

The Effect of Diet and 1,1,1-Trichloro-2,2-bis-(*p*-chlorophenyl)ethane (DDT) on Microsomal Hydroxylating Enzymes and on Sensitivity of Rats to Carbon Tetrachloride Poisoning

BY A. E. M. McLEAN AND ELIZABETH K. McLEAN
Toxicology Research Unit, Medical Research Council Laboratories, Woodmansterne Road, Carshalton, Surrey, and Department of Pathology, Royal Free Hospital, London, W.C. 1

(*Received* 1 *February* 1966)

1. Protein-depleted rats are resistant to the lethal effects of carbon tetrachloride. The LD_{50} is 6·4ml./kg. in stock rats and 14·7ml./kg. in rats fed on protein-free diets. 2. Protein-depleted rats are resistant to carbon tetrachloride in its effect on the liver as judged by histology, accumulation of liver water, and plasma enzyme and bilirubin measurement. 3. The protection is present after feeding rats on a no-protein diet for 4 days. It is present after feeding rats on a 3%-casein diet, and partly found after feeding rats on a 6%-casein diet. 4. The activities of the microsomal enzymes that demethylate Pyramidon and hydroxylate benzopyrene in the liver fall by over 80% in rats fed on the no-protein diet for 4 days or more, or in rats fed on a 3%-casein diet. A 50% fall is found in rats fed on a 6%-casein diet. 5. A single dose of DDT or three doses of phenobarbitone cause increased microsomal enzyme activity in protein-depleted rats. 6. The animals are then sensitive to the lethal and liver-damaging effects of carbon tetrachloride. 7. DDT dosage also leads to increased sensitivity to carbon tetrachloride in rats fed on stock diets. 8. These findings support the hypothesis that carbon tetrachloride is metabolized by microsomal enzymes to form the true toxic compound.

There is a widely held belief that animals fed on a diet deficient in protein are especially susceptible to poisons that affect the liver (Miller, 1948; Drill, 1952; Rouiller, 1964; for review see McLean, McLean & Judah, 1965). In some experiments on veno-occlusive disease of the liver (McLean, Bras & McLean, 1965) carbon tetrachloride was given to rats fed on a low-protein diet. Unexpectedly, the animals proved resistant to the poison and we decided to investigate this phenomenon. Campbell & Kosterlitz (1948) made histological observations that also indicated that protein-depleted rats were resistant to carbon tetrachloride. Carbon tetrachloride is known to be metabolized by liver microsomes (Rubinstein & Kanics, 1964). It has been suggested that metabolism might be necessary for the toxic effects (McCollister, Beamer, Atchison & Spencer, 1951; Butler, 1961; Wirtschafter & Cronyn, 1964; Snodgras & Pirias, 1965; Reynolds, 1963; Slater, 1965, 1966; Kondos & McClymont, 1965).

One mechanism by which rats could become resistant during protein depletion was that they might lose enzymes needed to metabolize carbon tetrachloride. Magee (1964) showed that liver injury caused by dimethylnitrosamine depends on microsomal demethylation of the compound, and Roberts & Plaa (1965) found evidence for the same result for α-naphthyl isothiocyanate toxicity. Protein-free diets affect the microsomal hydroxylating enzyme systems that are responsible for the metabolism of many drugs (Kato, Chiesara & Vassanelli, 1962; Conney & Burns, 1962; Ernster & Orrenius, 1965). Use was made of the effect discovered by Fouts (1963) that dicophane (DDT*) is a potent and long-lasting inducer of synthesis of microsomal hydroxylating enzymes (Hart & Fouts, 1965; Ernster & Orrenius, 1965).

METHODS

Young male rats of a Wistar-derived Porton strain were reared under specific pathogen-free conditions and brought out to a conventional animal house at weaning. When they weighed 120–150g., they were given various diets *ad libitum*. Water was freely available and mesh floors were used to limit coprophagy. The diets used were stock pellets (diet 41B of Bruce & Parkes, 1956) and no-protein and 3%-, 6%-, 10%- and 30%-casein diets (Table 1).

Dosing with carbon tetrachloride. The CCl_4 (AnalaR) was

* Abbreviation: DDT, 1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane.

given orally as a 1:1 (v/v) mixture in liquid paraffin (Light Liquid Paraffin, sp.gr. 0·83–0·87; British Drug Houses Ltd., Poole, Dorset). The rats were lightly anaesthetized with ether and the CCl_4 was given intragastrically by a fine soft-rubber Jaques catheter (no. 3 English gauge). The dose was always given between 10 and 11 a.m.

Rats were killed by anaesthetizing with ether and exsanguination from the carotid artery.

Determination of LD_{50}. For estimations of LD_{50} preliminary experiments were undertaken to assess the range of doses to be used. Then four or five groups each of five rats were given oral CCl_4 in doses that increased geometrically by a factor 1·2 or 1·44. Deaths were observed for 1 week and the LD_{50} values calculated by the method of Weil (1952). Where the animals were starved the diet was removed at 4 p.m. on the day before dosing and food returned at 2 p.m., 4hr. after dosing.

Determination of isocitrate dehydrogenase in liver and plasma. Blood was collected into a heparin-treated tube.

Table 1. *Composition of basal diets*

Diets containing casein in amounts between 0 and 30% were made by mixing the two diets in appropriate proportions. The diets were made up in 3kg. batches and stored at 4°.

	Composition (g./kg. of diet)	
	No-protein diet	30%-Casein diet
Sucrose	250	250
Olive oil B.P.	50	50
Salt mixture (Glaxo Laboratories Ltd., Greenford, Middx.)	30	30
Corn starch (Globe Brand; Brown and Polson Ltd., Esher, Surrey)	664	364
Casein (low-vitamin content; Genatosan Ltd., Fison Scientific, Loughborough, Leicestershire)	—	300
Choline hydrochloride	6	6

	Vitamin composition of all diets (mg./kg. of diet)
Thiamine	10
Riboflavine	16
Nicotinic acid	100
Pyridoxine	10
Calcium pantothenate	20
Inositol	200
p-Aminobenzoic acid	100
Folic acid	2
Menaphthone	10
Vitamin B_{12}	0·1
Vitamin E (α-tocopherol acetate)	200
Vitamin A (given as Rovimix A and D, 1mg./kg.; Roche Products Ltd., Welwyn Garden City, Herts.)	500 i.u.
Vitamin D	325 i.u.

Liver and plasma isocitrate dehydrogenase was estimated by measuring the formation of $NADPH_2$ by determining E_{340} on a Unicam SP.800 automatic recording spectrophotometer at 25°. Each cuvette contained 0·2–0·4ml. of plasma or 2mg. of homogenized liver in a final volume of 3ml. The incubation solution contained NaCl (225μmoles), tris buffer, pH7·4 (100μmoles), isocitrate (10μmoles), $MnCl_2$ (2μmoles) and NADP (0·4μmole). The reaction was started by adding isocitrate. The earlier experiments showed no detectable activity in serum or liver in the absence of substrate, so that in the later experiments water was used as a blank. The results are expressed as mμmoles of NADP reduced/min. per ml. of plasma or mg. of liver.

The logarithms of the serum enzyme values were distributed in a normal fashion with a standard deviation similar to those reported by other workers (Zimmermann, Kodena & West, 1965; Dinman, Hamdi, Fox & Frajola, 1963). Arithmetic means and standard deviations cannot be used because serum enzyme values are not normally distributed.

Determination of bilirubin. Total bilirubin in plasma was measured by a modification of the method of King & Coxon (1950) by using the diazo reagent of Lathe & Ruthven (1958). Plasma (0·3ml.) was mixed with 2·5ml. of 85% (v/v) ethanol and 0·3ml. of diazo reagent. Then 5min. later 0·2ml. of 50%-saturated $(NH_4)_2SO_4$ was added; 30min. later the supernatant was taken and E_{540} measured in a spectrophotometer. A standard curve was made with bilirubin dissolved in chloroform, diluted daily with ethanol. The diazo reagent was made daily by mixing 10ml. of 1% (w/v) sulphanilic acid in 0·2N-HCl with 0·3ml. of 0·5% $NaNO_2$.

Liver composition. The liver was removed from the exsanguinated animals and rinsed with water, blotted and weighed. A piece was taken for histological examination.

A 2g. portion of liver was weighed into 6ml. of ice-cold 0·15M-KCl and homogenized in an all-glass homogenizer of the Dounce type by using 12 strokes of the pestle. A sample (about 500mg.) was cut off the liver for estimation of water and fat content. This piece was dried overnight at 105° for water content, and then extracted with diethyl ether, light petroleum (b.p. 40–60°) and ether again for 24hr. each. It was then oven-dried again and weighed.

Determination of glycogen. Liver glucose plus glycogen was measured by the method of Kemp & Kits Van Heijningen (1954).

Dosing with DDT. DDT was a commercial sample, 99% pure. It was dissolved in olive oil in a concentration of 50mg./ml. and given to rats orally, subcutaneously or intraperitoneally. The growth rates of the rats were not affected by an injection of 100mg./kg.

Dosing with phenobarbitone. Phenobarbitone (80mg./kg.) was given intraperitoneally. It was dissolved in N-NaOH, neutralized to pH9 with HCl and diluted to 20mg./ml. for injection.

Estimation of hydroxylating enzymes. (*a*) Demethylation of Pyramidon. Rat liver was homogenized in ice-cold 0·15M-KCl in a Dounce-type homogenizer. A 1ml. portion of 25% homogenate was added to 4ml. of incubation medium containing the following: KH_2PO_4 buffer (adjusted to pH7·4 with KOH), 70μmoles; glucose 6-phosphate, 10μmoles; nicotinamide, 50μmoles; $MgCl_2$, 25μmoles; NADP, 0·5μmole; KCl, 500μmoles; Pyramidon (4-dimethylamino-1,5-dimethyl-2-phenyl-3-pyrazolone),

5 μmoles. The reaction was started by addition of the homogenate, and blanks were taken at zero time. The mixture was incubated at 37° for 30 min. in a shaking incubator with air as the gas phase. The reaction was ended by addition of 2·5 ml. of 18% (w/v) trichloroacetic acid.

The reaction product, 4-aminoantipyrene, was measured by the method of Brodie & Axelrod (1950) as modified by La Du, Gaudette, Trousof & Brodie (1955).

(*b*) Hydroxylation of benzopyrene. A modification of the methods of Wattenberg, Leong & Strand (1962) and A. H. Conney (personal communication) was used. A 1 ml. portion of 5% liver homogenate in 0·15 M-KCl was added to 2 ml. of ice-cold incubation mixture containing the same substances and amounts as were used for Pyramidon demethylation, except for the omission of the KCl and Pyramidon. The final addition was 60 μg. of benzopyrene in 25 μl. of acetone, and the reaction was started by taking flasks from the ice bath to 37° in a shaking incubator. After 12 min. incubation the flasks were returned to ice, and 3 ml. of acetone was added to each. For blanks, flasks with the complete reaction system with 3 ml. of acetone added to each at zero time, and kept cold, were used. Then 9 ml. of hexane (b.p. 67–70°) was added to each, the flasks were capped with foil and the mixtures were returned to the shaking incubator at 37° for 10 min. The flasks were then returned to the cold and kept dark for 2 hr. A 1 ml. portion of the hexane phase of each was put into 10 ml. of N-NaOH in a stoppered tube. Each tube was inverted once, left for 5 min. and inverted again, so that the unstable hydroxybenzopyrene should not be destroyed by shaking the alkaline solution with air. The fluorescence of the soda layer was immediately measured in an Aminco–Bowman spectrofluorimeter, by using excitation wavelength 400 mμ and emission wavelength 522 mμ. As a standard 0·3 μg. of quinine/ml. in 0·1 N-H_2SO_4, diluted daily from a concentrated standard, was used at wavelengths 350–450 mμ.

To ensure that this standard was reproducible in spite of the change of wavelengths between the quinine standard and hydroxybenzopyrene unknown, a second standard that fluoresces at the hydroxybenzopyrene wavelengths was used. This was xanthurenic acid (60 μg./ml. in 8 N-NaOH). This has the same fluorescence at 400–522 mμ as quinine (0·3 μg./ml.) has at 340–450 mμ. The results are expressed as units: 100 units of activity are present when 50 mg. of liver produces material that is as fluorescent as 0·3 μg. of quinine/ml. in the final soda phase of the assay.

RESULTS

Effects of diets. Male rats weighing 140–160 g. were fed on no-protein and 3%-, 6%-, 10%- and 30%-casein diets. After an initial check to growth, or loss of weight, the group on the no-protein diet lost about 0·8 g. body wt./day, whereas all the other groups made steady gains of 0·7, 2·1, 4·4 and 5·2 g. body wt./day.

A mild fatty liver of high glycogen content developed in the groups on no-protein and 3%-casein diets. The groups on 6%-, 10%- and 30%-casein diets did not develop fatty livers, apart from a few individual animals on the 6%-casein diet. The group on no-protein diet remained in good condition, in spite of weight loss, for more than 5 weeks. Liver weight as a percentage of body weight did not alter significantly from normal in the groups on no-protein diet with or without DDT (Table 8). The fat content of the liver rose about threefold, so that on a wet-weight basis the controls fed on diet 41B contained 2% of fat, whereas the rats fed on no-protein diets had 5% of fat. There was, however, a considerable variability in the fat content of the group on no-protein diet that did not depend on the length of time for which the diet had been given.

Lethal effects of carbon tetrachloride. Rats were fed on the no-protein or casein diets for 1–3 weeks and then given oral carbon tetrachloride. The number of deaths during the next week were observed. The protein-depleted animals were resistant to the lethal effects of carbon tetrachloride (Table 2). Histological examination showed that liver damage was produced by a fatal dose of carbon tetrachloride in all groups. There were, however, differences in the histological pattern of the lesions (A. E. M. McLean & E. K. McLean, unpublished work).

Effect of DDT and phenobarbitone on carbon tetrachloride toxicity. When carbon tetrachloride was given to rats fed on no-protein diet the lethal

Table 2. *Lethal effects of carbon tetrachloride given orally to rats fed on various diets*

Male rats weighing 100–150 g. were given CCl_4 and observed for 1 week. The method of performing LD_{50} estimation and calculations are described in the Methods section. The duration of feeding diets did not affect the LD_{50} during the periods 8–22 days on diet.

	Diet 41B (fed)	Diet 41B (starved)	No-protein diet (fed)	No-protein diet (starved)	30%-Casein diet (fed)	6%-Casein diet (fed)	3%-Casein diet (fed)
LD_{50} (ml./kg.)	6·4	3·4	14·7	9·7	9·1	—	—
95% Confidence limits	5·4–7·6	3·0–3·8	13·4–16·1	8·3–11·3	8·1–10·2	—	—
Mortality at:							
8·6 ml./kg.	9/10	—	0/5	1/5	7/15	2/4	0/5
10·4 ml./kg.	5/5	—	0/6	4/8	8/12	3/4	0/5

effects were enhanced by DDT and by phenobarbitone. When the animals were fed on stock diet DDT had very little effect (Table 3).

Effect of diet and DDT on demethylation of Pyramidon and hydroxylation of benzopyrene. These enzymic activities were markedly decreased by feeding the rats on a protein-free diet for 4 days, or longer, and also by feeding them on a 3%-casein diet (Table 4). The 6%-casein diet allows considerable enzyme activity to remain, whereas the 10%- and 30%-casein diets did not significantly decrease activity in comparison with stock diets.

A single dose of DDT led to a sustained increase of both enzymic activities in rats fed on protein-free diets. Three doses of phenobarbitone had an even more marked effect. Similar relative values were obtained with the supernatant remaining after mitochondria had been removed by centrifugation, instead of the whole homogenate.

The addition of isocitrate (10 μmoles) to the incubation mixture of Pyramidon demethylation did not increase the enzyme activity where this is lowered by diet, and the effect is therefore not due to shortage of $NADPH_2$ (Ernster & Orrenius, 1965).

Liver isocitrate-dehydrogenase activity was decreased by about 30% in the rats fed on a no-protein diet for 5 days.

Effects of a single dose of carbon tetrachloride on bilirubin concentration and isocitrate-dehydrogenase activity. A number of rats were given a single oral dose (2·5 ml./kg.) of carbon tetrachloride and killed 24 hr. later. Liver damage was assessed by measurement of isocitrate-dehydrogenase activity and bilirubin in the plasma, and by estimation of the water content of the liver, as well as by histological examination.

The expected rise of bilirubin concentration and isocitrate-dehydrogenase activity was found in the plasma of protein-fed animals given carbon tetrachloride (Table 5). In protein-depleted animals much smaller changes were found. The protective action of the diet was observable after 4 days of feeding and was found to a smaller extent in animals fed on a 3%-casein diet. The protection persisted as long as has been tested, at least up to 40 days of no-protein diet.

Starvation for 18 hr. increased the susceptibility of the animals as judged by plasma isocitrate dehydrogenase and bilirubin measurement but the

Table 3. *Effect of DDT and phenobarbitone on the lethal effects of carbon tetrachloride*

The method of determining LD_{50} is described in the Methods section. DDT was given as a single subcutaneous dose of 75 mg./kg. in olive oil, 1 week before CCl_4. Olive oil or 0·9% NaCl injections alone had no significant effect. Phenobarbitone (80 mg./kg.) was given daily for 3 days before CCl_4. There was no significant difference in LD_{50} between male and female rats fed on diet 41B. The results in the Table are all for male rats except for the groups given diet 41B+DDT, which were females.

Diet	LD_{50}	95% Probability range
Diet 41B	6·4	5·4– 7·6
Diet 41B+DDT	4·2	3·3– 5·4
No-protein diet	14·7	13·4–16·1
No-protein diet+DDT	4·3	3·7– 5·0
No-protein diet+phenobarbitone	<2·9	—

Table 4. *Effect of diet and DDT on Pyramidon demethylation and hydroxylation of benzopyrene*

Enzyme assays are described in the Methods section. DDT (75 mg./kg.) was given subcutaneously in olive oil 1 week before enzyme assays; phenobarbitone (80 mg./kg.) was given intraperitoneally for 3 days before enzyme assays. There was no significant alteration in enzymic activity on feeding rats on experimental diets beyond 1 week.

	Demethylation of Pyramidon		Hydroxylation of benzopyrene	
Diet	No. of animals	Mean ± S.D. (μmole/hr./g. wet wt.)	No. of animals	Mean ± S.D. ('quinine' units/g. wet wt.)
Diet 41B	9	0·68±0·28	9	46±23
30%-Casein diet (7–20 days)	4	0·45±0·29	3	43±—
10%-Casein diet (7–20 days)	4	0·66±0·35	4	56±14
6%-Casein diet (7–20 days)	6	0·36±0·10	6	22±11
3%-Casein diet (7–20 days)	8	0·07±0·01	8	8± 4
No-protein diet (2 days)	4	0·29±0·02	4	30± 7
No-protein diet (4 days)	4	0·06±0·04	4	9± 6
No-protein diet (7–20 days)	12	0·05±0·04	10	3± 2
No-protein diet (12–20 days)+DDT	12	0·40±0·12	6	25±12
No-protein diet (12–20 days)+phenobarbitone	4	0·80±0·13	4	31± 2

Table 5. *Plasma isocitrate-dehydrogenase activity and bilirubin concentration 24 hr. after a single oral dose of carbon tetrachloride (2·5 ml./kg.) in rats fed on various diets*

The use of logarithmic means and standard deviations is discussed in the Methods section. Values marked * differ significantly from the group fed on diet 41B+CCl_4 ($P<0·05$) by using Student's *t* test and by using the numbers in the smaller group only for the estimation of degrees of freedom. In the absence of CCl_4, none of the diets caused significant changes in plasma isocitrate-dehydrogenase activity or bilirubin concentration.

	Plasma isocitrate-dehydrogenase activity			Plasma concn. of bilirubin	
Diet	No. of animals	Geometric mean (mμmoles/ ml./min.)	Logarithm of mean ± S.D.	No. of animals	Mean ± S.D. (mg./100 ml.)
Diet 41B (fed)	23	133	2·13 ± 0·54	20	0·59 ± 0·44
Diet 41B (starved for 18 hr.)	10	361	2·56 ± 0·21*	10	1·51 ± 0·35*
No-protein diet (8–20 days)	34	25	1·39 ± 0·45*	23	0·48 ± 0·15
No-protein diet (starved)	6	295	2·47 ± 0·18	6	0·64 ± 0·22
No-protein diet (2 days)	9	151	2·18 ± 0·42	6	0·91 ± 0·46
No-protein diet (4 days)	9	35	1·54 ± 0·39*	9	0·32 ± 0·12
3%-Casein diet (8 days)	12	62	1·79 ± 0·24	12	0·43 ± 0·10
6%-Casein diet (8 days)	10	79	1·90 ± 0·35	10	0·45 ± 0·26
10%-Casein diet (8 days)	6	279	2·45 ± 0·32	6	0·67 ± 0·32
30%-Casein diet (8 days)	15	175	2·44 ± 0·44	7	0·69 ± 0·80
Controls: diet 41B, no CCl_4	8	1·4	0·15 ± 0·14	18	0·19 ± 0·02

Table 6. *Effect of DDT and phenobarbitone on plasma isocitrate-dehydrogenase activity and bilirubin concentration 24 hr. after a single oral dose of carbon tetrachloride (2·5 ml./kg.) in rats fed on various diets*

Animals were pretreated with a single dose of DDT or with three doses of phenobarbitone (see Table 4). In the absence of CCl_4 neither DDT nor phenobarbitone caused a significant change in plasma isocitrate-dehydrogenase activity or bilirubin concentration. Values marked * differ significantly from the group fed on diet 41B ($P<0·01$). Values marked † differ significantly from the group fed on no-protein diet ($P<0·01$). Injection of 0·9% NaCl or olive oil alone did not increase isocitrate-dehydrogenase activity or bilirubin concentration after the administration of CCl_4.

	Plasma isocitrate-dehydrogenase activity			Plasma concn. of bilirubin	
Diet	No. of animals	Geometric mean (mμmoles/ ml./min.)	Logarithm of mean ± S.D.	No. of animals	Mean ± S.D. (mg./100 ml.)
Diet 41B	23	133	2·13 ± 0·54	20	0·59 ± 0·44
Diet 41B + DDT	9	898	2·79 ± 0·27*	9	1·35 ± 0·24*
No-protein diet (8–22 days)	34	25	1·39 ± 0·45*	23	0·48 ± 0·15
No-protein diet + DDT	13	221	2·35 ± 0·31†	14	1·26 ± 0·29†
No-protein diet + phenobarbitone	7	125	2·10 ± 0·30†	7	1·53 ± 0·60†

starved group previously given diet 41B remained more susceptible than the starved group previously given the no-protein diet.

Effect of DDT and phenobarbitone on indices of liver damage after carbon tetrachloride. Pretreatment with a single dose of DDT or three doses of phenobarbitone led to increased plasma isocitrate-dehydrogenase activity and bilirubin concentrations after carbon tetrachloride dosage (Table 6). Rats fed on diet 41B showed the effect and so did the rats fed on the no-protein diet. DDT alone had no effect on liver or plasma isocitrate-dehydrogenase activity, nor did it affect liver fat, water or glycogen content. Carbon tetrachloride increased the content of liver water. This water influx was less in animals on the no-protein diet, and was increased by pretreatment with phenobarbitone or DDT (Table 7).

Effect of carbon tetrachloride on liver fat and glycogen content. The wet weight of liver as a percentage of body weight was not significantly altered by feeding rats on the no-protein diet, even

Table 7. *Effect of various diets on liver water content of rats 24hr. after a single oral dose of carbon tetrachloride* (2·5*ml./kg.*)

DDT (75mg./kg.) was given subcutaneously in oil 7 days before CCl_4. Phenobarbitone (80mg./kg.) was given intraperitoneally on three successive days before giving CCl_4. The differences between the group given no-protein diet (8–20 days)+CCl_4 and those groups marked * were significant ($P<0·01$).

	Water content				
	Control		CCl_4-treated		
Diet	No. of animals	Mean ± S.D. (g./g. fat-free dry wt.)	No. of animals	Mean ± S.D. (g./g. fat-free dry wt.)	Change of water content (g./g. fat-free dry wt.)
Diet 41B	15	2·61 ± 0·10	20	3·83 ± 0·32*	1·22
No-protein diet (8–20 days)	23	2·80 ± 0·17	23	3·28 ± 0·24	0·48
3%-Casein diet (8 days)	7	2·64 ± 0·10	12	3·29 ± 0·21	0·65
No-protein diet (4 days)	5	2·68 ± 0·20	9	3·28 ± 0·25	0·60
30%-Casein diet (8 days)	6	2·53 ± 0·09	11	3·65 ± 0·21*	1·12
No-protein diet + DDT	12	2·79 ± 0·15	9	3·88 ± 0·33*	1·09
No-protein diet + phenobarbitone	3	3·03 ± —	7	4·26 ± 0·26*	1·23

Table 8. *Effect of diet and DDT on liver weight, and on liver fat and glycogen content, before and 24hr. after a single oral dose of carbon tetrachloride* (2·5*ml./kg.*)

Liver fat and glycogen contents were measured as described in the Methods section. The group given no-protein diet had been fed on the diet for 8–14 days. The group given no-protein diet + DDT had been given a single subcutaneous injection of DDT in oil (75mg./kg.) 1 week beforehand.

		Liver fat content			Liver glycogen + glucose content		
			Mean ± S.D. (g./kg. fat-free dry wt.)			Mean ± S.D. (g. of glucose/100g. wet wt.)	
Diet	Mean liver weight (g./100g. body wt.) control	No. of animals	Control	CCl_4-treated	No. of animals	Control	CCl_4-treated
Diet 41B	4·9	15	71 ± 23	371 ± 75	7	7·4 ± 0·7	0·8 ± 0·5
No-protein diet	4·8	5	213 ± 146	509 ± 207	9	10·4 ± 2·4	5·3 ± 2·2
No-protein diet + DDT	5·0	5	194 ± 38	716 ± 157	6	11·8 ± 4·0	1·0 ± 0·7

with addition of DDT (Table 8). The increase in fat content of the liver after carbon tetrachloride was approximately the same in rats fed on the no-protein as in those fed on diet 41B. Giving DDT led to a slightly more severe fatty liver after giving carbon tetrachloride. Glycogen depletion after carbon tetrachloride was much more marked in rats fed on diet 41B, or in those given DDT, than in the rats on the no-protein diet.

Time-course of carbon tetrachloride injury. Protection of rats against carbon tetrachloride injury by cordotomy has been shown to be due to delay in onset rather than decrease in total damage (Larson & Plaa, 1965).

In our own experiments groups of animals were given carbon tetrachloride (2·5ml./kg.) and then killed at 24, 48 and 72hr. There was no evidence of increased liver damage at later times in the rats on the no-protein diet. Histological examination of the livers of these animals showed that, though the usual extensive liver-cell necrosis followed by repair was present in rats fed on diet 41B and given carbon tetrachloride, the rats on the no-protein diet showed only a few dead cells and slight centrilobular glycogen loss 24hr. after the carbon tetrachloride; by 48hr. there was hardly any evidence of damage.

DISCUSSION

Young rats fed on a protein-free or 3%-casein diet become resistant to the acute effects of carbon tetrachloride, whether measured by mortality or by indices of liver damage. This protective effect

can be observed from 4 days of feeding onwards, and is found despite the presence of a fatty liver. The sensitivity of rats on a normal or no-protein diet is enhanced by starvation and by two drugs that are known to increase activity of liver microsomal enzymes. The effect of the protein content of the diet on two microsomal hydroxylation enzymes seems clear-cut. Protein deprivation leads to an 80% fall of activity in 2–4 days, and the critical protein concentration in the diet is between 3 and 6%.

The protective effects can be reversed by giving DDT or phenobarbitone. The close correspondence between the factors that influence microsomal hydroxylating enzyme activity, and the way these factors influence sensitivity to carbon tetrachloride, supports the evidence that the microsomal hydroxylating enzymes may be necessary for carbon tetrachloride toxicity.

The wet weight of the liver as a percentage of body weight is not altered by feeding rats on the no-protein diet. This is because the extra weight of fat and glycogen compensates for the decrease in protein weight. As a result, the use of a wet-weight basis for expressing liver enzyme activity reflects the activity available to the animal. Moreover, the changes in enzyme activity are of a different order of magnitude from the changes in gross liver composition, so that even if the results were expressed in terms of dry weight the differences would still be present.

Of the various measurements that can be made to quantitate the degree of liver damage all are open to criticism. What determines clearance of enzymes from plasma is unknown. Bilirubin accumulation is not dependent on cell necrosis, but on many other cell functions. Potassium concentrations fall in necrotic cells, but inflammatory tissue has a high K^+/dry weight ratio (McLean, 1960). Calcium accumulation in a damaged organ reflects a balance between influx into dead cells and efflux as these cells are phagocytosed. In all, water content has been found to be the measurement that correlates best with histological observations of damage and necrosis.

The relation between carbon tetrachloride toxicity and the activity of microsomal hydroxylating enzymes is not quantitatively simple. The enzyme activities differ tenfold between the group on no-protein diet and the group on diet 41B, but LD_{50} and indices of liver damage alter only by about twofold. Similarly, when DDT is given the enzyme activity rises to only about 60% of that found in the animals fed on stock diet, but sensitivity to carbon tetrachloride becomes greater in the group fed on the no-protein diet and given DDT than in the group fed on the stock diet. It is thus possible that the activation of carbon tetrachloride does not follow the same sequence of enzymic steps that are necessary for metabolism of Pyramidon or benzopyrene.

Studies of carbon tetrachloride metabolism may show that the same quantity of toxic intermediate is required to cause the same amount of damage in the various physiological states of the liver. It is also possible that when the toxic intermediate is identified it will be found that the protein-depleted animal is more sensitive to the true toxin.

Rubinstein & Kanics (1964) have shown that it is in the microsomal fraction that carbon tetrachloride is metabolized to carbon dioxide. Perhaps an intermediate step, or side reaction in the sequence from carbon tetrachloride to carbon dioxide, is the true toxic agent that causes liver-cell necrosis.

REFERENCES

Brodie, B. B. & Axelrod, J. (1950). *J. Pharmacol.* **99**, 171.
Bruce, H. M. & Parkes, A. S. (1956). *J. Anim. Tech. Ass.* **7**, 54.
Butler, T. C. (1961). *J. Pharmacol.* **134**, 311.
Campbell, R. M. & Kosterlitz, H. W. (1948). *Brit. J. exp. Path.* **29**, 149.
Conney, A. H. & Burns, J. J. (1962). *Advanc. Pharmacol.* **1**, 31.
Dinman, B. D., Hamdi, E. A., Fox, C. F. & Frajola, W. J. (1963). *Arch. environ. Hlth*, **7**, 630.
Drill, V. A. (1952). *Pharmacol. Rev.* **4**, 1.
Ernster, L. & Orrenius, S. (1965). *Fed. Proc.* **24**, 1190.
Fouts, J. R. (1963). *Ann. N.Y. Acad. Sci.* **104**, 875.
Hart, L. G. & Fouts, J. R. (1965). *Biochem. Pharmacol.* **14**, 263.
Kato, R., Chiesara, E. & Vassanelli, P. (1962). *Biochem. Pharmacol.* **11**, 211.
Kemp, A. & Kits Van Heijningen, A. (1954). *Biochem. J.* **56**, 646.
King, E. J. & Coxon, V. J. (1950). *J. clin. Path.* **3**, 248.
Kondos, A. C. & McClymont, G. L. (1965). *Nature, Lond.*, **206**, 846.
La Du, B. N., Gaudette, L., Trousof, N. & Brodie, B. B. (1955). *J. biol. Chem.* **214**, 741.
Larson, R. E. & Plaa, G. L. (1965). *J. Pharmacol.* **147**, 103.
Lathe, G. H. & Ruthven, C. R. J. (1958). *J. clin. Path.* **11**, 155.
McCollister, D. D., Beamer, W. H., Atchison, G. J. & Spencer, H. C. (1951). *J. Pharmacol.* **102**, 112.
McLean, A. E. M. (1960). Ph.D. Thesis: University of London.
McLean, A. E. M., McLean, E. K. & Judah, J. D. (1965). *Int. Rev. exp. Path.* **4**, 127.
McLean, E. K., Bras, G. & McLean, A. E. M. (1965). *Brit. J. exp. Path.* **46**, 367.
Magee, P. N. (1964). *Ciba Symp.: Cellular Injury*, p. 1. Ed. by De Reuck, A. V. S. & Knight, J. Boston, Mass.: Little, Brown and Co.
Miller, L. L. (1948). *Occup. Med.* **5**, 194.
Reynolds, E. S. (1963). *J. Cell Biol.* **19**, 139.
Roberts, R. J. & Plaa, G. L. (1965). *Fed. Proc.* **24**, 518.

Rouiller, C. (1964). In *The Liver*, vol. 2, p. 335. Ed. by Rouiller, C. New York and London: Academic Press Inc.
Rubinstein, D. & Kanics, L. (1964). *Canad. J. Biochem.* **42**, 1577.
Slater, T. F. (1965). *Biochem. J.* **97**, 22c.
Slater, T. F. (1966). *Nature, Lond.*, **209**, 36.
Snodgras, P. J. & Pirias, M. M. (1965). *Fed. Proc.* **24**, 395.
Wattenberg, L. W., Leong, J. L. & Strand, P. J. (1962). *Cancer Res.* **22**, 1120.
Weil, C. S. (1952). *Biometrics*, **8**, 249.
Wirtschafter, Z. T. & Cronyn, M. W. (1964). *Arch. environ. Hlth.* **9**, 180.
Zimmermann, H. J., Kodena, Y. & West, M. (1965). *J. Lab. clin. Med.* **66**, 324.