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The Effect of Diet on Carbon Tetrachloride Metabolism

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1. Blood and liver concentrations of carbon tetrachloride were measured, at intervals after an oral dose, in rats given stock and protein-free diets. The values did not correlate with the resistance to poisoning found in the rats on protein-free diets. 2. The metabolism of carbon tetrachloride to carbon dioxide *in vivo* and in liver microsomal preparations was depressed in animals given protein-free diets. 3. Rats given a single dose of DDT [1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane] were highly sensitive to carbon tetrachloride poisoning. The livers of such animals had an increased microsomal protein content and greatly increased microsomal activity in the demethylation of Pyramidon (aminopyrine) and in the conversion of $^{14}CCl_4$ into $^{14}CO_2$. 4. The incorporation of [^{14}C]leucine into protein by liver slices was depressed by carbon tetrachloride. This effect was decreased by addition of SKF 525A (2-diethylaminoethyl 2,2-diphenyl-2-propylacetate) and in slices from rats given protein-free diets. It is suggested that the toxicity of carbon tetrachloride is closely linked to its metabolism.

McLean & McLean (1965, 1966) have previously shown that rats given a protein-free diet for 4 days become resistant to carbon tetrachloride poisoning. This resistance was correlated with loss of hydroxylating-enzyme activity from the liver microsomes. The activity of the hydroxylating enzymes could be restored by injection of phenobarbitone or DDT [1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane]. The animals then regained their sensitivity to carbon tetrachloride.

Paul & Rubinstein (1963) found that when rats are given $^{14}CCl_4$ about 1% of the dose is breathed out as $^{14}CO_2$ and most of the rest is breathed out unaltered. Rubinstein & Kanics (1964) showed that rat liver microsomes in the presence of the supernatant fraction convert $^{14}CCl_4$ into $^{14}CO_2$. It seems likely that the toxic action of carbon tetrachloride is due to a metabolite formed in the microsomes (Wirtschafter & Cronyn, 1964; McLean & McLean, 1965, 1966; Slater, 1966).

The present study was carried out to find out whether the decrease in carbon tetrachloride toxicity brought about by giving a protein-free diet was associated with a decreased ability of the microsomes to metabolize carbon tetrachloride. In addition, the relation of the inhibition of protein synthesis that occurs when liver slices are incubated in the presence of carbon tetrachloride (Smuckler, 1966) to the metabolism of carbon tetrachloride was studied.

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METHODS

Rats. Male Wistar rats of a Porton strain and weighing usually 130–150g. were given stock or protein-free diets (McLean & McLean, 1966) for 7 days or more. Some were injected with DDT (100mg./kg.) once subcutaneously 1 week before death, and some with phenobarbitone (80mg./kg./day) intraperitoneally for 3 days before death, as previously described (McLean & McLean, 1966).

The rats given the protein-free diet lost about 30% of their body weight in 7 days, and thereafter lost weight at about 1%/day. They remained active and in good condition for more than 4 weeks. Injection of DDT or phenobarbitone did not influence weight loss.

Chemicals. Phenergan (promethazine hydrochloride) was bought from May and Baker Ltd., Dagenham, Essex; SKF 525A (2-diethylaminoethyl 2,2-diphenyl-2-propylacetate) was a gift of Smith, Kline and French Ltd., Welwyn, Herts.; DDT was a commercial sample, 99% pure, the gift of Geigy Chemical Co., Basle, Switzerland. Highly purified isocitrate dehydrogenase from pig heart was bought from Sigma Chemical Co. Ltd., London, S.W. 6. Other reagents were of A.R. quality and were bought from British Drug Houses Ltd., Poole, Dorset. Pyramidon (aminopyrine; 4-dimethylamino-1,5-dimethyl-2-phenyl-3-pyrazolone) was of laboratory grade.

Metabolism of $^{14}CCl_4$ in vivo. The rats were lightly anaesthetized and given $^{14}CCl_4$ (specific activity 8μc/ml.; dose 2·5ml./kg.), diluted in an equal volume of paraffin oil, intragastrically by means of a soft rubber catheter. Each animal was placed in a metabolism cage, and air was drawn through the cage and passed through two towers containing

10% (w/v) NaOH and through three tubes containing toluene, to absorb CO_2 and unchanged CCl_4 respectively. The air flow rate was about 600ml./min. Carbonate was precipitated with $BaCl_2$, washed with acetone and water and suspended in Cab-O-Sil gel. Its radioactivity was measured in a Packard liquid-scintillation counter. Addition of internal standards allowed conversion of the counts into disintegrations/min. The concentration of $^{14}CCl_4$ in the blood and liver was measured at intervals by the method of Dawkins (1963). The animals were allowed food throughout the experiment.

The $^{14}CCl_4$ was obtained from The Radiochemical Centre, Amersham, Bucks. Some samples contained appreciable amounts of $^{14}CO_2$; this was removed after dilution with carrier CCl_4 by equilibrating with 0·5M-Na_2CO_3 until less than 0·01% of the radioactivity could be extracted as carbonate.

Metabolism of $^{14}CCl_4$ in vitro. Liver microsomes were prepared from rats that had been starved overnight and killed by exsanguination under ether anaesthesia. The method of Kato & Gillette (1965) was followed. The liver was homogenized by hand in 0·15M-KCl in a Dounce all-glass homogenizer, 3ml. of KCl/g. of liver being used. The homogenate was spun for 10min. at 10000$g_{av.}$ at 4° to remove debris, nuclei, erythrocytes and mitochondria. The supernatant was spun at 104000g for 1hr. and the microsomal pellet resuspended in 0·15M-KCl.

The microsome preparation, together with a source of purified isocitrate dehydrogenase, was incubated in a Warburg flask in 3ml. of medium containing isocitrate (10μmoles), $MgCl_2$ (25μmoles), nicotinamide (50μmoles), NaCl (300μmoles), $NADP^+$ (0·5μmole), 0·1M-phosphate buffer made to pH7·4 with NaOH (60μmoles) and 3μl. of $^{14}CCl_4$ (specific activity 100μc/ml.).

The amount of isocitrate dehydrogenase present was that which would reduce 0·3μmole of $NADP^+$/min. at 25°. In one experiment (Table 4) the supernatant fraction remaining after separation of the microsomes was used as a source of isocitrate dehydrogenase.

The CCl_4 was added last to the side arm by a Hamilton syringe. The flasks were stoppered and left on ice for 10min. while the CCl_4 evaporated into the main compartments. The reaction was started by moving the flasks to an incubator at 37°. The usual gas phase was air and the CO_2 evolved was collected in 20% (w/v) KOH contained in the centre well. The flasks were shaken at 110 strokes/min. and the incubation time was usually 30min. To bring the reaction to an end, 25μmoles of Na_2CO_3, followed by 1ml. of 25% (w/v) trichloroacetic acid, were added. The flasks were then reincubated for 15min. to allow complete absorption of CO_2 in the centre well.

Toluene was added to the outer compartment at the end of the experiment to decrease the amount of CCl_4 in the gas phase. The centre well was washed out with Na_2CO_3, and the CO_2 was precipitated, washed and counted as $BaCO_3$. Flasks without tissue were always incubated at the same time as the test flasks and the radioactivity in these control experiments was subtracted.

Preliminary experiments showed that recovery of [^{14}C]-carbonate added to the incubation mixture was better than 95%. They also showed that it was necessary to add CCl_4 to the incubation mixtures in the gas phase. When CCl_4 was added into the liquid medium after the liver preparation, it did not dissolve but remained as a droplet covered with a film of liver at the bottom of the flask. The rate of conversion of CCl_4 into CO_2 was then only one-third as high as when CCl_4 was added in the gas phase. The saturating concentration of CCl_4 is approx. 5mM, which is equivalent to 1·5μl. of CCl_4 in 3ml. When this amount of CCl_4 was dissolved in the aqueous medium, before addition of liver, the same amount of CCl_4 was converted into CO_2 as when 1·5μl. of CCl_4 was added last to the side arm of the Warburg flask and allowed to distil into the main compartment. Addition by the gas phase has the advantage that it is not limited by the solubility of CCl_4 in the incubation medium.

Demethylation of Pyramidon to 4-aminoantipyrine by microsomes. This was measured under the conditions used for studyng CCl_4 metabolism *in vitro*. The analyses were carried out as stated by McLean & McLean (1966).

Inhibition of amino acid incorporation in liver slices. The effect of CCl_4 on [^{14}C]leucine incorporation into protein was measured in liver slices (150mg.) incubated in 2ml. of Ringer phosphate buffer, pH7·4 (McLean, 1963), by methods previously described (Seawright & McLean, 1966; Hultin, Arrhenius, Löw & Magee, 1960). CCl_4 was added from the side arm of a Warburg flask as in the CCl_4-metabolism experiments. The slices were incubated for 45min. at 38° with oxygen as the gas phase. [^{14}C]Leucine (0·05μc) was then added and the incubation was carried on for another 1hr. Radioactivity in protein from the liver slices was measured by dissolving the purified protein in Hyamine, adding toluene scintillation fluid and counting in a liquid-scintillation counter. Internal standards were added and all the samples were counted again to allow conversion of counts into disintegrations/min.

RESULTS

Conversion of $^{14}CCl_4$ *into* $^{14}CO_2$ in vivo. The results in Fig. 1 show that animals given a protein-free diet breathe out only about one-half as much $^{14}CO_2$ after dosing with $^{14}CCl_4$ as do animals given a laboratory stock diet. The expiration of unchanged $^{14}CCl_4$ is similar in the two groups.

Table 1 shows the blood and liver concentrations of carbon tetrachloride at 1hr. and 3hr. after dosing, in rats given a stock diet, a protein-free diet and a protein-free diet together with DDT treatment. Liver concentrations of carbon tetrachloride were higher in the groups given the protein-free diets.

Conversion of $^{14}CCl_4$ *into* $^{14}CO_2$ in vitro. The rate of carbon tetrachloride metabolism by rat liver microsomes is dependent on the amount of carbon tetrachloride added, and in the present experiments saturating values were not reached. The rate of carbon tetrachloride metabolism rose from 1·1μmoles/g. of liver/hr. when 1μl. of carbon tetrachloride was added, to 2·3μmoles/g. of liver/hr. with the standard addition of 3μl., and 3·2μmoles/g. of liver/hr. with 10μl. Further increases in the amount of carbon tetrachloride led to the formation of a film of carbon tetrachloride on the surface of the incubation medium.

Fig. 2 shows that the rate of conversion of carbon

tetrachloride into carbon dioxide decreased after 20min. Tables 2 and 3 show that the conversion of $^{14}CCl_4$ into $^{14}CO_2$ is carried out by microsomes, requires oxygen and an NADPH-generating system, and is inhibited by SKF 525A, Phenergan or aniline.

Effects of diet on carbon tetrachloride metabolism and Pyramidon demethylation. Table 4 shows the ability of microsomes from rats given stock and protein-free diets to convert $^{14}CCl_4$ into $^{14}CO_2$ and to demethylate Pyramidon. The effect of diet on the ability of liver homogenates to demethylate Pyramidon has been described previously (McLean & McLean, 1966). The present experiments were designed to show whether there was any marked difference between the effects of diet and DDT on metabolism of the two substrates, carbon tetrachloride and Pyramidon.

Liver microsomes from animals given the stock diet metabolize Pyramidon and carbon tetrachloride about three times as fast as do those from animals given the protein-free diet. Administration of DDT results in a twofold increase in the metabolism of carbon tetrachloride in the animals given stock diets and a threefold increase in the animals given protein-free diets. Pyramidon demethylation increases threefold and tenfold respectively.

Effect of carbon tetrachloride on the incorporation of [^{14}C]leucine. Table 5 shows that when carbon tetrachloride is added to liver slices through the gas phase very marked inhibition of the incorporation of [^{14}C]leucine into protein is observed after 1hr. Smuckler (1966) has shown that carbon

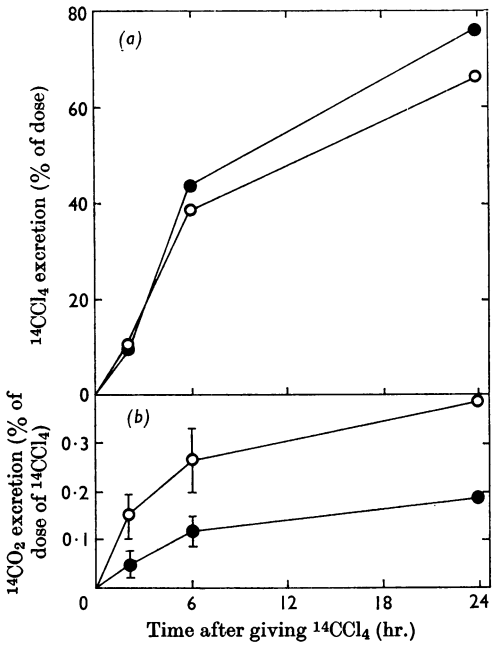

Fig. 1. Expiration of $^{14}CCl_4$ (*a*) and $^{14}CO_2$ (*b*) after rats given stock (○) or protein-free (●) diets had been given $^{14}CCl_4$ (2·5ml./kg.). Each point marks the mean and range of measurements made on at least three rats. The techniques of measurement are described in the Methods section.

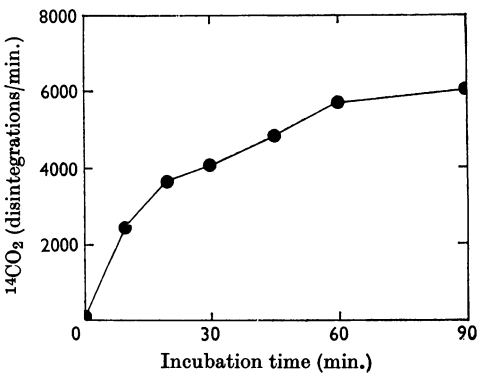

Fig. 2. Time-course of conversion of $^{14}CCl_4$ into $^{14}CO_2$ by liver microsomes. The incubation conditions are described in the Methods section. $^{14}CCl_4$ (3μl., 675000 disintegrations/min.) was added to each flask. At each time, two flasks each containing 0·4ml. of microsome suspension (20mg. of protein/ml., equivalent to 200mg. of tissue) were analysed together with control flasks containing no microsomes. The control values were subtracted.

Table 1. *Carbon tetrachloride concentrations in blood and liver*

Young male rats were given the diets for 1 week with or without DDT treatment, and then dosed with $^{14}CCl_4$ (2·5ml./kg.). Analyses were performed as described in the Methods section. Results are given as the means ± S.D. for five animals at each time.

Diet and treatment	Time after dose (hr.)	CCl_4 in blood (μg./ml.)	CCl_4 in liver (μg./g. wet wt.)
Stock	1	26±11	296±168
	3	38±19	385±283
Protein-free	1	49± 7	574± 96
	3	49±13	575±178
Protein-free+DDT	1	39±12	455±159
	3	45± 9	497±115

Table 2. *Activity of tissue fractions in the conversion of* $^{14}CCl_4$ *into* $^{14}CO_2$ *and in the demethylation of Pyramidon*

Rat liver, from a rat that had been treated with DDT, was homogenized and fractionated as described in the Methods section. The precipitate from the first centrifugation at 10000*g* was suspended in the original volume of 0·15M-KCl. The amount of each fraction incubated was equivalent to 200mg. of tissue. The incubation time was 30min.

Fraction	Conversion of $^{14}CCl_4$ into $^{14}CO_2$ (μmoles/g. of liver/hr.)	Pyramidon demethylation (μmoles/g. of liver/hr.)
Homogenate	1·7	1·32
First precipitate (10000*g*)	0·4	0·23
Post-mitochondrial supernatant	1·8	1·84
Microsomes + isocitrate dehydrogenase	2·6	1·30
Post-microsomal supernatant	0·04	0·06

Table 3. *Effect of incubation conditions and inhibitors on the conversion of* $^{14}CCl_4$ *into* $^{14}CO_2$

Microsomes were prepared from the liver of a rat treated with DDT and were washed by resuspension in KCl followed by centrifugation at 105000*g*. The incubation conditions for the complete system are described in the Methods section. A microsome suspension (0·4ml., 20mg. of protein/ml.) equivalent to 200mg. of liver was used in each flask. The $^{14}CCl_4$ added contained $1·03 \times 10^6$ disintegrations/min. In the complete system 9500 disintegrations/min. were recovered as carbonate, corresponding to a conversion of 2·8μmoles of CCl_4/g. of liver/hr. In the control systems without tissue 170 disintegrations/min. were recovered as carbonate. Results are expressed as percentages of the complete system.

	Conversion of $^{14}CCl_4$ into $^{14}CO_2$ (% of control)
Complete system	(100)
Complete system in N_2	7
Complete system − isocitrate	0·2
Complete system − NADP+	3
Complete system + SKF525A (0·1mM)	55
Complete system + Phenergan (0·1mM)	56
Complete system + EDTA (0·1mM)	94
Complete system + aniline (5mM)	12
Complete system + ethanol (0·25M)	100
Complete system + acetone (0·15M)	100

tetrachloride does not inhibit the activation or uptake of amino acids. The inhibition of the incorporation of [^{14}C]leucine into protein is much less when the liver slices were obtained from rats given the protein-free diet. The inhibiting effect of carbon tetrachloride is also decreased by the addition of SKF525A to the medium. The administration of phenobarbitone to the protein-depleted rats renders the slices more sensitive to carbon tetrachloride.

DISCUSSION

The present experiments were designed to explore the association between carbon tetrachloride metabolism and carbon tetrachloride toxicity. The observation that rats given a stock diet and those given a protein-free diet plus subcutaneous doses of DDT were susceptible to the poison, whereas rats given a protein-free diet alone were resistant (McLean & McLean, 1966), gave us an experimental situation in which to work.

Excretion of unchanged $^{14}CCl_4$ after oral dosage is similar in rats given stock and protein-free diets, but excretion of $^{14}CO_2$ is strikingly less in the resistant group given a protein-free diet (Fig. 1).

The amounts of carbon tetrachloride in the blood and liver at 1hr. and 3hr. after dosing do not correspond with the pattern of susceptibility (Table 1). There are some increases in carbon tetrachloride content in rats given protein-free diets.

The microsomal conversion of $^{14}CCl_4$ into $^{14}CO_2$ *in vitro* resembles typical microsomal hydroxylation reactions. It requires an NADPH-generating system and oxygen, and is inhibited by SKF525A and by substrates for hydroxylation. The response to DDT injection is similar for carbon tetrachloride metabolism and Pyramidon metabolism.

We have confirmed that carbon tetrachloride inhibits [^{14}C]leucine incorporation into proteins in liver slices. Our results are more striking than those of Smuckler (1966), perhaps because addition of carbon tetrachloride in the gas phase provides a more effective technique. This effect of carbon tetrachloride is blocked by SKF525A, and by using slices from rats given a protein-free diet. Thus the inhibiting effect on the liver slice parallels both carbon tetrachloride metabolism and carbon tetrachloride toxicity *in vivo*.

Recknagel & Litteria (1960) found that a peak concentration of 1mg. of carbon tetrachloride/g. of liver was reached 1½hr. after carbon tetrachloride had been given orally to starved rats. Our rats were not starved and this probably accounts for the lower blood and liver carbon tetrachloride concentrations. The percentage conversion of $^{14}CCl_4$ into $^{14}CO_2$ *in vivo* was less in our experiments than in the experiments of Paul & Rubinstein (1963).

Table 4. *Effect of diet and DDT injection on the metabolism of carbon tetrachloride and of Pyramidon by liver microsomes*

Rats weighing 100–140g. were starved overnight after being given a stock or a protein-free diet for the previous week. Injections of DDT were given 1 week before the animals were killed. Methods of preparation of the microsome suspension and of incubation and analysis are described in the Methods section. Microsomes equivalent to 200mg. of liver were used for each flask. The incubation time was 30min. The source of isocitrate dehydrogenase in this experiment was the supernatant fraction remaining after removal of the microsomes from the rat liver homogenate. Samples (0·2ml.) of a single supernatant fraction, derived from a liver homogenate prepared from rats that had been given a stock diet and injected with DDT, were used in all the tests. The activity of each microsomal preparation was measured in duplicate. Results are given as means ±S.D.

Diet	No. of rats	Liver wt. (g./100g. body wt.)	Microsomal protein content (mg. of protein/g. of liver)	Pyramidon demethylation (μmole/g. of liver/hr.)	Conversion of $^{14}CCl_4$ into $^{14}CO_2$ (μmoles/g. of liver/hr.)
Stock	5	3·8	19·2±1·7	0·16±0·10	1·10±0·19
Stock+DDT	5	4·2	29·0±5·2	0·63±0·21	2·62±0·35
Protein-free	4	3·9	16·8±1·3	0·05±0·03	0·38±0·05
Protein-free+DDT	4	4·0	24·8±2·3	0·55±0·22	1·27±0·29

Table 5. *Effect of carbon tetrachloride on the incorporation of* [^{14}C]*leucine into protein in liver slices*

The incubation conditions and the dietary treatment of the animals are described in the Methods section. Liver slices were incubated with CCl_4 for 45min., after which [^{14}C]leucine was added and the incubation continued for a further 1hr. The results are expressed as the means ±S.E.M. of the percentage incorporation found in control slices not exposed to CCl_4. Control slices from animals given a stock diet incorporated 1230±370 disintegrations min./mg. of protein (mean ±S.D.). Control slices from rats given protein-free diets incorporated 1610±650 disintegrations/min./mg. of protein.

Diet and treatment	Addition to incubation medium	Incorporation of [^{14}C]leucine (% of control)			
	CCl_4 dose.....	0	1 μl.	2 μl.	3 μl.
Stock	—	(100)	67±12	38±2	25±2
Stock	SKF525A (0·1mM)	(100)	—	74±5	—
Protein-free	—	(100)	—	72±5	67±4
Protein-free+phenobarbitone	—	(100)	—	—	34±3
Protein-free+DDT	—	(100)	—	55±5	—

However, these workers used a smaller dose of carbon tetrachloride and gave it by intraduodenal injection at laparotomy. It seems likely that they would achieve high liver carbon tetrachloride concentrations by this route, and hence increase the percentage yield of $^{14}CO_2$.

The rates of conversion of carbon tetrachloride into carbon dioxide are much higher in the experiments *in vitro* described in Tables 3 and 4 than in the experiments of Rubinstein & Kanics (1964). This may be because these workers used incubation periods of 2hr. The finding that the enzymic activity of microsomes falls off rapidly applies to many substrates, and cannot be used to suggest that carbon tetrachloride damages the microsomes (Gram & Fouts, 1966).

Carbon tetrachloride metabolism and carbon tetrachloride toxicity are affected in a similar manner by diet, and by stimulation of microsomal hydroxylation by DDT and phenobarbitone. The link between metabolism and toxicity may well be an intermediate in the conversion of carbon tetrachloride into carbon dioxide. Two suggestions are current, either that a free radical such as $\cdot CCl_3$ may be the damaging agent (Wirtschafter & Cronyn, 1964; Slater, 1966), or alternatively that some substance such as carbonyl chloride may be produced inside the cell. The present experiments have not led to any evidence that would allow us to decide between these possibilities.

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