Br. J. Pharmac. (1969), **37**, 733–737.

Carbon tetrachloride metabolism in the rabbit

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1. Carbon tetrachloride was administered by stomach tube to rabbits and its distribution in fat, liver, kidney and muscle studied by gas liquid chromatography, during the next 48 hr.

2. Chloroform, hexachloroethane and two unidentified chlorinated metabolites were detected in the tissues.

3. Hexachloroethane may arise by dimerization of free trichloromethyl radicals.

The hepato- and nephro-toxic actions of carbon tetrachloride have been recognized for many years (Graham, 1915 ; Meyer & Pessoa, 1923) but it was not realized until recently that these actions may be linked with the metabolism of the drug.

The progressive de-chlorination of the drug was suggested by Butler (1961), who demonstrated the presence of chloroform and postulated the formation of an intermediate trichloromethyl radical (CCl_3^-). Free trichloromethyl radicals may be expected to have high electron affinity (Gregory, 1966), to disrupt essential cellular processes (Albert, 1968) and to combine with unsaturated lipids (Gordis, 1969).

Although it seems likely that an active metabolite may be responsible for the toxicity of carbon tetrachloride, free trichloromethyl radicals have not been demonstrated. Using the electron capture detector in conjunction with gas liquid chromatography some evidence is presented supporting the hypothesis of free radical formation following carbon tetrachloride administration. Extracts from tissues of rabbits which had received carbon tetrachloride were examined by gas chromatography for high boiling point chlorinated metabolites.

Methods

To rabbits (1·5–3·0 kg) maintained on British Pelletted Diet, *ad libo*, carbon tetrachloride (1 ml./kg) was administered by stomach tube as a 20% (v/v) solution in olive oil B.P.

Sampling and extraction technique

Five rabbits were killed by stunning and bleeding 6, 24 and 48 hr after receiving carbon tetrachloride ; two rabbits which received olive oil were killed as controls.

Samples of liver, kidney, perinephric fat, muscle (gracilis) and the gall-bladder with contents were weighed and frozen at −20° C.

Tissues were ground with acid-washed silversand in a glass pestle and mortar or chopped with a homogenizer. The macerated tissue was extracted by a single heptane partition with shaking for 10 min followed by centrifugation at −5° C and RCF 910 for 1 hr to break emulsions and freeze the aqueous layer. The heptane extract was decanted and examined by gas-liquid chromatography.

Examination of samples by gas-liquid chromatography

A concentric tube electron capture detection system was used on the Aerograph Hi-Fi 600-C model gas chromatograph and aliquots of heptane extracts were injected with a Hamilton syringe. Retention times of extracted material were compared on four columns:

Column 1. 2·0 m × 3 mm stainless steel tubing packed with firebrick 60/80 mesh coated with 1·5% (w/w) SE-30 and 2% (w/w) polyethylene glycol 20 M. Column temperature 97° C. Carrier gas N_2 at 25 p.s.i. corresponding to a flow rate of 32 ml./min (soap-bubble meter).

Column 2. 3·0 m × 3 mm stainless steel tubing packed with Chromosorb G 100/120 mesh coated with 3% (w/w) di(2-ethylhexyl) sebacate. Column temperature 99° C. N_2 pressure 27·5 p.s.i. Flow rate 28 ml./min.

Column 3. 1·5 m × 3 mm stainless steel tubing packed with Celite 60/72 mesh coated with 5% (w/w) SE-30. Column temperature 76° C. N_2 pressure 25 p.s.i. Flow rate 80 ml./min.

Column 4. 6·0 m × 3 mm stainless steel tubing packed with Celite 60/72 mesh coated with 5% (w/w) SE-30. Column temperature 102° C. N_2 pressure 27 p.s.i. Flow rate 21 ml./min.

Chloroform and carbon tetrachloride were estimated on column 4 by comparison of peak heights in extracts with standard solutions of chloroform and carbon tetrachloride in heptane. Hexachloroethane was estimated on column 3 by calculation of peak area ratios to an internal standard (hexachlorobut-1,3-diene).

It was possible to separate hexachloroethane in concentrations greater than 0·5 ng/ml. Extracts were diluted when necessary to obtain a linear response from the electron capture detector. A Pyrex injector port liner was used to remove non-volatile materials from samples.

Identification of peaks from extracts

Apart from carbon tetrachloride and chloroform the materials extracted produced peaks only at high detector sensitivity. Three unknown peaks were encountered, in order of elution, " W ", " Z " and " HCE ". The HCE peak was identical in all conditions to that produced by a standard solution of hexachloroethane. The W and Z peaks were not identified. Retention times of the following were determined in an effort to identify W and Z: chloroethane ; 1,1-dichloroethane ; 1,2-dichloroethane ; 1,1,1-trichloroethane ; 1,1,2,2-tetrachloroethane ; pentachloroethane ; tetrachloroethylene ; trichloroethylene ; 1,2-dichloroethylene ; 2-chloroethanol ; 2,2,-di-chloroethanol ; 2,2,2-trichloroethanol ; dichloroacetic acid ; trichloroacetic acid ; trichloromethylsulphenyl chloride ; trichloromethylsulphonyl chloride* ; trichloromethyl mercaptan* ; hexachlorodimethylthioether* ; hydrogen sulphide, chloroacetone, trichloroacetone.

* Preparative methods used and yields were not satisfactory (see *Reagents*).

In a further attempt to identify peaks W and Z several reagents were added to heptane extracts from rabbit tissues: ammonia (SG 0·880); water; alcoholic silver nitrate solution; sodium hydroxide; nascent hydrogen; concentrated nitric acid. The extract was also evaporated at 50° C (water bath) to determine stability or relative boiling points of W and Z.

Reagents

Commercial grade reagents were used with the following exceptions: trichloromethylsulphonyl chloride (preparation attempted by the method of Sosnovsky, 1961); trichloromethyl mercaptan (preparation attempted by the method of Vogel, 1961); hexachlorodimethylthioether (Vogel, 1961); hydrogen sulphide (sodium sulphide and hydrochloric acid). Available *n*-heptane was contaminated with 4×10^{-9}–10^{-7} parts carbon tetrachloride and 0–2×10^{-9} parts hexachloroethane. A sample containing 4×10^{-9} parts CCl_4 and 0×10^{-9} C_2Cl_6 was washed with sulphuric acid (SG 1·84) and slowly redistilled until CCl_4 could not be detected by the unattenuated EC detector. Samples were analysed for recontamination before use.

Carbon tetrachloride B.P.C. (1959) quality was washed with sulphuric acid (SG 1·84) and contained not more than 125×10^{-9} parts of hexachloroethane (limit of sensitivity of method).

Direct injection into the gas-chromatograph of olive oil (B.P. grade) gave no peaks.

Results

Gas-liquid chromatographic analysis of heptane extracts of rabbit tissues using the electron capture detector enabled separation of carbon tetrachloride and some chlorinated metabolites.

The following peaks were resolved from liver, kidney, fat, muscle and gall-bladder bile of rabbits which received carbon tetrachloride:

Column 1. $CHCl_3$ (RT = 18 sec); CCl_4 (RT = 24 sec); W (RT = 76 sec); Z (RT = 144 sec); HCE (hexachloroethane, RT = 190 sec).

Column 2. $CHCl_3$ (RT = 24 sec); CCl_4 (RT = 32 sec); W (RT = 56 sec); Z (RT = 226 sec); HCE (RT = 346 sec).

Column 4. $CHCl_3$ (RT = 130 sec); CCl_4 (RT = 150 sec); W (RT = 215 sec); Z (RT = 273 sec); HCE (RT = 1,260 sec).

Highest concentrations of carbon tetrachloride were in fat (6 hr sample) and lowest were in muscle (Table 1); chloroform concentrations were high in liver and in fat in the 6 hr sample (Table 1). Concentrations of chloroform were also high in liver of a rabbit which died 44 hr after receiving carbon tetrachloride (Table 1).

Column 3. W (RT = 56 sec); Z (RT = 70 sec) HCE (RT = 119 sec); internal standard (RT = 260 sec).

The concentrations of HCE in extracts were calculated on this column (Table 1). Fat contained the highest concentration of HCE and muscle the lowest; the concentrations in all tissues were high in the 24 hr sample. The rabbit which died 44 hr after CCl_4 administration also showed high tissue concentrations of HCE

(Table 1). Tissue extracts from two control rabbits produced no response on gas-liquid chromatography.

Concentrations of W as reflected by peak area were highest in muscle and were increased when extracts were treated with concentrated ammonia. Peak Z was reduced by treatment of extracts with ammonia and could not be detected in gall-bladder bile.

Identification of W and Z

None of the compounds investigated had identical retention times to W and Z on both column 1 and column 2. Addition of several reagents (Table 2) did little to aid identification.

Peak Z was reduced by addition of concentrated solutions of ammonia, sodium hydroxide and nitric acid. Peak W increased as peak Z was reduced by concentrated ammonia (Table 2), and the relationship was linear over a period of 90 min.

Concentration of the extract at 50° C increased the concentration (as reflected by peak area) of Z and HCE and it was assumed that Z was therefore less volatile than

TABLE 1. *Concentrations of carbon tetrachloride* (CCl_4, $\mu g/g \pm$ S.D.), *chloroform* ($CHCl_3$, $\mu g/g \pm$ S.D.) *and hexachloroethane* ($CCl_3.CCl_3$, $ng/g \pm$ S.D.) *in rabbit tissues following administration of carbon tetrachloride* (1 *ml./kg*)

Tissue and sample time		No. of rabbits	CCl_4	$CHCl_3$	$CCl_3.CCl_3$
6 hr	Fat	5	787±289	4·7±0·5	4·1±1·2
	Liver	5	96±11	4·9±1·5	1·6±0·5
	Kidney	5	20±13	1·4±0·6	0·7±0·2
	Muscle	5	21±12	0·1±0·1	0·3±0·2
24 hr	Fat	5	96±11	1·0±0·2	16·5±1·6
	Liver	5	7·7±1·3	1·0±0·4	4·2±1·8
	Kidney	5	6·9±3·9	0·4±0·2	2·2±1·1
	Muscle	5	1·3±0·6	0·1±0·1	0·5±0·2
44 hr	(Died)				
	Fat	1	23	1·4	10·0
	Liver	1	1·1	4·4	3·1
	Kidney	1	0·5	0·4	2·2
	Muscle	1	0·3	Trace	9·2
48 hr	Fat	4	45±12	0·4±0·1	6·8±2·4
	Liver	4	3·8±0·1	0·8±0·2	1·0±0·3
	Kidney	4	0·5±0·3	0·2±0·0	Trace
	Muscle	4	0·5±0·3	0·1±0·1	Trace

TABLE 2. *Treatment of heptane extracts with simple reagents, followed by gas chromatography of peaks W, Z and HCE (hexachloroethane)*

Reagent	Change in gas chromatographic peak		
	W	Z	HCE
Ammonia (SG 0·880)	+++	−−	+
Water	nc	nc	nc
Concentration NaOH		−−	nc
EtOH+$AgNO_3$	nc	nc	nc
Nascent hydrogen	nc	nc	nc
Concentration at 50°		++	++
Concentration HNO_3		−−−	nc

nc, No change.
\+ ++ +++, Peak augmented.
− −− −−−, Peak diminished.

heptane (b.p. 97°–98°). Peak Z may arise from a trichloromethylated lipid as described by Gordis (1969), and Z has some properties characteristic of an ester. The peak due to standard solutions of hexachloroethane, and attributed to hexachloroethane in extracts of rabbit tissues, was not reduced by any of the reagents used (Table 2).

Discussion

Distribution of carbon tetrachloride and metabolites

The highest concentrations of carbon tetrachloride in tissues were in fat samples 6 hr after administration, but these diminished rapidly during the subsequent 42 hr. Chloroform was identified in four tissues together with hexachloroethane. Hexachloroethane was detected in all tissues, but fat contained the highest concentrations as in sheep given the drug (Fowler, 1969). The amount of hexachloroethane in rabbit tissues probably represented only a fraction of the total formed, since hexachloroethane is metabolized by the liver (Fowler, unpublished). Concentrations of chloroform and hexachloroethane were high in the tissues of a rabbit which died 44 hr after receiving carbon tetrachloride. This is in accordance with the results of Garner & McLean (1969) which suggest that toxicity of carbon tetrachloride is related to the absolute quantity metabolized.

Although hexachloroethane is an anthelmintic and causes hepatotoxicity it seems unlikely that it is responsible for these properties of carbon tetrachloride. It may, however, be formed by the dimerization of trichloromethyl radicals to hexachloroethane.

My thanks are due to Professor F. Alexander and Mr. J. Nicholson for advice throughout this work and to Mr. J. Allen for technical assistance.

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(*Received June* 24, 1969)