An investigation into the fate of certain halogenated hydrocarbon compounds in domesticated animals.

by

J. S. L. Fowler

A thesis presented for the degree of Doctor of Philosophy in the Department of Veterinary Pharmacology, Faculty of Veterinary Medicine, University of Edinburgh, May 1970.

to my family

Declaration.

In compliance with Edinburgh University regulation 2.4.15 I, the undersigned, hereby declare that this thesis has been composed by myself and that the material in it is my own.

In compliance with regulations 2.4.11 and 5.7 reprints of two papers and a communication to the British Pharmacological Society describing some of the material in this thesis are enclosed as an appendix.

Other parts of the experimental work have been accepted for publication; that concerning cockerels and ducks, by the Editors of the Journal of Comparative Pathology; that concerning drug actions on liver flukes by the Editors of the British Journal of Pharmacology.

J. S. L. Fowler

ABSTRACT OF THESIS

Name of Candidate J. S. L. Fowler

Address .....

Degree Ph. D. *Date* 26th May 1970

Title of Thesis An investigation into the fate of certain halogenated hydrocarbon compounds in domesticated animals.

The fate of carbon tetrachloride and hexachloroethane was studied in sheep, rabbits, cockerels, ducks and liver flukes. A new method was described for detection and estimation of hexachloroethane and its metabolites pentachloroethane and tetrachloroethylene in biological extracts using a multi-column gas-liquid chromatographic technique. Pentachloroethane had not prieviously been reported as a metabolite of hexachloroethane and was itself metabolised to tetrachloroethylene.

A new metabolite of carbon tetrachloride, hexachloroethane, was described and may have arisen by dimerisation of free trichloromethyl radicals. Detection of the dimer of such radicals may provide evidence for the formation of trichloromethyl radicals from carbon tetrachloride ; a current hypothesis suggests that the toxicity of the drug is due to liberation of these radicals in vivo.

The biliary excretion of carbon tetrachloride and hexachloroethane in several species including sheep was studied.

The effect of carbon tetrachloride, hexachloroethane, pentachloroethane, tetrachloroethylene, tetrachlorodifluoroethanes and bistrichloromethylbenzene on plasma enzyme activities in sheep was studied. Carbon tetrachloride and hexachloroethane were hepatotoxic in sheep, but probably not in cockerels and ducks. Hepatotoxicity due to hexachloroethane had not prieviously been described and was detected by elevation of plasma enzyme activity and reduced bromsulphthalein dye transfer rates.

The hepatotoxicity of carbon tetrachloride for sheep and tolerance of cockerels and ducks to the drug was discussed.

The in-vitro toxicity on liver flukes of various substances was studied. Liver flukes metabolised carbon tetrachloride and hexachloroethane in vitro. Carbon tetrachloride, carbon tetrachloride treated liver lipid and a carbon tetrachloride-methyl oleate complex were toxic to liver flukes in vitro, in the presence of sheep bile.

The fasciocidal action of carbon tetrachloride was discussed and related to the toxicity of the drug in mammals.

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Summary.

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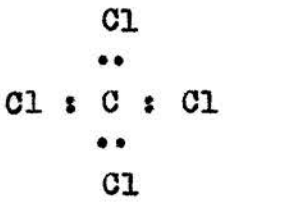
Structures of drugs.



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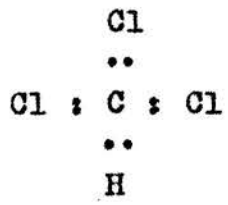

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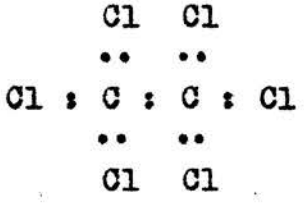

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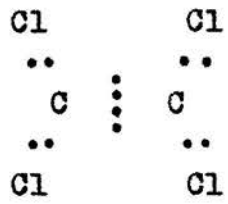

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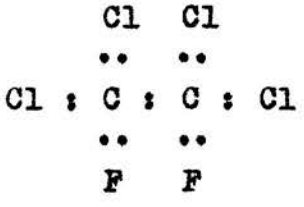

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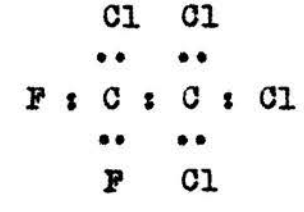


1,1-difluorotetrachloroethane (Freon 112a)

bistrichloromethylbenzene (Hetol)

Introduction.

Halogenated hydrocarbons are probably the oldest group of synthetic drugs used in veterinary anthelmintic therapy.

In 1918, Hall and Foster reviewed the available anthelmintics and noted the value of chloroform against hookworms in dogs.This was replaced by carbon tetrachloride (Hall,1921) * and in 1923 Hall and Shillinger described the use of this drug against Haemonchus contortus and other helminths of sheep. The first report of large scale medication of food animals with an halogenated hydrocarbon was probably that of Hall and Shillinger (1925)(53). In the same year workers in Eastern European countries described the use of proprietary mixtures containing carbon tetrachloride against Fasciola hepatica, the common liver fluke of cattle and sheep (117)(118)(30)(31)(93). Ernst also recorded the use of carbon tetrachloride alone against F. hepatica of sheep (32).

Also in 1925 the unsaturated chlorinated hydrocarbon, tetrachloroethylene, was used to treat hookworm infestation in dogs (54).

The first accounts in Britain of the use of a chlorinated hydrocarbon as an anthelmintic were from Montgomerie (1926) and Norris (1926) who reported the use of carbon tetrachlor-

* Author names quoted in key references only otherwise a number system has been adopted.

ide against F. hepatica of cattle and sheep.

A related chlorinated hydrocarbon, hexachloroethane, was found to be active against F. hepatica of cattle when dissolved in tetrachloroethylene ('Neoserapis')(Thienal,1926). Tetrachloroethylene alone was said to be active against stomach worms of sheep (106) but this has not been confirmed by more recent work (36).

Use of chlorinated hydrocarbons as anthelmintics was accompanied by reports of acute toxic reactions to these drugs. Toxicity under certain conditions has been partly responsible for the recent decline in their use. The acute toxicity of the newer drugs which are replacing the chlorinated hydrocarbons is less; however, the chronic effects have not been fully evaluated. Moreover, persistance in the tissues of some compounds, such as the nitro- compounds niclofolan and nitroxynil, may present a hazard when they are used in food animals. The halogenated hydrocarbons have the advantage that a large proportion of the dose is rapidly excreted through the lungs since these agents are volatile.

Carbon tetrachloride as a fasciocidal agent.

Carbon tetrachloride has been used as a fasciocidal agent in sheep in a wide range of doses (117)(118)(30)(31)(93) (32).Although doses up to 2 ml per kg have been used, the current B.Vet.C. recommendation is for a total dose of only 2 to 5 ml by mouth for cattle and sheep (13). This dose is

effective against adult liver flukes such as are encountered in 'chronic fascioliasis', the commonest manifestation of liver fluke infection. Higher doses are required when treating 'acute fascioliasis', which is caused by immature liver flukes 4 to 10 weeks after their entry into cattle or sheep; doses with reasonable 'larvicidal activity' (84 per cent of 4 week old flukes removed) may be two to three times the B. Vet. C. recommendation.

Acute toxicity may follow administration of high doses of carbon tetrachloride and toxicity has been observed after low doses when animals are on certain dietary regimes (80). For example, toxic signs have been observed in sheep receiving supplementary feeding (88)(69) and high mortality occurred in a flock of ewes receiving cotton-cake (18 to 21 per cent crude protein) at the time of carbon tetrachloride administration (115). Parenteral administration of the drug may reduce the toxicity (63); large numbers of animals have been medicated by this route (89) and a wide range of doses ~~rates~~ has been found satisfactory against F. hepatica (7). The site of parenteral administration influences blood concentrations of carbon tetrachloride; intramuscular injection delayed distribution and reduced blood concentrations of the drug when compared with intra-ruminal or intra-abomasal administration (67). Intramuscular injection of the drug is also effective against F. hepatica (68).

The toxicity of carbon tetrachloride for man is of interest since the drug is widely used as an industrial solvent. Van Oettingen regards carbon tetrachloride as 'a dangerous and insidious industrial poison' (90). Toxic effects following exposure to the vapour have included depression of the central nervous system, irritation of mucous membranes and defatting of the skin leading to dermatitis. Exposure to carbon tetrachloride in vapour form is most likely to occur when used in industrial and drycleaning processes(Weir,1969). The drug is also toxic to man by the oral route and has been used successfully in attempts at suicide (90). Oral ingestion may give rise to gastro-intestinal haemorrhage, abdominal pain and coma. Jaundice frequently occurs accompanied by ascites and general oedema; congestive heart failure with cyanosis leading to collapse has been described in severe cases (90). Hepatic effects are exacerbated by prievious ingestion of alcohol (90). Renal dysfunction is frequently seen in human cases of carbon tetrachloride poisoning (128) (85)(46). An initial polyuria is followed by oliguria or anuria: urine passed may contain blood, tubular casts, high albumen concentrations and bile pigments; in man, renal lesions can occur in absence of gross hepatic disorder (90).

In domesticated species, toxic symptoms usually arise as a result of administration of carbon tetrachloride as an anthelmintic. In sheep toxic signs following oral administration

of the drug may include deranged calcium metabolism (104); wool-shedding, diarrhoea, depression and gastro-enteritis (103); or combined kidney and liver dysfunction (111). However, the most frequent finding is disturbed liver function (Alexander and Macdonald,1960; Ford and Lawrence, 1965).

The toxicity of carbon tetrachloride for sheep and cattle when administered by the oral route and, to a lesser extent by the parenteral routes, has led to a recent decline in use of the drug. Boray and Happich (1968) consider carbon tetrachloride inferior to niclofolan, nitroxynil and cliox-anide for treatment of acute fascioliasis in sheep.

Since its introduction carbon tetrachloride has been regarded as especially toxic for cattle (79)(13). As a treatment for fascioliasis in cattle, carbon tetrachloride was superceded first by hexachloroethane, and recently by hexachlorophene, oxyclozanide and nitroxynil.

Despite its limitations, there would seem no reason to discontinue use of carbon tetrachloride as a routine prophylactic and chemotherapeutic agent for chronic fascioliasis in sheep held at a low plane of nutrition. Carbon tetrachloride is cheap and its efficacy against mature liver flukes compares favourably with the newer drugs (8).

Carbon tetrachloride is also effective against Paramphistomum spp. (the rumen flukes)(72)(49)(71);Dicrocoelium sp. (the lanceolate fluke)(48)(47); against gastro-intest-

inal parasites and F. hepatica of goats (18) and against F. hepatica of pigs (130).

In poultry carbon tetrachloride has been used against Ascaridia galli but has been replaced by piperazine compounds (40); it is also suitable for treatment of Amidostomum anseris infections in turkeys (40).

Hexachloroethane against Fasciola hepatica.

Since the introduction of hexachloroethane (Thiemal,1926) it has been (until recently) the drug of choice for treatment of F. hepatica infestations of cattle. Hexachloroethane is well tolerated by cattle (92) and was also used extensively in sheep after its introduction in 1927 (86)(37) (91) although there have been accounts of poisoning,possibly involving calcium metabolism (114)(16)(40). Its potency against F. hepatica and toxicity to sheep offered no advantage over carbon tetrachloride.

Hexachloroethane is not suitable for parenteral administration as it is not very soluble and if injected may taint the carcase.

Toxic reactions to hexachloroethane have included intoxication, inco-ordination, muscle tremors and death (91)(92) (114)(16) and as with carbon tetrachloride, the toxicity of hexachloroethane is increased by a high protein diet and withdrawal of such feedstuffs at the time of dosage has been advocated (40).

Related halogenated hydrocarbons.

Tetrachloroethylene.

Tetrachloroethylene is an important industrial solvent with anthelmintic properties. It is used in drycleaning processes, for degreasing metals, extraction of natural oils and as an anthelmintic agent (90).

Since its introduction as an anthelmintic in 1925 (54), tetrachloroethylene has been widely used for eradication of hookworms in dogs, cats and humans (82)(13)(90). Tetrachloroethylene is also active against Haemonchus placei of cattle (102) and has some action against stomach worms of sheep (106). There are no records of activity against F. hepatica.

The hepatotoxic action of tetrachloroethylene is considered to be slight. In man, exposure to the vapour may result in 'burns' due to severe defatting of the skin and ingestion may be followed by giddiness, dizziness, vertigo, inebriation, nausea or even coma, although these reactions are not common (90). In domesticated species toxic reactions observed have included cloudy swelling of liver cells (in calves)(108)(107); coughing, choking, giddiness, anaesthesia and death (in cattle) (102); deaths in sheep 2 to 10 days after treatment (114) and in dogs debilitated by distemper. Healthy dogs tolerated anthelmintic doses of tetrachloroethylene without discomfort (55)(54).

Although some other halogenated hydrocarbons possess anthelmintic activity, none ~~have~~ has achieved wide popularity as fasciocidal agents.

Tetrachlorodifluoroethanes.

The tetrachlorodifluoroethanes ('Freons') are fully halogenated compounds homologous with hexachloroethane in two isomeric forms: 1,2-difluoro and 1,1-difluoro tetrachloroethane ('Freon 112' and 'Freon 112a'). The presence of fluorine on a carbon atom stabilises other halogen atoms and metabolism of the Freons may be less than that of fully chlorinated compounds (14)(45)(95).

Tetrachlorodifluoroethane is not particularly toxic to domesticated species; although ruminitis has been reported in cattle (59), liver damage is said not to occur (9)(44). Prolonged administration or very high dose rates have however, produced pulmonary damage in rats (administered by inhalation) although they can tolerate an oral dose of 2 g per kg daily for 23 to 33 days'without pathologic change in any organ' (44). A dose of 1.5 to 1.7 g per kg killed two out of three sheep which received it (94).

Tetrachlorodifluoroethane is active against mature F.hepatica and Paramphistomum spp. (10)(94)(59).

Bistrichloromethylbenzene.

Bistrichloromethylbenzene ('Hetol') may be regarded as a

'stretched' hexachloroethane molecule, the trichloromethyl groups situated in the 1,4- positions of a benzene ring. Although Hetol is active against mature F. hepatica of sheep and cattle (8)(29) it is less efficient and more toxic to sheep than carbon tetrachloride or hexachloroethane (8); however, it is reported to be well tolerated by rats (135) and even by sick and gravid cattle at the dose employed for removal of F. hepatica (29).

It seems unlikely that Hetol will compete successfully with the newer fasciocidal drugs, at least in the Western Hemisphere.

Metabolism of halogenated aliphatic hydrocarbons.

Dehalogenation commonly occurs in vitro when halogenated compounds are incubated with tissue extracts.

Removal of halogen has been attributed to enzymatic activity and'enzymatic dehalogenation' has been described for several compounds including brominated and chlorinated aliphatic hydrocarbons (57). The halogenated anaesthetics halothane and methoxyflurane are also debrominated, dechlorinated and reduced by microsomal enzymes with a requirement for $NADPH_2$ and oxygen, but the carbon - fluorine bond is apparently stable (14)(45)(95).

Non-enzymatic dehalogenation may also occur, at least in

in-vitro systems. Free halogen was detected and -SH content reduced when cysteine was incubated with several halogenated compounds (12). Enzymes containing -SH groups may be attacked by halogenated hydrocarbons in vivo since urease, succinic dehydrogenase and papain are inactivated in vitro by methyl bromide (73).

Dehydrochlorination occurs during metabolism of aromatic chlorinated hydrocarbons such as the insecticide dicophane (DDT) and may be a general method of detoxification of halogenated compounds; in this case, removal of H-Cl leaves a double bond and DDT gives rise to DDE. Resistant houseflies rapidly convert DDT to DDE and introduction of a double bond has been suggested as a detoxification mechanism (133).

Metabolism of carbon tetrachloride and hexachloroethane.

It seems likely that, although the mechanism involved is not understood, the toxicity of carbon tetrachloride can be related to the metabolism of the drug (Butler, 1961; Slater, 1966). It was thought that carbon tetrachloride was directly toxic because of its lipid solvent action but this did not account for the potent hepatotoxic action of the drug which was greater than that of related lipophilic compounds (45). Nor did it account for the centrilobular location of lesions, the susceptibility of the endoplasmic reticulum to early damage or even the extreme sensitivity of the liver to its toxic

action (Slater,1966). The'direct solvent action theory' was then clearly untenable.

A second possibility was that the products of dechlorination of carbon tetrachloride and hexachloroethane may be toxic in their own right. Thus it was suggested that free hydrochloric acid may be liberated in vivo after chemical dissociation of carbon tetrachloride (Graham,1915).Graham found that carbon tetrachloride was more toxic than chloroform and dichloromethane and he suggested that this was a result of the higher chlorine content, which might have been expected to give rise to greater concentrations of halogen acid. He also suggested that the toxic action of phosgene might be due to hydrolysis in vivo with production of hydrochloric acid and carbon dioxide.

In support of this hypothesis, areas of centrilobular necrosis produced by carbon tetrachloride administration gave an acid reaction and took up the indicator neutral red. However, against the hypothesis, Graham was not able to produce centrilobular lesions with hydrochloric acid, which in fact produced perilobular lesions. A further objection to Graham's hypothesis is that an equimolar dose of hexachloroethane is not more toxic than carbon tetrachloride, despite its greater chlorine content.

In-vivo dechlorination of carbon tetrachloride and hexachloroethane has given rise to organic chlorinated metabol-

ites. Thus, in dogs, carbon tetrachloride was metabolised to chloroform, an example of reductive dechlorination (Butler,1961) and, in rabbits, several metabolites of labelled hexachloroethane ($^{14}C_2Cl_6$) were reported (Jondorf,Parke and Williams,1957), including toxic compounds such as sym-tetrachloroethane and monochloracetic acid (Williams,1959).

Although some of the metabolites of hexachloroethane were known to be toxic to mammals, a major metabolite, tetrachloroethylene, was of low toxicity to domesticated species (90) and may be analogous to the ethylenic metabolite of dicophane (DDE) which arose by dechlorination of the parent compound (129).

Metabolism of hexachloroethane may thus involve progressive dechlorination with reduction to yield products with a wide range of toxicity and the quantitative yield of each metabolite is likely to be of importance when assessing the toxicity of the drug in vivo.

Although there was some suggestion that metabolites of carbon tetrachloride might be responsible for its toxicity, none was known with such marked hepatotoxic properties; as far as hexachloroethane was concerned, several of the proposed metabolites were more toxic in equimolar doses (Williams,1959).

A third, and the current hypothesis on the mode of action of carbon tetrachloride is really an extension of the second 'dechlorination hypothesis'. In this case the product of de-

chlorination has not been identified but may be a highly active free radical of short half-life presumably arising from a homolytic cleavage of the carbon tetrachloride molecule (Butler,1961; Wirtschafter and Cronyn,1964;Slater, 1966). Because of its unpaired electron, such a small,free radical would be extremely reactive. The 'trichloromethyl radical' has been suggested as an activated form of carbon tetrachloride (15)(112) although,as yet, no electron spin resonance data has been presented to confirm the existence of such radicals in vivo (Albert,1968).

In the absence of marked toxic reactions to hexachloroethane, a free radical metabolite has not been suggested for this compound although the related compound, pentachloroethane, may react chemically through a free radical intermediate (Walling, 1957). DDT, which in some ways may be regarded as an analogue of pentachloroethane, also gives rise to free radicals, at least in vitro (84).

The toxicity of carbon tetrachloride in domestic animals.

Carbon tetrachloride is toxic to many species. Hall and Shillinger (1923) quoted the following tolerances: fowl 20 ml per kg; dog 16 ml per kg; cat 8 ml per kg; monkey 6 ml per kg; rabbit 5 ml per kg; fox 2.7 ml per kg; hog 1.66 ml per kg; sheep 1.3 ml per kg; turkey, at least 1 ml per kg; cattle 0.88 ml per kg (52). These doses are higher than recently

determined values but illustrate a wide range of susceptibility to the drug. Carbon tetrachloride is a potent hepatotoxin; as little as 16 mg per kg body weight depressed glucose-6-phosphatase activity (100) and doubled liver triglycerides in rats (97): 30 to 100 mg per kg killed parasitic liver flukes in the bile ducts of sheep (B.Vet.C.), reduced bromsulphthalein dye clearance and caused fatty infiltration , centrilobular necrosis and congestion of the liver (Alexander and Macdonald,1960). Hepatic dysfunction is probably always induced when fasciocidal doses of carbon tetrachloride are administered to sheep (4).

Renal damage, such as is reported in man (von Oettingen, 1964) probably only occurs when sheep receive massive doses of carbon tetrachloride (111). However, kidney damage has been observed in dogs (78) and in cats (132) after exposure to the drug.

Correlation of metabolism with toxicity of the drug.

The marked species differences in tolerance to carbon tetrachloride indicate that the fate of the drug is different in, for example, chickens and rats. This, of course, could relate to rate of absorption, elimination, to an absolute difference in sensitivity or distribution of the drug. Thus when Cameron and Karunaratne (1936) observed that young (6 to 10 g) rats were resistant to carbon tetrachloride the tolerance was attributed to circulatory differences between adult and neonatal

animals. Although this now seems unlikely to be a major factor in rats (95), distribution of the drug can be related to toxicity of the drug in ruminants (68).

It is well known that the metabolism of a drug may differ markedly in various species, even in populations within a species. Differences may be genetic, for example the inability of the Gunn strain of rats to conjugate bilirubin (95), or environmental. It has long been recognised that carbon tetrachloride may be toxic under certain conditions such as high protein intake (88)(115)(69) and this has recently been rationalised in terms of metabolic studies. Well-fed sheep showed lower bromsulphthalein dye clearance rates after carbon tetrachloride administration than did poorly fed sheep (4) and poorly fed rats were also resistant to the toxic effects of the drug. Rats fed protein depleted diets for at least 4 days became resistant to carbon tetrachloride poisoning as measured by LD_{50} , liver enzymes and liver fat content (McLean and McLean, 1966). A protein intake provided by 6 per cent casein in the diet led to a 50 per cent reduction in demethylation and hydroxylation enzyme activities; a 3 per cent casein diet reduced enzyme activities by 80 per cent. Conversely, administration of DDT or phenobarbitone increased the toxicity of carbon tetrachloride and increased microsomal enzyme activity (77). Concentrations of carbon tetrachloride in blood and liver tissue are little affected

by protein depletion or DDT administration and so metabolic differences appear to be implicated (109). Phenobarbitone also enhanced the toxicity of carbon tetrachloride for sheep (110) and quantitative results relating metabolism to toxicity in rats have recently been published. Administration of phenobarbitone sodium (1 mg per ml) in the drinking water for 14 days prior to carbon tetrachloride administration reduced the LD_{50} from 3.6ml per kg (control) to 0.5 ml per kg (test). Rats which received 0.25 ml per kg suffered similar liver damage to control rats which received 2.5 ml carbon tetrachloride per kg; both groups metabolised 12 mg per kg in a 6 hr period. This was the first report which linked toxicity with the absolute quantity of drug metabolised; the extent of the liver damage was related to the amount of the drug metabolised, not the amount administered (Garner and McLean, 1969). This supports the 'activation theory' for the toxicity of carbon tetrachloride (Slater, 1966).

Assessment of hepatotoxicity.

Although the classical work concerning carbon tetrachloride toxicity employed histopathological techniques (Graham, 1915; Meyer and Pessoa, 1923; Cameron and Karunaratne, 1936), in recent years, increasing emphasis has been placed on detection of functional or 'biochemical' lesions.

Increased plasma activity of tissue enzymes as a means of

referring damage to certain tissues has recently beoome popular. Few enzymes are organ specific or even tissue specific; however the relative differences in enzyme activity of various tissues is often large and clinical interpretations based on plasma enzyme activity have gained acceptance. Increased plasma activity of enzymes may result from ~~either~~ an increased rate of cell attrition or necrosis induced by a chemical, physical or biological cell toxin, or by a decreased rate of inactivation of enzymes in plasma (King, 1965).

Normal plasma activity of so-called 'tissue enzymes' may be due to leakage of soluble enzymes from the microsomes and cellular cytoplasm (66). These soluble or unbound enzymes may be expected to show rapidly in the circulation after the administration of cytotoxic agents; mitochondrial and nuclear enzymes in the plasma may be evidence of more severe cell damage.

Although choice of suitable enzyme tests may enable localisation of damage to a particular organ, the rate of clearance of a dye, bromsulphthalein, may also be determined to further assess hepatic function.

Bromsulphthalein dye is injected intravenously and passes from the plasma into the hepatic polygonal cells by which it is excreted into the bile; 85 per cent of a dose has been recovered from the hepatic duct of sheep within 4½ hr of administration (24).

Bromsulphthalein dye does not undergo enterohepatic circulation in dogs and has been used as an intestinal marker in absorption studies (74). There are few extra-hepatic binding sites; however, 24 hr is usually allowed before repeating a test.

The dye half-life in the circulation of normal cattle is 4.18 min (60) and in sheep is 2.03 ± 0.34 min (23); as an example of the extent that this may be changed in hepatic dysfunction, in sheep suffering from pregnancy toxaemia the dye half-life range was from 7 to 62 min (23).

When large doses of bromsulphthalein are administered to normal animals, or when liver function is impaired, the plasma disappearance curve for the dye may be resolved into two components (101). The first component represents removal of bromsulphthalein from the plasma to saturate the liver cells, the second component also represents removal of dye from plasma but in this case is directly related to the rate at which liver cells can excrete bromsulphthalein into the bile.

Thus a measure of the excretory capacity of the liver may be obtained as a bromsulphthalein dye transfer rate (20).

Although aspartate aminotransferase (GOT) activity and alanine aminotransferase (GPT) activity have been useful in assessing hepatic and myocardial damage in humans (99), they are not satisfactory when applied to ruminants (11).

GOT determinations may be employed but glutamate dehydrogenase (GD), sorbitol dehydrogenase (SD) and ornithine carbamyl transferase (OCT) activity are particularly suitable (Ford,1967). In sheep, GD activity is highest in liver and kidney; SD activity is also high in these tissues but OCT activity is highest in liver and small intestine and low in kidney. A simultaneous rise in plasma activity of GD, SD and OCT is seen as a result of administration of hepatotoxic agents to ruminants (33) and may be useful when assessing experimental hepatic lesions.

Methods

Carbon tetrachloride, hexachloroethane, bistrichloromethylbenzene and the tetrachlorodifluoroethanes are linked by their common fasciocidal actions and part of the object of the experimental work was to determine whether any further similarities (apart from the obvious similarities of structure) could be elucidated, particularly those that might relate to the mode of action of these drugs on the common liver fluke, Fasciola hepatica.

A series of experiments was designed and conducted to study metabolism and toxicity of carbon tetrachloride. The drug was administered to sheep, rabbits, cockerels, ducks and liver flukes in an attempt to relate toxicity and metabolism in a wide range of species.

For comparison, hexachloroethane was administered to sheep, cockerels, liver flukes and several laboratory species.

The hepatotoxicity of 1,4-bistrichloromethylbenzene (Hetol) and tetrachlorodifluoroethanes (Freon 112 and Freon 112a) were studied in sheep.

Management of animals.

Sheep

Metabolism of carbon tetrachloride was studied in sheep 37,38,60 (Scottish Blackface); 21,23,24,33,34,35,36,39a,39b, 40 (Cheviot Cross). Sheep 39a,39b and 40 were female and the other sheep were castrated males.

Metabolism of hexachloroethane was studied in sheep 1,2,3, 4,27,28 (Scottish Blackface); 5,6,7,8,9,10,11,12,17,18,19,20, 22,25 (Cheviot Cross). Sheep 6 and 8 were entire males; the other sheep were castrated males.

Sheep weighed from 15 to 33 kg and were housed with hay and water available ad lib..

Sheep 27 and 28 were used for bile collection whilst anaesthetised and did not regain conciousness; sheep 40 and 60 received rumen fistulae and bile duct cannulae.

Rabbits

Rabbits weighing 1.5 to 3.0 kg were maintained on British Pelleted Diet in wire cages with water available ad lib..

Cockerels

White Leghorn cockerels (1.4 to 1.6 kg) were maintained in battery cages (40 x 35 x 35 cm) on layers mash (16 per cent protein) with water available ad lib..

Ducks

Khaki Campbell ducklings of both sexes, weighing 1.6 to 1.9 kg were maintained indoors on straw with water and layers mash available ad lib..

Liver flukes

Viable liver flukes were obtained from sheep bile ducts within 1 hr of slaughter and were washed with Hedon-Fleig solution at room temperature(see p. 40).

Entire motile flukes were incubated one per tube with emulsions of drugs or various media. Incubation was at 37°C. in the dark, in screw top containers containing 8 ml medium (or containing 20 ml medium in studies of hexachloroethane metabolism).

Other experiments

Small laboratory animals were obtained as required, from the usual sources.

Scottish Blackface sheep 29,30,31,32 (23 to 32 kg) received tetrachlorodifluoroethanes; Cheviot Cross sheep 48 (28 kg) and 49 (19kg) received bistrichloromethylbenzene.

Administration of drugs.

Carbon tetrachloride

Carbon tetrachloride was diluted with 4 parts of olive oil (20 per cent solution) before administration to rabbits or sheep. Cockerels and ducks received olive oil 2 parts, carbon tetrachloride 1 part (33 per cent solution).

The oily solutions were administered from an all-glass syringe through a silicone rubber stomach tube of suitable diameter.

Carbontetrachloride was administered to liver flukes as an emulsion in the incubation medium (Table 7, page 60). Acacia powder (0.1 per cent w/v) was used as the emulsifying agent and the disperse phase droplet size was 1.4 to 5.0 µm (diameter of not less than 95 per cent of the droplets) as measured by a microscope stage micrometer.

Hexachloroethane

Hexachloroethane in olive oil (15 per cent w/v) was administered to cockerels by stomach tube or injected direct to the alimentary tract in anaesthetised animal experiments.

Before administration to unanaesthetised sheep a (15% w/v) solution of hexachloroethane in olive oil was emulsified with water using powdered acacia (12.5 per cent w/v) and powdered tragacanth (0.7 per cent w/v) as emulsifying agents to produce a final volume of about 300 ml.The emulsion was administered from a drenching bottle.

Hexachloroethane was administered to flukes as an emulsion (Table 15, page 73).

Pentachloroethane and tetrachloroethylene

Pentachloroethane and tetrachloroethylene were diluted with 2 parts olive oil (33 per cent v/v solution). The solution was administered from a syringe with a four inch tubular extension.

'Hetol' and 'Freons'

Hetol and Freons were administered by stomach tube as, respectively, 10 per cent (w/v) and 20 per cent (v/v) solutions in olive oil.

Doses of drugs

Carbon tetrachloride:

2 ml Sheep 21,23,24

3 ml Sheep 33,34,35,36,37,38,39a,39b,40,60

6 ml Cockerels and ducks

1 ml per kg Rabbits

Hexachloroethane:

0.5 g per kg Sheep 1,2,3,4,5,6,11,12,27,28

0.75g per kg Sheep 7

1.0 g per kg Sheep 8

4 g (total) Cockerels

Pentachloroethane: 0.3 ml per kg (Sheep 19 and 25)

Tetrachloroethylene: 0.3 ml per kg (Sheep 20 and 22)

Freons: 0.33 ml per kg (Sheep 29,30,31,32)

Hetol: 0.15 g per kg (Sheep 48 and 49)

A dose of 2 to 3 ml carbon tetrachloride by mouth is within the B.Vet.C. recommended range for treatment of liver fluke infection in sheep; in terms of ml per kg the doses of the drug were as follows:

0.08 ml per kg Sheep 23,24

0.1 ml per kg Sheep 21,33,36,37,38

0.11 ml per kg Sheep 34

0.12 ml per kg Sheep 39a,39b,40

0.13 ml per kg Sheep 35

0.15 ml per kg Sheep 60

Sheep 9,10,17,18 received olive oil only.

The concentrations of carbon tetrachloride and hexachloroethane in liver fluke incubation media are shown in Tables 7 and 15 (pages 60 and 73).

Collection of samples.

Blood:

Sheep Blood samples (5 ml) were taken from the jugular vein with evacuated glass tubes containing sodium heparin as anticoagulant. Plasma and erythrocytes were seperated by centrifugation for 20 min at RCF 1600. Plasma was used immediately for various analyses or stored frozen at -20°C for subsequent use. Erythrocytes were washed three times with 0.15 M sodium chloride solution before use or storage.

Cockerels and ducks Blood samples (2 ml) were taken from

the median vein with a heparinised disposable syringe and 26 g needle. Plasma was seperated as above for use or storage.

Urine and faeces:

Urine and faeces samples were collected from sheep 11 and 12 while they were confined in metabolism cages. Faeces were collected on polyethylene sheeting and recovered at 24 hr, 48 hr, 72 hr and 96 hr for examination. Urine was collected from male sheep into a receiver cooled by an ice-water jacket (Warwick,1969). Urine was collected from female sheep (39a,39b) by use of a silicone rubber retention catheter (Folatex 60 80G).

Bile:

Anaesthetised sheep

Sheep 27 and 28 were fasted for 24 hr and anaesthetised with pentobarbitone sodium. The bile duct was cannulated with polyvinyl tubing (4 mm o.d.) and the cystic duct ligated. The common duct was also ligated and the duodenum occluded above and below the sphincter of Oddi with bowel clamps to avoid any chance of samples becoming directly contaminated with hexachloroethane. After collection of a sample of bile, hexachloroethane solution was injected at a dose of 0.5 g per kg into the rumen and lower duodenum (divided dose). Bile was collected continuously and 2 ml retained each 30 min for analysis; any remaining bile was returned to the lower duodenum after recording its volume.

The preparation was artificially respired with a mixture of 95 per cent oxygen and 5 per cent carbon dioxide for 1 min every 30 min to supplement normal respiration and to reduce any tendency to hypoxia. Anaesthesia was maintained by intravenous infusion of pentobarbitone sodium and the experiments were terminated 8½ hr after administration of hexachloroethane.

Non-anaesthetised sheep

A silicone rubber cannula (3.2 mm o.d.) was inserted in the bile duct of sheep 40 and sheep 60. In sheep 60 the gall-bladder and cystic duct were functionally obliterated by a silicone rubber cannula (5 mm o.d.) introduced through the gall-bladder towards the cystic duct which allowed flushing out of the system. Bile was returned to the duodenum by a further cannula (3.2 mm o.d.)(Plate 1). Bile duct and duodenal cannulae were exteriorised to the right sub-lumbar fossa and connected by a polyethylene non-return valve (Griffin S 42- 630) with adaptors. This obviated blockage of bile cannulae by aspiration of duodenal contents and reduced risk of ascending biliary infection by preventing reversal of bile flow during duodenal activity. A rumen cannula with fenestrated flange (3) was also inserted.

Bile was collected in sterile 2 litre polyethylene bags supported in a non-crushable harness on the sheep.

The operation was conducted under thiopentone sodium and cyclopropane anaesthesia; two weeks of post-operative recovery were allowed before collection of bile.

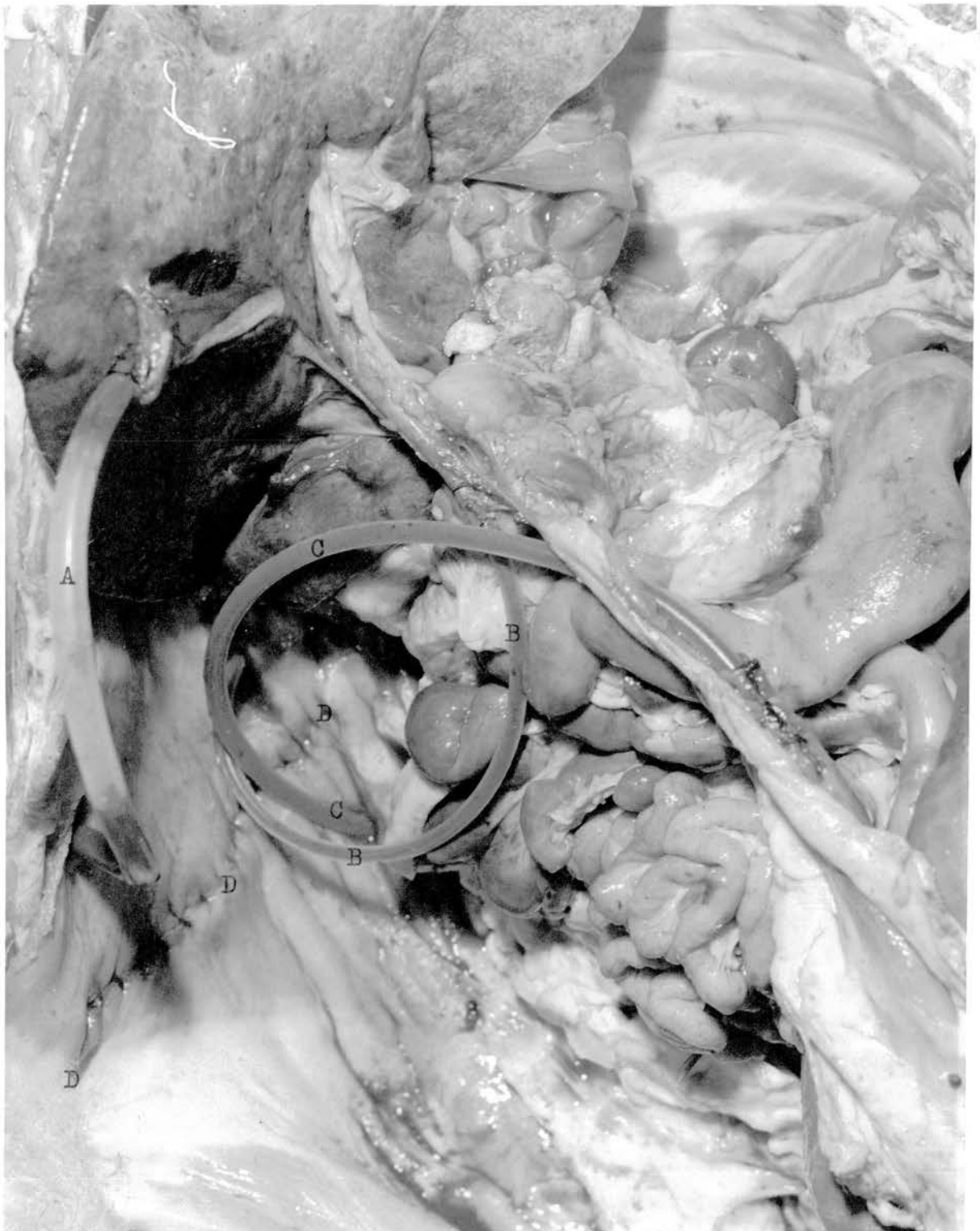


Plate 1. Cystic duct (A), bile duct (B) and duodenal (return) (C) cannulae. Sheep with 6-week F.hepatica infection. Right paracostal incision (D). 6/5th actual size.

Rabbit gall-bladder bile

The contents of the gall-bladders of five rabbits were taken 6 hr, 24 hr and 48 hr after carbon tetrachloride administration.

Other species

Bile was collected through polyvinyl cannulae from preparations anaesthetised with urethane.

Tissue samples.

Sheep Samples (3 to 4 g) of brain, liver, kidney, muscle; perinephric, subcutaneous and inguinal fat were taken from sheep 27 and 28 within 10 min of death, weighed and stored frozen at -20°C until required.

Rabbits Samples of liver, kidney, perinephric fat and muscle (gracilis) were taken 6 hr, 24 hr and 48 hr after administration of carbon tetrachloride.

Ducks and cockerels Fresh samples of liver, kidney, spleen, cardiac muscle, skeletal muscle, gizzard wall, section of small intestine, brain, lung and pancreas were weighed in 25 ml screw top glass containers.

Liver flukes Liver flukes were blotted dry and used immediately or stored frozen at -20°C.

Storage of biological samples.

Urine and faeces samples were used as procured. Aliquots of plasma, erythrocytes, bile or tissues were stored frozen in glass tubes closely covered by metal foil secured by a screw top or elastic ring.

Before use, bile, blood or erythrocytes samples were stood at room temperature until liquid: tissue samples were ground from the frozen state (sheep, rabbit, liver fluke) or fresh (rabbit, liver fluke, cockerel, duck).

Preparation of extracts for gas chromatography.

Hexane extraction.

Bile, plasma and red blood cells Samples (1 ml) of bile or plasma, or weighed samples of red blood cells, were extracted in closed tubes with hexane (2 ml) containing hexachlorobut-1,3-diene as internal standard. Ammonium sulphate solution (1 ml 2.5 M) was included to reduce the extent of emulsion formation and saponin (2 ml 0.01 per cent w/v) solution was added to red blood cell extraction tubes to aid haemolysis.

The tubes were tightly covered with metal foil, shaken for 40 min and centrifuged to break down any emulsion. Rapid cooling to -20°C followed, which enabled the hexane to be

decanted from the frozen aqueous portion.

Hexane extracts were examined within 24 hr by gas chromatography. When prolonged storage was required, samples were sealed into glass ampoules.

Urine and faeces Urine samples were extracted by a similar technique. Urine (100 ml) was extracted by a single partition with hexane (20 ml) which was then washed to remove interfering substances. Faecal samples were weighed, macerated under warm hexane, and washed in a similar manner. Interfering substances were removed from extracts of urine and faeces by successive washings with equal volumes of: water, sodium hydroxide (1 N), water, hydrochloric acid (1 N) and finally water, followed by drying with anhydrous sodium sulphate.

Tissues Tissue samples were weighed and ground with silversand using a glass- pestle and mortar. Extraction was by three partitions with hexane containing internal standard.

To reduce contamination of gas chromatographic columns, the fat content of samples was reduced by chilling (-20°C for 3hr) and a pyrex glass trap was incorporated in the injection port of the gas chromatograph.

Heptane extraction.

Hexane was not suitable for extraction of carbon tetrachloride and chloroform from samples. Gas chromatographic seperation of these compounds from the solvent was difficult with simple

columns and low boiling point metabolites were extracted with a high boiling point solvent (n-heptane).

Bile, plasma, blood and urine Samples of sheep bile, plasma, whole blood or urine and cockerel or duck plasma were extracted with n-heptane. The methods were similar to those described for hexane extraction, omitting an internal standard.

Tissues Sheep tissues and whole liver flukes were ground with acid washed silversand in a glass pestle and mortar or chopped with a homogeniser. The macerated tissue was extracted by a single heptane partition with shaking for 10 min followed by centrifugation (for 1 hr at -5°C and RCF 910) to break emulsions and freeze the aqueous layer.

Heptane extracts were then examined by gas chromatography.

Gas chromatography of samples.

Extracts of samples were analysed on an Aerograph Hi-Fi gas chromatograph (model 600 C) with a concentric tube electron capture (EC) detector head. Aliquots of extracts were injected with a 10µl Hamilton syringe.

Samples were diluted when necessary to obtain a linear response from the EC detector (utilising less than 30 per cent of the available standing current). Standard reference samples were analysed frequently during analysis of experimental material.

Columns.

Columns were made from stainless steel tube (3.2 mm o.d.) and oxygen-free nitrogen was used as carrier gas. Rate of flow of carrier gas (F/R) was measured by means of a soap bubble meter.

Column 1	2.0 m packed with firebrick 60/80 mesh		
	Coated with SE-30 (1.5% w/w);polyethylene glycol 20 M (2% w/w)		
	Column temp. 97°C	N_2 pressure 25psi	F/R 32 ml/min
Column 2	3.0 m	Chromosorb G 100/120	
	Di (2-ethylhexyl) sebacate (3% w/w)		
	Temp. 99°C	N_2 pressure 27.5psi	F/R 28 ml/min
Column 3	1.5 m	Celite 60/72	
	SE-30 (5% w/w)		
	Temp. 76°C	N_2 press. 25psi	F/R 80 ml/min
Column 4	6.0 m	Celite 60/72	
	SE-30 (5% w/w)		
	Temp. 102°C	N_2 press. 27psi	F/R 21 ml/min
Column 5	2.12 m	Celite 60/72	
	SE-30 (5% w/w)		
	Temp. 95°C	N_2 press. 25psi	F/R 17 ml/min

Column 6 1.5 m Firebrick 60/80

SE-30 (1.5% w/w) PEG 20 M (2% w/w)

Temp. 78°C N_2 press. 28psi F/R 60 ml/min

Column 7 1.25 m Chromosorb G 60/80

Dinonylphthalate (3% w/w)

Temp. 92°C N_2 press. 20psi F/R 32 ml/min

Samples were injected separately onto each column.

Samples from animals which received carbon tetrachloride.

Carbon tetrachloride and its metabolites were separated on columns 1,2,3 and 4. Carbon tetrachloride and chloroform in extracts were estimated on column 4 by comparison of peak heights or peak areas (Disc units) with heights or areas of peaks due to standard solutions of chloroform and carbon tetrachloride in heptane.

Apart from carbon tetrachloride and chloroform, extracted materials produced peaks at high detector sensitivity only. Three unknown peaks were encountered, all had longer retention times than carbon tetrachloride. In order of elution these were designated 'W', 'Z' and 'HCE'. The 'HCE' peak was identical under all conditions with that produced by a standard solution of hexachloroethane. The 'W' and 'Z' peaks were not identified.

In an attempt to identify 'W' and 'Z' several reagents were added to heptane extracts from rabbit tissues: ammonia

(Specific gravity 0.88); water; alcoholic silver nitrate; sodium hydroxide; nascent hydrogen; concentrated nitric acid. The extract was also evaporated on a water bath (50°C) to determine stability or relative volatility of 'W' and 'Z'.

HCE (hexachloroethane) was estimated on column 3 by calculation of peak area ratios to an internal standard (hexachlorobut-1,3-diene). Retention times of the following were determined in an attempt 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-dichloroethanol; 2,2,2-trichloroethanol; dichloroacetic acid; trichloroacetic acid; trichloromethylsulphenyl chloride; hydrogen sulphide; chloroacetone; trichloroacetone. Trichloromethylsulphonyl chloride; trichloromethyl mercaptan and hexachlorodimethylthioether were prepared by general methods (Sosnovsky,1961; Vogel,1961); they did not match 'W' and 'Z' but it was not certain that the reactions had proceded satisfactorily since yields were very poor.

Samples from animals which received hexachloroethane.

Hexachloroethane and its metabolites were separated on columns 5,6 and 7. Retention times of extracted materials were compared with standard solutions of hexachloroethane, pentachloroethane and tetrachloroethylene in hexane. Retention

times for 1,2-dichloroethane and 1,1,2,2-tetrachloroethane (reported metabolites of hexachloroethane) were also determined; there were no unidentified peaks in extracts and none corresponding to these compounds.

Apart from a peak due to hexachloroethane, two peaks designated 'X' and 'Y' were detected in hexane extracts of sheep and cockerel blood; sheep bile,urine and faeces and in tissue extracts from liver flukes. The 'X' and 'Y' peaks were identical under all conditions tested with peaks produced by standard solutions of tetrachloroethylene and pentachloroethane in hexane (compared on columns 5,6 and 7). Sheep which received pentachloroethane exhibited peaks 'X' and 'Y'; those receiving tetrachloroethylene peak 'X' only. 'X' and 'Y' were identified as tetrachloroethylene and pentachloroethane.

The peak area ratios of 'X' and 'Y' to internal standard were determined and the concentrations of tetrachloroethylene and pentachloroethane in biological extracts calculated.

Drug toxicity.

Hepatic function was studied by means of plasma enzyme estimations and bromsulphthalein dye clearance tests; renal function was studied by means of urine analysis and phenol-sulphonephthalein dye clearance.

Plasma enzyme activity.

Glutamate dehydrogenase (EC 1.4.1.3)(GD) activity was determined by measuring the rate of utilisation of $NADH_2$ by plasma in the presence of ammonium ion and oxoglutarate at pH 7.4 and 25°C. (Ford and Boyd,1962). Disappearance of $NADH_2$ was determined by spectrophotometer (Unicam SP 500) at 340 nm using a constant temperature cell housing which maintained the reaction cuvette at 25 ± 0.2°C. Plasma containing high enzyme activity was diluted with phosphate buffer (pH 7.4) until reactions proceded in a linear fashion for at least 3 min.

Sorbitol dehydrogenase (EC 1.1.1.14)(SD) activity was determined by a similar method (Ford,1967).

Ornithine carbamyl transferase (EC 2.1.3.3)(OCT) activity was determined by measurement of the breakdown of (±)-citrulline in the presence of arsenate (Moore,1967). In this method ammonia produced was displaced by a microdiffusion technique (Conway,1957) and determined by a modified Bertholet reaction. The intensity of the blue colour produced was measured at 640 nm using an EEL spectrophotometer.

Aspartate aminotransferase (EC 2.6.1.1)(GOT) activity and alanine aminotransferase (EC 2.6.1.2)(GPT) activity were determined colorimetrically (Reitman and Frankel,1957) using a shortened incubation period when samples of high activity were encountered (Wootton, 1964).

Fresh or recently unfrozen plasma samples were used for determinations.

Cockerels and ducks.

In order to determine the normal distribution of enzyme activity in avian tissues, fresh samples of liver,kidney, spleen, cardiac muscle, skeletal muscle, gizzard wall, section of small intestine, brain, lung and pancreas were taken from cockerels and ducks. Weighed samples were homogenised with distilled water by 25 strokes of an all-glass hand homogeniser. 10 per cent extracts were centrifuged (20 min at RCF 1600) to remove particulate matter; the supernatant was stored at -20°C.

Samples were usually diluted to 1 per cent extracts for determination of enzyme activity.

Bromsulphthalein dye test .

Bromsulphthalein dye (BSP) clearance tests were conducted on sheep,cockerels and ducks.

Sheep BSP sodium (50 mg per ml solution) was injected intravenously at a dose of 5 mg per kg. Six blood samples were collected at accurately timed intervals between 2 and

30 min after injection of BSP. Optical density (OD) of diluted aliquots of plasma (1 ml) was determined at 575 nm using an EEL spectrophotometer.

The control reading was obtained by dilution of plasma with hydrochloric acid (2 ml 0.05 N); the test reading by dilution of plasma with ammonium hydroxide (2 ml 0.5 N).The concentration of BSP in samples was calculated by reference to a standard curve relating OD (at 575 nm) to several concentrations of a standard solution of bromsulphthalein sodium. A water blank and a standard solution of BSP were read with each diluted plasma sample.

Cockerels and ducks BSP tests were conducted as above with the following differences: the concentration of BSP solution injected was 10 mg per ml; 0.5 ml aliquots of plasma were diluted with acid or alkali (2.5 ml).

In sheep, chickens and ducks BSP (5 to 10 mg per kg) disappears from plasma in a biphasic manner. This allows determination of two transfer rates for BSP (Richards,Tindall and Young, 1959). Resolution of the plasma disappearance curve into two components was by a graphical 'trial-and-error' method (Clarkson and Richards,1967).

Sheep urine investigation.

Urine was collected from castrated male sheep (Warwick, 1969) for determination of volume, pH, SG, chloride ion concentration and chlorinated hydrocarbon content. The urine receiver was cooled by an ice-water bath.

Specific gravity was determined by hydrometer at laboratory temperature; chloride ion concentration was determined by an EEL Chloridemeter, pH by a Marconi TF 1093 pH meter.

PSP* clearance (Rowntree and Geraughty, 1910) was determined in sheep 35 and 36 24 hr, 48 hr and 11 days after carbon tetrachloride administration and compared with normal clearance rates. PSP solution (4 mg per kg) was injected into the jugular vein at zero time and urine was collected for 1½ hr in 15 min aliquots. Each sheep received a 4 litre water load by stomach tube 1 hr before injection of PSP. Aliquots of urine were diluted with sodium hydroxide (0.5 N) and OD was determined at 550 nm on an EEL spectrophotometer. The proportion of PSP excreted in each 15 min aliquot was determined.

* PSP = phenolsulphonephthalein

Action of drugs against liver flukes in vitro.

Washed, viable liver flukes were incubated, one per tube, with emulsions of drugs or various media. Inactivated flukes were obtained by heating viable flukes for 5 min at 100°C in 0.15 M sodium chloride solution.

The various incubation media and drug emulsions were made up with Hedon Fleig solution as the aqueous phase; freshly ~~unfrozen~~ thawed sheep bile was used in some experiments (Table 25; p. 88).

Flukes were examined frequently during incubation and especially 3 hr, 5 hr, 10 hr, 21 hr and 26 hr after the start of incubation for signs of movement. Non-motile flukes were stimulated with a 15 volt DC current at 60 pulses per sec for 3 sec to investigate their ability to respond.

Hedon Fleig solution (Gatenby, 1937) was prepared freshly for each series of experiments and contained glucose (0.005 M), procaine penicillin (5×10^5 units per litre) and streptomycin sulphate (0.6 g per litre).

Emulsions of drugs were prepared with acacia B.P. as emulsifying agent. A primary emulsion was prepared (oil 4 parts: acacia 2 parts: water 1 part) and diluted with Hedon Fleig solution, with shaking.

Oleic acid (methyl ester) - carbon tetrachloride addition

product ('trichloromethylated oleate') was prepared (Gordis,1969) and purged with nitrogen for 18 hr at 100°C to remove volatile contaminants.

Rabbit liver extracts were prepared as follows: of four New Zealand White rabbits (1.5 to 1.8 kg), two received carbon tetrachloride (2 ml per kg) by stomach tube. Two hours later, control and treated rabbits were stunned, bled, the livers removed and ground with acid-washed silversand in glass pestles-and-mortars under nitrogen. The macerated livers were extracted with three partitions of ether (total 300 ml), the extracts combined and the ether evaporated off at room temperature under a reduced pressure of nitrogen. The aqueous mass was centrifuged to remove tissue debris (30 min at RCF_{A}^{1600}) and the supernatant was stored frozen at -20°C. 25 ml of 'aqueous liver extract' was equivalent to about 12.5 g ether extracted liver tissue. Ether extracted material was also stored at -20°C but under nitrogen to protect unsaturated fatty acids from oxidation.

After incubation, liver flukes were blotted dry and extracted, for analysis by gas chromatography.

Reagents.

Analytical grade reagents were used where available. Carbon tetrachloride was B.P.C. (1959) quality and was distilled twice in glass apparatus, collected at 76°C (uncorrected) and washed with concentrated sulphuric acid (specific gravity 1.84). Analysis by gas chromatography confirmed that residual contamination was never greater than 33 ng chloroform per ml (determined on column 4) or 30 ng hexachloroethane per ml (column 3) if detected at all.

Technical grade hexachloroethane was purified by sublimation; pentachloroethane and tetrachloroethylene by two fractional distillations in all-glass apparatus. Fractions distilling at 158°C (uncorr.)(pentachloroethane) and 121°C (uncorr.)(tetrachloroethylene) were collected.

After purification, hexachloroethane and tetrachloroethylene each produced a single peak on the gas chromatograph; pentachloroethane was still contaminated with tetrachloroethylene at a concentration of 7.6 p.p.m.

Technical grade bistrichloromethylbenzene ('Hetol'), 1,2- and 1,1- tetrachlorodifluoroethanes were used without further purification. Olive oil was B.P. grade; direct injection gave no peaks on the gas chromatograph.

Commercially available solvents were not sufficiently pure for use with the electron capture detector of the gas chromatograph. When purchased, n-heptane was contaminated with

4 to 100 x 10^{-9} parts carbon tetrachloride and 0 to 2 x 10^{-9} parts hexachloroethane. Samples containing not more than 10 x 10^{-9} parts CCl_4 and 0 x 10^{-9} parts C_2Cl_6 were washed with concentrated sulphuric acid (s.g. 1.84) (10 per cent v/v); distilled water (50% v/v); distilled water (50% v/v); sodium bicarbonate solution (1.1 M; 50% v/v);distilled water (50% v/v); distilled water (50% v/v) and finally dried with anhydrous sodium sulphate (25% w/v). The washed solvent was slowly distilled at a rate of one drop per five seconds (approx.) until reduced to about 25 per cent of the original volume. The undistilled fraction was used for extraction of samples.

Hexane fraction of petroleum (b.p. 67 to 70°C) was purified in 200 ml aliquots by washing as described above.

Results.

Management of experimental animals

Blackface sheep barely maintained condition or lost up to 1 kg per month on the hay and water diet. Bile duct and rumen fistulated sheep also gradually lost condition and post-mortem examination revealed that degenerative and resorptive changes had occurred in the bone marrow. This indicated that digestion or absorption had been impaired although enterohepatic circulation had been maintained.

Cockerels and ducks thrived when housed.

Sampling techniques

Evacuated glass containers ('Vacutainers') containing sodium heparin were suitable for collection of liquid blood samples from sheep, but collapsed the veins of cockerels and ducks. Plastic syringes with the dead space filled by sodium heparin solution were convenient for use in these species although haematoma formation often occurred even with the small (26 G) needles used.

Extraction and analysis of samples

The simple extraction techniques used minimised losses of volatile materials and the high specificity of the electron capture detector made extensive clean-up of samples unnecessary. A pyrex glass injector-port lining (supplied by Varian Aerograph for pesticide analysis) was suitable for removal of

extracted, non-volatile contaminants.

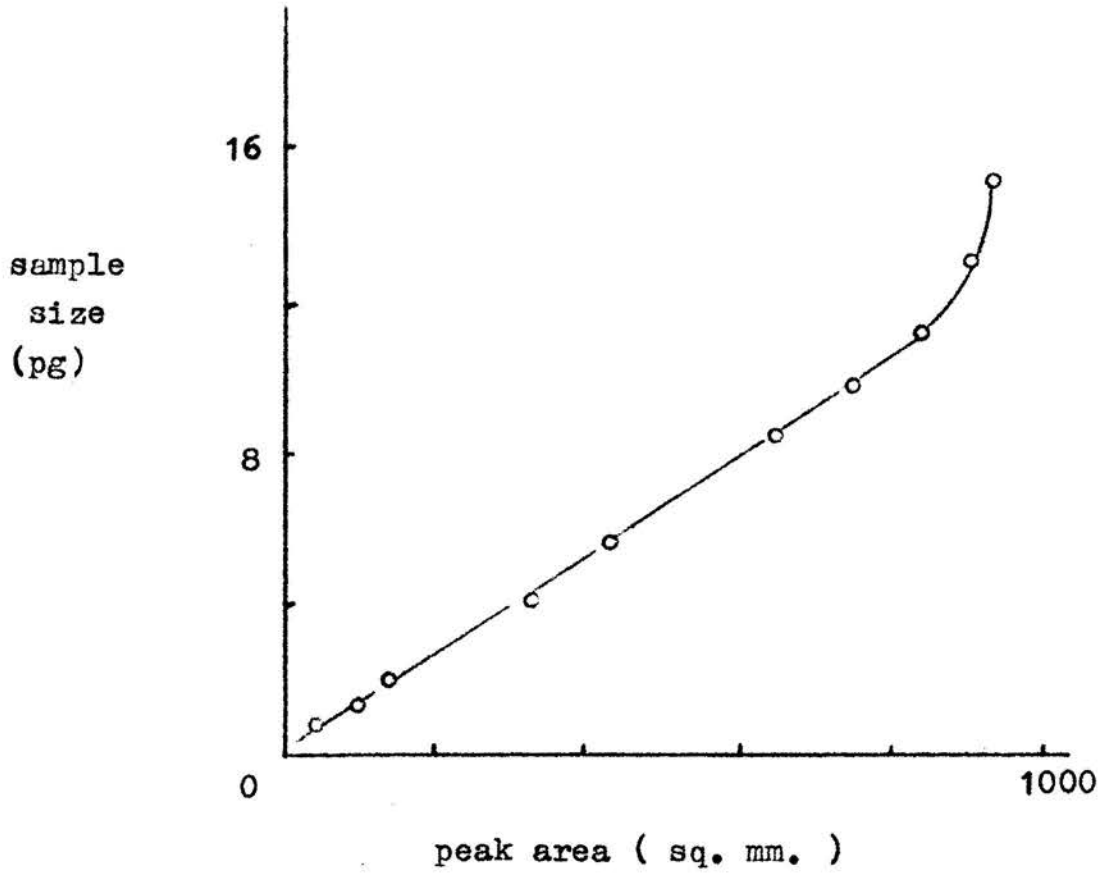
Gas chromatography provided a sensitive and quantitative method for determination of carbon tetrachloride, chloroform, hexachloroethane, pentachloroethane , tetrachloroethylene and other chlorinated, aliphatic hydrocarbons. The low concentrations and high volatility of these compounds did not allow study by conventional thin-layer or paper chromatographic techniques. Gas chromatography was quantitative within a limited range of drug concentrations; the response of the electron capture detector was linear within the following ranges:

carbon tetrachloride	0.1 to 10 picograms (pg)	(Figure 1)
chloroform	20 to 300 pg	(Fig 2)
hexachloroethane	0.5 to 200 pg	(Fig 3)
pentachloroethane	20 to 60 pg	(Fig 4)
tetrachloroethylene	200 to 1600pg	(Fig 5)

on columns 4 and 5.

Retention times of compounds on these columns could be related to boiling point (Fig 6); the resulting curve was used to aid identification of unknown compounds in extracts. Peaks 'X' and 'Y' from sheep which received hexachloroethane corresponded to pentachloroethane and tetrachloroethylene when fitted to Fig 6 by their retention times; this provided evidence in support of the identity of 'X' and 'Y'.

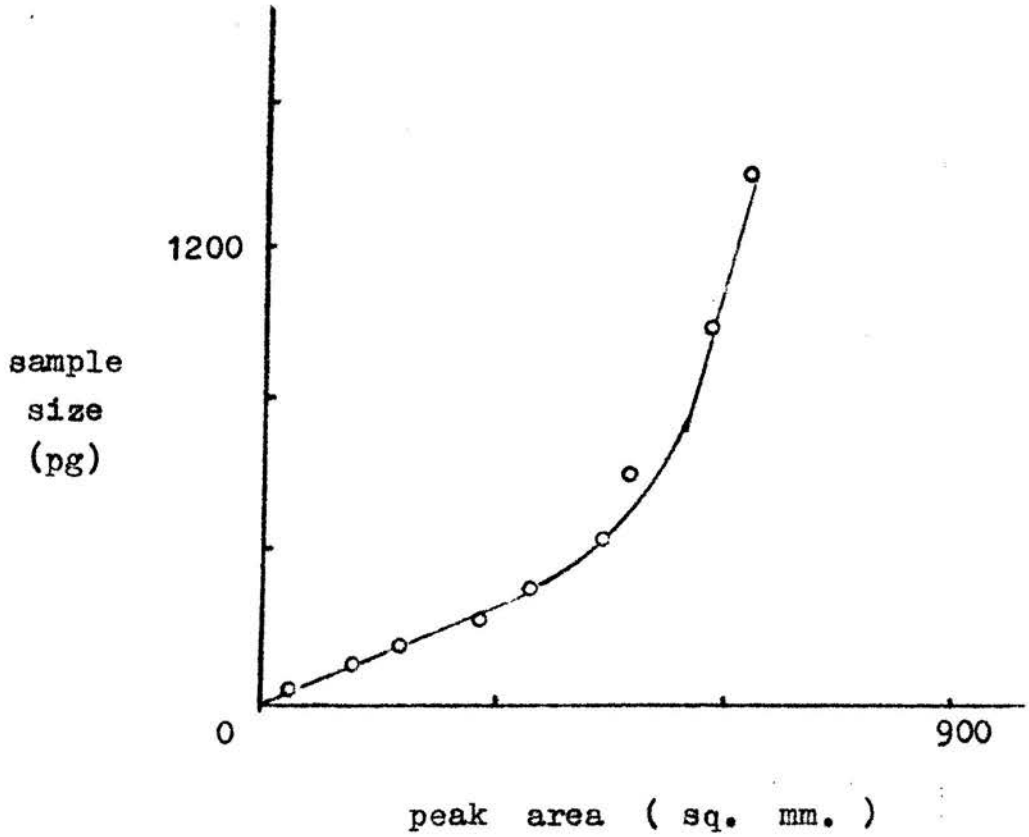
Figure 1.



Linearity of Carbon Tetrachloride

column 5% SE-30 on Celite 60/72

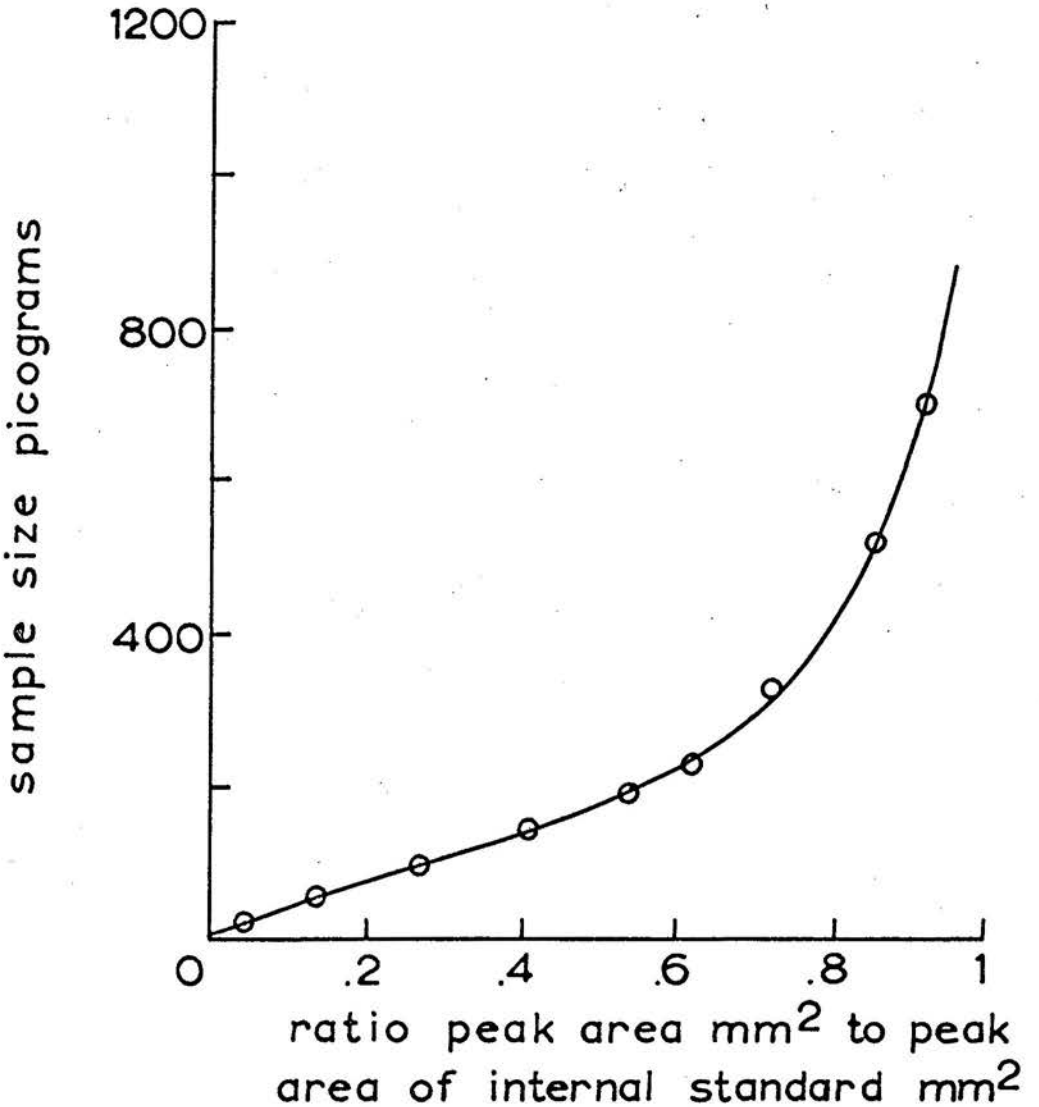
Figure 2.



Linearity of Chloroform

column 5% SE-30 on Celite 60/72

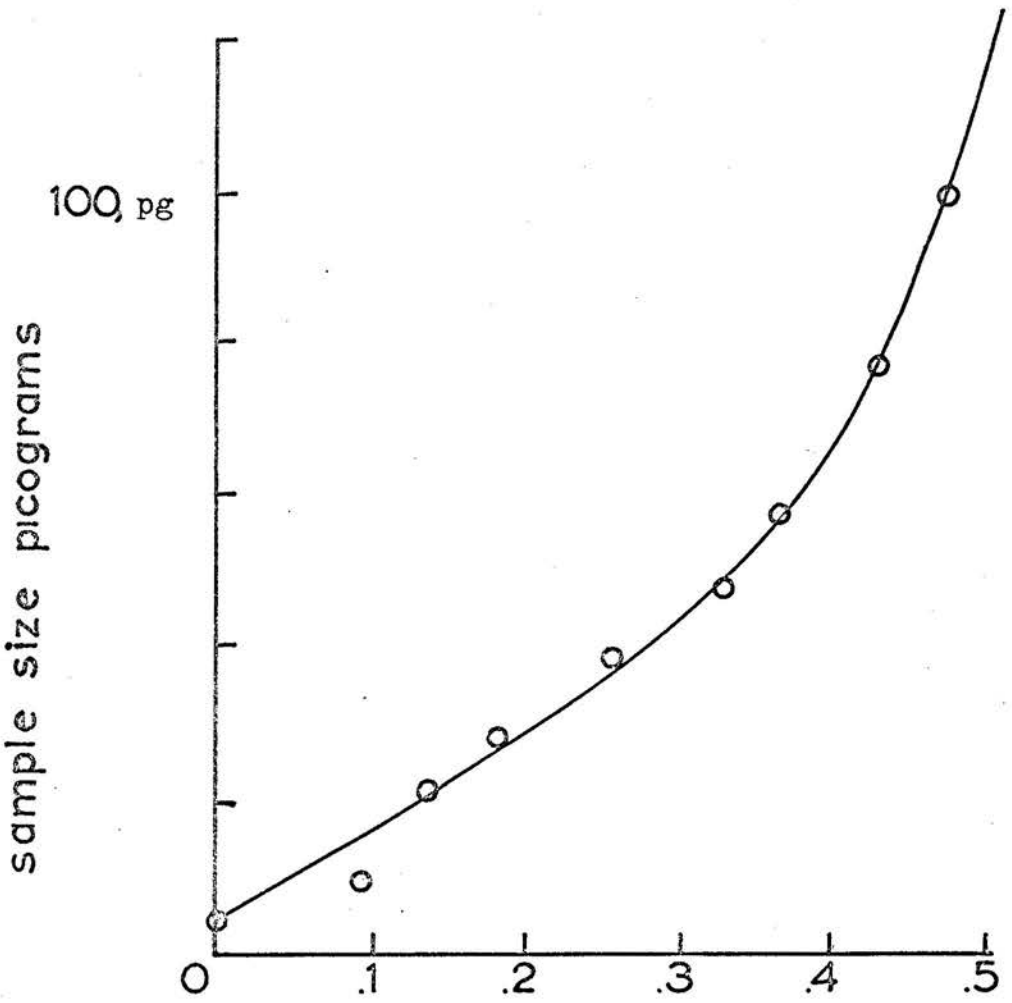
Figure 3.



LINEARITY OF HEXACHLOROETHANE

column 5% SE-30 on Celite 60/72
temp 95° N_2 flow rate 17mls/min

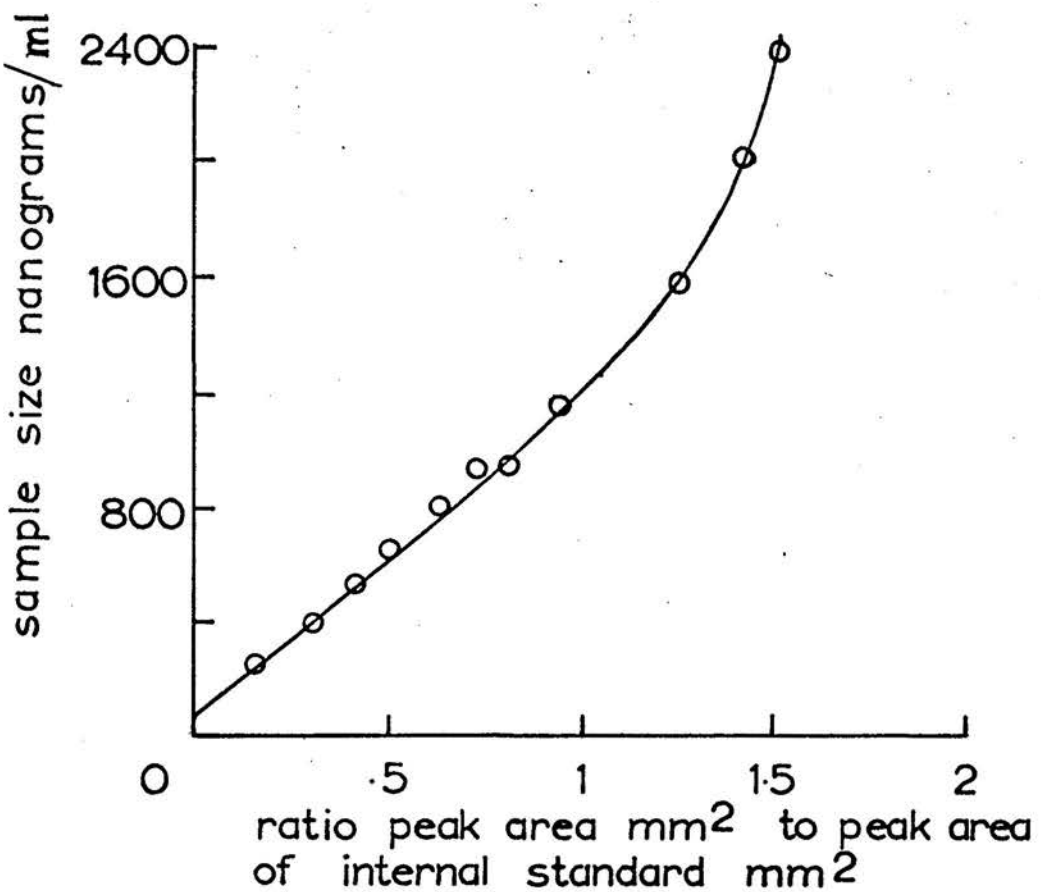
Figure 4.



LINEARITY OF PENTACHLOROETHANE

column 5% SE-30 on Celite 60/72
temp 95° N_2 flow rate 17mls/min

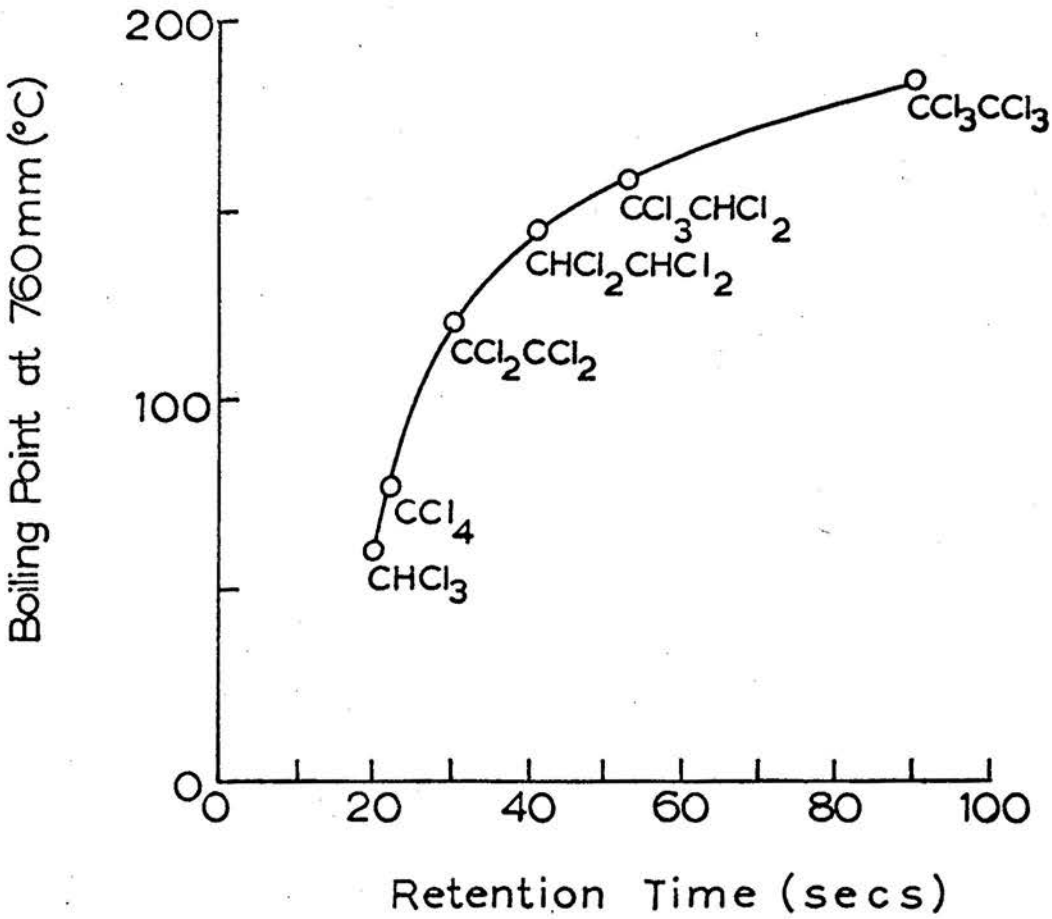
Figure 5.



LINEARITY OF TETRACHLOROETHYLENE

column 5% SE-30 on Celite 60/72
temp 95° N flow rate 17mls/min

Figure 6.



Column: 5% SE-30 on Celite 60/72
Temp. 95° N_2 Flow Rate 17ml/min

Simple chlorinated hydrocarbons: elution from a non-polar column according to b.p.

Metabolism of carbon tetrachloride.

Concentrations of carbon tetrachloride in blood of sheep which received the drug are presented in Table 1. Highest concentrations were encountered 6 hr after dosage and declined to less than 1/10th peak values within 3 days. In cockerels and ducks highest concentrations occurred at the 24 hr sample and were always less than those encountered in sheep blood although they declined relatively slowly (Table 2).

Carbon tetrachloride was excreted in the bile of sheep and the highest concentrations were encountered 1 to 2 hr after administration of the drug (Table 3). Carbon tetrachloride was also found in gall-bladder bile from rabbits which received the drug; concentrations were highest in rabbits killed 6 hr after administration of it (Table 4).

Traces of carbon tetrachloride were also excreted in urine of sheep but concentrations were very much lower than those observed in sheep bile (Table 5).

The distribution of carbon tetrachloride in liver, kidney, fat and muscle of rabbits was consistent with the lipophilic nature of the drug; highest concentrations were encountered in fat and lowest in muscle (Table 6).

Carbon tetrachloride was absorbed by liver flukes *in vitro* from aqueous emulsions of the drug which contained dispersed phase droplets 1.4 to 5.0 µm in diameter (Table 7).

Metabolism of carbon tetrachloride occurred in sheep, rabbits and liver flukes, but not in cockerels or ducks. In addition to detection of unchanged carbon tetrachloride, chloroform was identified in sheep blood (Table 1), bile and urine (Table 5) rabbit and liver fluke tissue (Tables 6 and 7) and rabbit gall-bladder bile (Table 4). A second metabolite, hexachloroethane, was detected in liver flukes and in extracts of tissues and gall-bladder bile from rabbits which had received carbon tetrachloride (Tables 7,6 and 4).

Other peaks detected in extracts of tissues from rabbits which received the drug were designated 'W' and 'Z'. In contrast to carbon tetrachloride, chloroform and hexachloroethane 'Z' was found mainly in muscle and liver extracts (Tables 8 and 9); treatment of extracts containing 'Z' with concentrated ammonia solution appeared to augment peak 'W' (Tables 8, 9 and 10) and concentrated sodium hydroxide solution removed 'Z' but did not augment 'W' . Concentration of extracts increased the size of peaks 'HCE', 'W' and 'Z' but decreased peaks due to carbon tetrachloride and chloroform; thus the volatility of 'W' and 'Z' was probably less than that of n-heptane.

Table 1. Blood concentrations of carbon tetrachloride and its metabolite chloroform in sheep (5) which received carbon tetrachloride at day 0 (µg/ml).

Day	CCl_4 $\bar{x} \pm$ s.d.		$CHCl_3$ $\bar{x} \pm$ s.d.	
0	0	0	0	0
¼	2.70	0.32	0.63	0.30
1	2.35	0.20	0.75	0.35
2	0.62	0.12	0.21	0.04
3	0.12	0.03	0.08	0.02
6	0.01	0.00	0.05	0.01

Table 2. Plasma concentrations of carbon tetrachloride (µg per ml) in ducks and cockerels which received the drug (6ml) at 0 hr (all ± s.d.).

Time	Cockerels (5)	Ducks (5)
0 hr	0.000 ± 0.000	0.000 ± 0.000
6 hr	0.590 ± 0.203	-
24 hr	1.706 ± 0.695	1.302 ± 0.292
42 hr	-	0.888 ± 0.076
48 hr	1.651 ± 0.858	-
3 day	-	0.672 ± 0.135
5 day	-	0.206 ± 0.344
6 day	0.158 ± 0.472	-
9 day	0.034 ± 0.025	0.009 ± 0.018

Table 3. Biliary concentrations of carbon tetrachloride (μg per ml) and total carbon tetrachloride passed in sheep bile (μg).

	Sheep 40				Sheep 60			
	Expt.1		Expt.2		Expt.1		Expt.2	
	Conc.	Total	Conc.	Total	Conc.	Total	Conc.	Total
Predosing	nil	nil	nil	nil	nil	nil	nil	nil
0 to 1 hr	0.52	64	0.68	78	1.12	81	1.06	81
1 to 2 hr	3.28	185	3.30	241	3.64	242	3.62	232
2 to 3 hr	3.04	73	3.10	99	1.89	53	1.82	55
3 to 4 hr	1.64	23	1.60	32	1.21	34	1.10	29
4 to 5 hr	0.78	21	0.76	23	0.71	17	0.80	21
5 to 6 hr	0.68	21	0.45	14	0.45	9	0.30	8
6 to 24hr	0.06	46	-	-	0.15	107	-	-
1 to 2day	0.006	7	-	-	0.01	9	-	-
2 to 3day	0.006	6	-	-	0.01	8	-	-
3 to 4day	0.005	5	-	-	0.004	2	-	-
4 to 5day	0.005	5	-	-	0.002	1	-	-

Table 4. Volatile chlorinated constituents of gall-bladder bile from rabbits which received carbon tetrachloride (1 ml per kg).

Time after dose	CCl_4 µg/g ± s.d.	$CHCl_3$ µg/g ± s.d.	$Cl_3C.CCl_3$ ng/g ± s.d.	n =
6 hr	37 ± 7	0.50 ± 0.12	trace	5
24 hr	7.8 ± 1.5	0.14 ± 0.02	5.5 ± 1.8	5
48 hr	1.1 ± 1.3	0.45 ± 0.21	trace	5

Table 5. Carbon tetrachloride and chloroform (µg) in sheep bile and urine after administration of carbon tetrachloride (3 ml).

	Sheep 37 Urine		Sheep 38 Urine		Sheep 40 Bile		Sheep 60 Bile	
	CCl_4	$CHCl_3$	CCl_4	$CHCl_3$	CCl_4	$CHCl_3$	CCl_4	$CHCl_3$
0 to 1day	19.2	3.7	1.2	6.6	433	241	543	210
1 to 2day	5.9	2.0	1.0	3.3	7	122	9	126
2 to 3day	4.6	1.8	0.7	2.2	6	95	8	120
3 to 4day	1.3	0.8	0.7	2.0	5	50	2	20
4 to 5day	0.6	0.2	0.5	0.2	5	nil	1	nil
5 to 6day	(+)	(+)	(+)	(+)	(+)	nil	nil	nil
6 to 7day	(+)	(+)	(+)	(+)	(+)	-	nil	nil

Table 6. Volatile chlorinated compounds identified in rabbit tissues following administration of carbon tetrachloride (1 ml per kg).

Hours after	Tissue	No.	CCl_4 µg/g ± s.d.	$CHCl_3$ µg/g ± s.d.	$Cl_3C.CCl_3$ ng/g ± s.d.
6	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	fat	5	96 ± 11	1.0 ± 0.2	16.5 ± 1.6
	liver	5	7.7 ± 1.3	1.4 ± 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
48	fat	5	45 ± 12	0.4 ± 0.1	6.8 ± 2.4
	liver	5	3.8 ± 0.1	0.8 ± 0.2	1.0 ± 0.3
	kidney	5	0.5 ± 0.3	0.2 ± 0.0	trace
	muscle	5	0.5 ± 0.3	0.1 ± 0.1	trace
44 (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

Table 7. Concentration of carbon tetrachloride, chloroform and hexachloroethane (µg per g ± s.d.) in liver fluke tissues after incubation in carbon tetrachloride emulsions.

CCl_4 in medium (µg/ml)	Fluke wt. (mg±s.d.)	No.	Tissue extracts		
			CCl_4	$CHCl_3$	$Cl_3C.CCl_3$
50 ± 5	102 ± 12	17	76 ± 8	0.38 ± 0.04	0.19 ±0.01
93 ± 14	161 ± 22	10	141 ± 5	0.57 ± 0.06	0.22 ±0.02
126 ± 19	160 ± 15	18	223 ± 71	1.91 ± 0.21	0.38 ±0.06

Table 8. Distribution of the unknown metabolites of carbon tetrachloride 'W', 'Z' and 'HCE' in rabbit tissues (gas chromatographic column 3).

Sample	Gas chromatographic peaks		
	'W'	'Z'	'HCE'
6 hr liver	-	++	+
6 hr muscle	-	++	(+)
48hr fat	-	-	++++
48hr liver	(-)	+++	+++
48hr kidney	-	++	++
48hr bile	-	-	+
48hr muscle	(+)	+++	(+)
48hr muscle + ammonia	+++	++	(+)

Table 9. As Table 8, but on column 4.

- absent
+ present

Sample	Gas chromatographic peaks		
	'W'	'Z'	'HCE'
6 hr muscle	(+)	++	+
6 hr kidney	-	+++	(+)
48hr fat	(+)	+	++
48hr liver	+	+++	+
48hr kidney	+	+	+
48hr bile	(+)	-	(+)
48hr muscle	+	+++	(+)
Rpt + ammonia	+++	++	(+)

Table 10. Treatment of heptane extracts of rabbit tissues with simple reagents to aid identification of the metabolites of carbon tetrachloride 'W','Z' and 'HCE' (gas chromatographic column 4).

Reagent	Change in gas chromatographic peak 'W'	'Z'	'HCE'
ammonia (SG 0.88)	+++	--	+
water	nc	nc	nc
concentrated NaOH		--	nc
ethanolic $AgNO_3$	nc	nc	nc
nascent hydrogen	nc	nc	nc
evaporation at 50°		++	++
concentrated HNO_3		---	nc

nc = no change
+ ++ +++ = peak augmented
- -- --- = peak diminished

Metabolism of hexachloroethane.

In unanaesthetised sheep, blood concentrations of hexachloroethane were high 24 hr after administration (Table 11; Figures 7,8 and 9) and were approximately 10 times greater than those in anaesthetised sheep (Table 12) or in plasma of cockerels (Table 13). The highest concentrations of the drug in cockerel plasma occurred 42 hr after administration of the drug.

Urine and faeces of sheep contained hexachloroethane; more than 80 per cent of the total faecal hexachloroethane (1 to 2 mg) was excreted in the 24 hr following administration of the drug (Table 14).

Hexachloroethane was also detected in the bile of anaesthetised sheep within 15 min of administration and at least 12 min before it was detected in jugular venous blood (sheep 28). The highest concentrations of hexachloroethane in bile were 8 to 10 times greater than maximum blood concentrations and the drug was widely distributed in sheep tissues; fat contained high and muscle low concentrations of the drug (Table 12). Muscle and fat from different sites did not show significant variation in drug content.

Liver flukes in vitro absorbed hexachloroethane from aqueous emulsions (Table 15).

Hexachloroethane was metabolised by sheep, cockerels and liver flukes and the metabolites were shown to be

tetrachloroethylene and pentachloroethane. Pentachloroethane was detected in venous blood of sheep and plasma of cockerels 24 hr and 72 hr after hexachloroethane administration (Tables 11 and 13; Figures 7,8 and 9). Relatively high concentrations of pentachloroethane were detected in sheep urine (Table 14); however, in anaesthetised sheep, blood, bile and tissue concentrations were very low (Table 12). When administered to sheep, pentachloroethane was rapidly absorbed and metabolised to tetrachloroethylene (Figure 10); tetrachloroethylene was the only metabolite of pentachloroethane detected and was also a metabolite of hexachloroethane.

Tetrachloroethylene was quantitatively a most important metabolite of hexachloroethane in sheep and cockerels. A high ratio of tetrachloroethylene to pentachloroethane was observed 24 to 48 hr after administration of the drug (Table 13;Figures 7, 8 and 9). In liver flukes, tetrachloroethylene was quantitatively a less important metabolite (Table 15).

After administration of hexachloroethane, concentrations of tetrachloroethylene were relatively high in sheep urine and faeces and the metabolite could be detected when hexachloroethane was absent (Table 14). Since the concentrations of tetrachloroethylene were also high in anaesthetised sheep which received hexachloroethane it is possible that although the rate of absorption of hexachloroethane was reduced during anaesthesia, metabolism was less affected. Tetrachloroethylene

was widely distributed in the tissues: brain and muscle contained less tetrachloroethylene than other tissues, but liver (sheep 28) and fat (sheep 27)contained high concentrations of the metabolite (Table 12).

Blood from sheep which had received tetrachloroethylene showed only one peak on gas chromatographic analysis, that due to tetrachloroethylene.

Liver flukes which had been inactivated by boiling before incubation in vitro did not metabolise hexachloroethane (Table 15).

Other metabolites of hexachloroethane are presumed to be water soluble and none were detected in hexane extracts.There were no unidentified peaks and none corresponding to 1,2-dichloroethane or 1,1,2,2-tetrachloroethane.(The most toxic of the chlorinated ethanes; the latter reported to be a metabolite of hexachloroethane in rabbits (Jondorf,Parke and Williams,1957;Williams,1959)).

Blood and plasma samples from sheep and cockerels which received olive oil and extracts of liver flukes incubated in olive oil emulsions produced no response when analysed by gas chromatography.

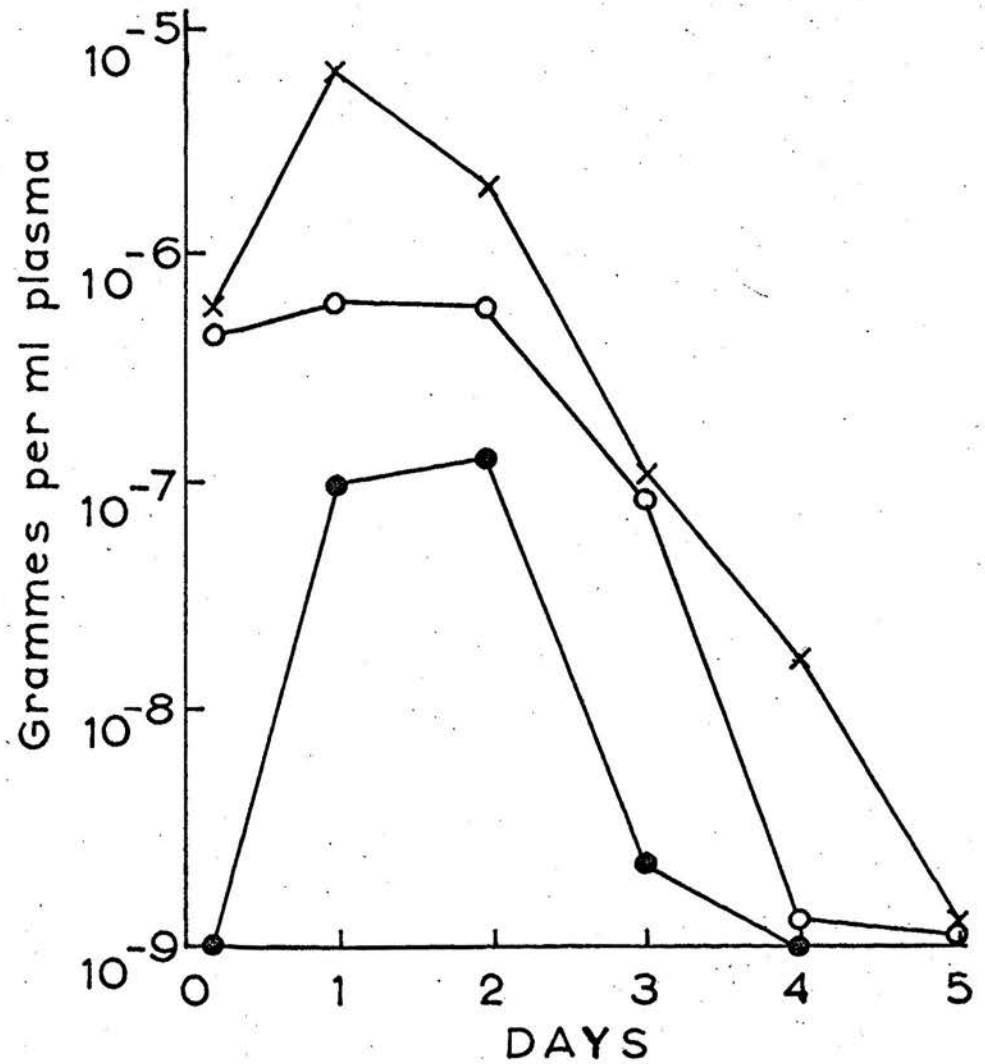
Rabbits,guinea-pigs and a rat were able to metabolise hexachloroethane (Table 16) to pentachloroethane and tetrachloroethylene. Fresh liver slices liberated the same metabolites in vitro whereas boiled sheep liver slices did not. Heated liver slices produced a different ratio of metabolites and suggested that at least two enzymes were concerned in the degradation of hexachloroethane and that these are present in the liver (Table 17).

Table 11. Blood concentrations of hexachloroethane (HCE), pentachloroethane (PCE) and tetrachloroethylene (TCE) as µg per ml. whole blood and change in the excretory capacity of the liver following administration of hexachloroethane.

Sheep No.	HCE dose g/kg	24 hr blood concentration HCE	TCE	PCE	72hr BSP transfer rate *
1	0.5	27	0.6	0.15	25
2	0.5	-	-	-	12
3	0.5	28	0.7	0.30	66
4	0.5	10	0.8	0.06	47
5	0.5	27	1.0	0.50	79
6	0.5	23	1.1	0.15	35
7	0.75	-	-	-	8
8	1.0	-	-	-	36
9	control	-	-	-	94
10	control	nil	nil	nil	105

* as percentage of the 0 hr transfer rate

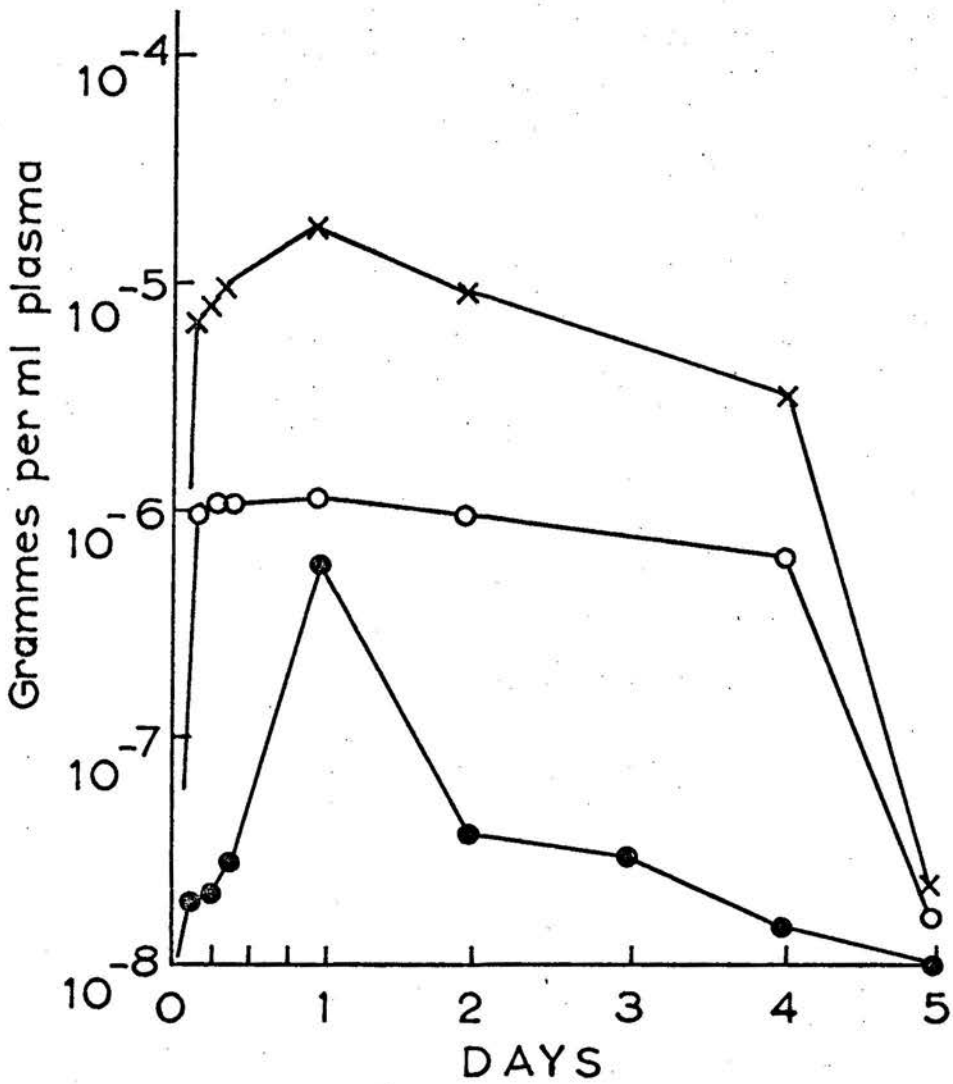
Figure 7.



SHEEP EXPT 1

Plasma concentrations of hexachloroethane (x----x), pentachloroethane (•-----•) and tetrachloroethylene (o----o) in a sheep which received hexachloroethane (0.5 G per Kg) at day 0.

Figure 8.



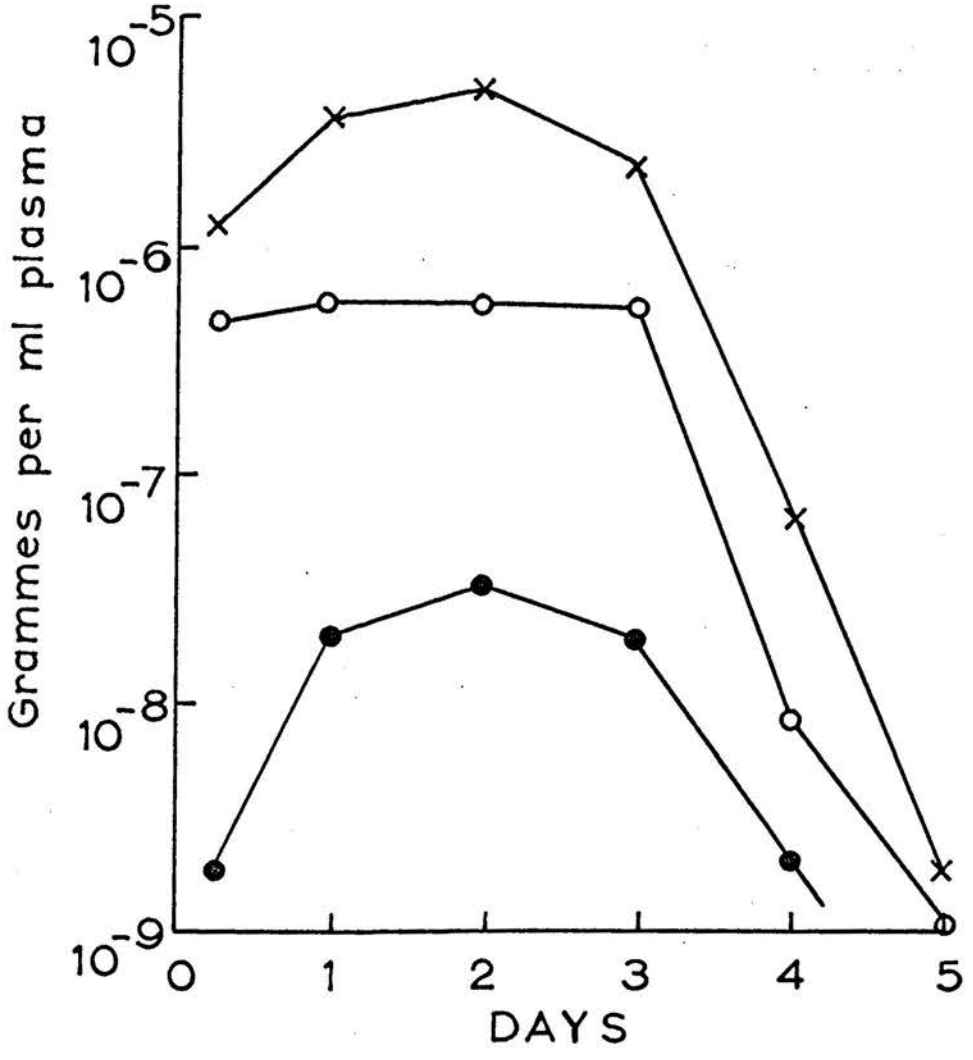
SHEEP EXPT 3

x----x = hexachloroethane

•----• = pentachloroethane

o----o = tetrachloroethylene

Figure 9.



SHEEP EXPT 4

x----x = hexachloroethane

●----● = pentachloroethane

o----o = tetrachloroethylene

Table 12. Concentrations (μg/G) of hexachloroethane (HCE), pentachloroethane (PCE) and tetrachloroethylene (TCE) in two anaesthetised sheep which received hexachloroethane. Post-mortem tissue samples (8.5 hr).

Tissue	Sheep 27			Sheep 28		
	HCE	TCE	PCE	HCE	TCE	PCE
Bile (4hr)	1.7	0.3	trace	2.2	0.5	nil
Blood (6hr)	0.2	0.4	trace	0.2	0.2	nil
Brain	0.2	0.9	0.02	trace	trace	trace
Fat	1.1	2.1	0.02	trace	0.6	nil
Kidney	0.1	1.2	trace	trace	0.6	trace
Liver	0.2	0.9	0.01	trace	2.8	trace
Muscle	0.04	0.5	0.01	trace	trace	trace

Table 13. Plasma concentrations (µg per ml) of hexachloroethane (HCE), tetrachloroethylene (TCE) and pentachloroethane (PCE: ng per ml) in cockerels 10 and 11 which received hexachloroethane (4g.) at 0 hr.

Time	HCE		TCE		PCE	
	10	11	10	11	10	11
0 hr	nil	nil	nil	nil	nil	nil
24 hr	1.432	1.380	1.40	2.32	10.1	8.2
42 hr	1.807	1.847	1.70	2.70	14.6	11.5
72 hr	1.657	0.735	0.63	0.85	15.2	7.0
5 day	0.064	0.027	0.07	0.04	trace	trace
9 day	0.000	0.000	0.00	0.00	0.0	0.0

Table 14. Total (µg) hexachloroethane (HCE), pentachloroethane (PCE) and tetrachloroethylene (TCE) in the urine and faeces of two sheep that received hexachloroethane.

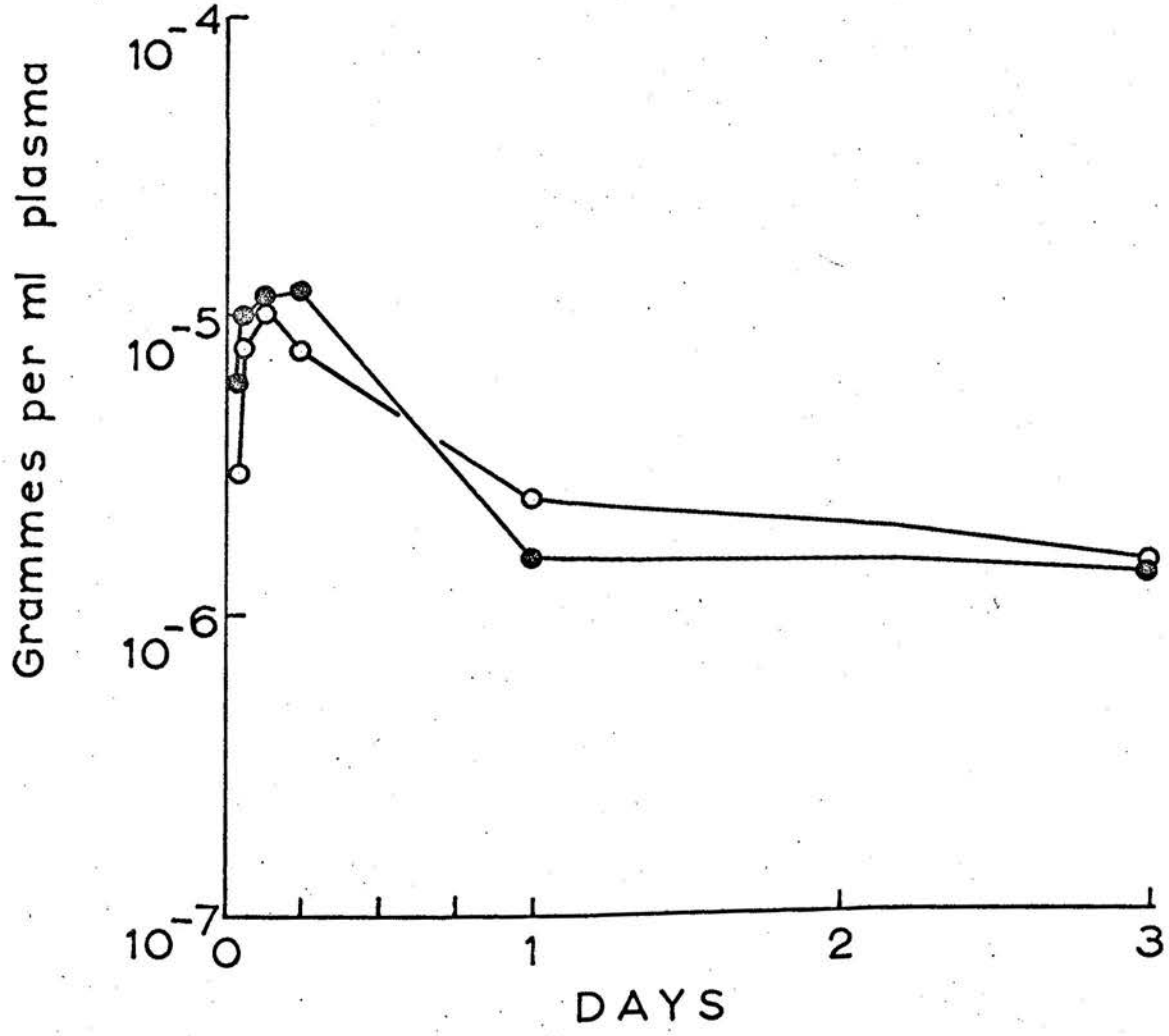
	Sheep 11			Sheep 12		
Faeces	HCE	TCE	PCE	HCE	TCE	PCE
0 - 24hr	780	854	trace	1260	1300	468
24- 48hr	trace	22	trace	280	440	trace
48- 72hr	trace	trace	trace	trace	15	trace
72- 96hr	nil	trace	nil	trace	trace	trace
	Sheep 11			Sheep 12		
Urine	HCE	TCE	PCE	HCE	TCE	PCE
0 - 24hr	50	25	20	70	29	25
24- 48hr	4.4	8.8	1.3	11	8.9	1.0
48- 72hr	0.4	6.5	0.4	trace	5.4	0.5
72- 96hr	nil	nil	nil	trace	5.4	trace

Table 15. Concentrations of hexachloroethane (HCE), pentachloroethane (PCE) and tetrachloroethylene (TCE) (µg/g ± s.d.) in liver fluke tissues after incubation in hexachloroethane emulsions.

HCE in medium (µg/ml)	Fluke wt. (mg±sd)	No.	Tissue extracts HCE	PCE	TCE
14.2 ±0.3	290 ±58	19	8.51 ±0.40	0.19 ±0.06	0.55 ±0.14
16.0 ±2.0	263 ±82	10*	13.8 ±0.90	nil	nil
21.8 ±0.3	257 ±57	16	17.5 ±2.45	0.16 ±0.04	0.64 ±0.17

* flukes inactivated by boiling

Figure 10.



SHEEP EXPT 19

●----● = pentachloroethane

o----o = tetrachloroethylene

Table 16. Distribution of hexachloroethane (HCE) and its metabolites pentachloroethane (PCE) and tetrachloroethylene (TCE) in various species under urethane anaesthesia (rectal temperature 37 ± 0.5°C.).

Species	HCE mg/kg	HCE	PCE	TCE
Rat	500	bi,bl	bl	bi,bl
Guinea-pig	control	nil	nil	nil
Guinea-pig	515	bi,bl	bl	bi,bl
Guinea-pig	424	bi,bl	bl	bi,bl
Rabbit	control	nil	nil	nil
Rabbit	control	nil	nil	nil
Rabbit	566	bi,bl,f,u	bl	bi,bl,f
Rabbit	300	bi,bl,f,u	bl	bi,bl,f
Rabbit	255	bl,f	-	bl
Rabbit	420	bi, f	-	-
Rabbit	413	bi, f	-	bi
Rabbit	417	bi,bl,f,u	bl	bi,bl

(bi = bile; bl = blood; f = fat; u = urine)

Table 17. Liberation of tetrachloroethylene (TCE) and pentachloroethane (PCE) from hexachloroethane (HCE) by sheep liver as fresh, heated (70°C for 5 min) or boiled (100°C for 5 min) slices incubated at 37°C for 4 hr. (µg/g ± s.d.).

(1) Olive oil emulsion diluted with Hedon-Fleig

Tissue	No.of expts	HCE	TCE	PCE
Fresh	10	nil	nil	nil
Boiled	10	nil	nil	nil
(2) Emulsion + hexachloroethane 18µg per ml				
Fresh	10	13.3 ± 0.5	9.1 ± 0.1	0.76 ± 0.01
Heated	10	50.8 ± 0.1	2.4 ± 0.8	1.74 ± 0.66
Boiled	10	58.4 ± 0.9	nil	nil
None	4	15.9 ± 0.8	nil	nil
(3) Emulsion + hexachloroethane 54µg per ml				
Fresh	10	56.4 ± 3.0	56.4± 8.6	0.95 ± 0.35
Heated	16	20.2 ± 1.5	0.36±0.01	0.12 ± 0.01
Boiled	4	50.3 ± 1.5	nil	nil

Drug toxicity.

Plasma enzyme activity determinations provided a sensitive means of assessing experimental drug-induced hepatic damage. The methods employed for estimation of GD and SD activity were simple and in conjunction with a constant temperature cell housing and recording spectrophotometer would be ideal for routine assay of plasma enzyme activities. Assay of plasma OCT activity was extremely tedious and the widely used method of Reichard and Reichard (1958) required large samples of plasma or serum. However, the micromethod used (Moore, 1967) was satisfactory and economical of plasma but was also time consuming and required a supply of ammonia-free reagents and glassware.

Determination of GOT and GPT activity by a colorimetric method (Reitman and Frankel, 1957)(Woctton, 1964) whilst said to be less accurate than spectrophotometric methods (Karmen, 1955) was simple and easily conducted with minimum instrumentation.

The distribution of GOT, GD and SD activity in normal avian tissue extracts is presented in Table 18. OCT activity was not detected, but activities of other enzymes were high; in particular, kidney and liver extracts contained high enzyme activity and suggested that plasma GOT, GD and SD activity may reflect hepatotoxic or nephrotoxic syndromes in birds.

In cockerels, GOT activity was highest in extracts from liver and kidney; all tissue extracts contained appreciable activity with the exception of lung, which contained almost none (Table 18). GOT activity was also evenly distributed in duck tissues (Table 18). GD activity was high in cockerel liver and kidney and was also found in other tissues, particularly brain, but was almost absent from duck tissues other than liver and kidney (Tables 19,20). Activity of SD was was higher in cockerel tissues than in duck tissues but was not always detected in cockerel plasma (Tables 18,26,19).

The bromsulphthalein dye test also provided evidence of hepatic dysfunction and was easily adapted for use in sheep but was technically difficult in avian subjects. A minimum of six accurately timed blood samples between 2 to 30 min after BSP administration was easily obtained from sheep but difficulty was experienced with birds due to the fragile nature of avian veins and the ease with which haematoma formation occurred. Interpretation of plasma disappearance curves for BSP by the method of Clarkson and Richards (1967) was suitable for experimental assessment of hepatic dysfunction.

Toxicity of carbon tetrachloride.

Administration of carbon tetrachloride to sheep was followed by simultaneous increases in plasma GOT, GD, SD and OCT activity which reached a maximum 24 hr after dosage and returned to normal within 7 days (Tables 21,22). Plasma GOT activity increased by up to three times; GD activity 40 to 1000 times, SD activity 13 to 850 times and OCT activity 5 to 67 times (Tables 21,22). There was no significant difference in plasma enzyme activities of sheep which had received 2 ml and those which had received 3 ml of carbon tetrachloride (Tables 21,22).

Carbon tetrachloride administration to cockerels was accompanied by an increase in plasma activity of GOT which reached a maximum 6 to 48 hr after dosage ($p = 0.01$)(Table 19);this was not accompanied by a rise in plasma GD or SD activity.A similar response occurred in ducks which received the drug (Table 20). Plasma GD and SD activity did not increase significantly, but plasma GOT activity increased to reach a maximum 5 days after dosage ($p = 0.05$)and returned to normal nine days later.

Cockerels and ducks showed no significant changes in BSP transfer rates 48 hr after receiving carbon tetrachloride.

Changes in bile and urine of sheep occurred after administration of carbon tetrachloride: the pH of bile increased and returned to normal 6 to 7 days later; biliary volumes decreased sharply and remained depressed for 12 to 14 days. pH of sheep urine did not change markedly although an initial,transient

diuresis was observed; no change in specific gravity of urine was seen but that of bile increased after administration of the drug. The concentration of chloride ion decreased in bile and in urine (Table 23).

Phenolsulphonephthalein dye clearance was slightly reduced by administration of carbon tetrachloride (Table 24).

No gross clinical abnormality was observed in sheep or ducks which received carbon tetrachloride. The drug induced light narcosis in cockerels and reduced water intake for 48 to 72 hr. Anorexia was observed in cockerels and rabbits which received the drug. Rabbits which were killed 6, 24 and 48 hr after administration of carbon tetrachloride showed characteristic signs of toxicity due to the drug: the liver was fatty, friable and showed 'nutmeg' pattern; the kidneys were pale and occasionally showed fatty change; the intestine was inflamed, congested and often contained mucoid material.

Liver flukes <u>in vitro</u> were little affected by emulsions of carbon tetrachloride unless sheep bile was added to the medium; the addition of 25 per cent sheep bile rapidly led to death of the flukes (Table 25). The diameter of 95 per cent of the dispersed phase droplets was within the range 1.4 to 5.0 µm.

Table 18. Enzyme activity (i.u. per litre) in extracts (1 per cent) of tissues from cockerels (6,7,8,9) and ducks (19,20,21,22).

Cockerels (4)	GOT ± s.d.	GD ± s.d.	SD ± s.d.
liver	126 ± 42	67 ± 1.6	36 ± 3.3
kidney	113 ± 2.9	50 ± 11	38 ± 2.5
spleen	28 ± 8.2	10 ± 3.5	3.6 ± 1.2
cardiac muscle	93 ± 15	7.2 ± 2.0	2.4 ± 2.0
skeletal muscle	83 ± 4.4	0.5 ± 0.5	1.5 ± 3.0
small intestine	42 ± 9.5	4.9 ± 6.1	2.9 ± 3.1
gizzard	35 ± 15	1.8 ± 1.2	2.6 ± 0.6
pancreas	82 ± 4.2	7.2 ± 5.9	6.0 ± 3.1
lung	14 ± 9.1	4.8 ± 3.7	0.0 ± 0.0
brain	99 ± 8.2	31 ± 5.9	2.2 ± 2.5
Ducks (4)			
liver	95 ± 19	84 ± 7.4	5.5 ± 0.5
kidney	107 ± 8.7	115 ± 6.9	2.6 ± 0.1
spleen	38 ± 2.9	4.8 ± 2.2	0.0 ± 0.0
cardiac muscle	118 ± 28	5.5 ± 1.5	1.1 ± 0.1
skeletal muscle	119 ± 9.9	1.7 ± 0.7	0.7 ± 0.3
small intestine	19 ± 6.3	1.2 ± 0.7	0.0 ± 0.0
gizzard	52 ± 2.7	0.0 ± 0.0	0.4 ± 0.2
pancreas	64 ± 18	5.8 ± 5.0	2.4 ± 1.1
lung	26 ± 5.0	1.9 ± 0.5	1.2 ± 0.4
brain	99 ± 4.1	15 ± 1.7	1.2 ± 0.1

Table 19. Plasma activities of tissue enzymes (i.u.) in cockerels (5) which received carbon tetrachloride (6ml) at 0 hr (all ± s.d.).

Time	GOT	GD	SD
-48 hr	55.6 ± 11.8	3.0 ± 3.5	nil
-24 hr	54.6 ± 5.0	1.0 ± 2.2	nil
0 hr	55.8 ± 8.4	1.0 ± 1.0	nil
6 hr	119.4 ± 21.6	1.8 ± 1.1	nil
24 hr	145.4 ± 23.1	0.4 ± 0.9	nil
48 hr	143.8 ± 36.0	1.6 ± 0.9	nil
6 day	49.8 ± 13.3	2.3 ± 1.0	nil
Significance of deviation from normal			
6 hr	p = 0.01	p = 0.4	-
24 hr	p = 0.01	p = 0.5	-
48 hr	p = 0.01	p = 0.5	-
6 day	p = 0.5	p = 0.2	-

Table 20. Plasma activities of tissue enzymes (i.u.) in ducks (5) which received carbon tetrachloride (6 ml) at 0 hr (all ± s.d.).

Time	GOT	GD	SD
0 hr	4.3 ± 1.8	2.9 ± 2.0	3.1 ± 2.3
42 hr	7.4 ± 9.9	5.0 ± 5.6	4.6 ± 2.7
72 hr	11.2 ± 9.0	5.3 ± 5.6	5.3 ± 2.5
5 day	12.4 ± 5.0	4.3 ± 5.6	2.4 ± 2.7
9 day	2.4 ± 2.9	1.4 ± 2.2	1.4 ± 2.2
Significance of deviation from normal			
42 hr	p = 0.6	p = 0.6	p = 0.5
72 hr	p = 0.3	p = 0.3	p = 0.3
5 day	p = 0.05	p = 0.7	p = 0.8
9 day	p = 0.8	p = 0.4	p = 0.4

Table 21. Plasma enzyme changes following administration of carbon tetrachloride to sheep.

Sheep	Day	CCl_4 (ml)	GOT (i.u.)	GD (i.u.)	SD (i.u.)	OCT (i.u.)	Breed and Sex
21	0	2	32	1.2	9.6	0.07	Scottish
	1		58	530	336	1.11	Blackface
	2		54	430	336	0.43	
	3		34	48	43	0.16	Castrated
	4		30	24	24	0.11	male
	7		32	0.0	2.4	0.08	
23	0	2	28	0.0	12	0.10	Scottish
	1		98	310	12	3.92	Blackface
	2		84	190	1310	0.62	
	3		48	144	290	0.36	Castrated
	4		36	24	216	0.19	male
	7		29	1.2	5.0	0.08	
24	0	2	26	5.0	12	0.50	Scottish
	1		40	1310	960	5.60	Blackface
	2		84	290	432	1.20	
	3		52	210	120	0.60	Castrated
	4		48	48	144	0.50	male
	7		30	0.0	2.4	0.40	
33	-7		100	8.0	36	0.75	Scottish
	-6		100	2.0	40	0.60	Blackface
	-5		96	3.0	34	0.70	
	-4		106	4.0	32	0.90	Castrated
	-3		82	2.0	24	0.36	male
	0	3	54	3.0	8.0	0.11	
	¼		62	20	30	0.07	
	1		120	120	300	2.46	
	2		86	80	140	1.20	
	3		70	12	35	0.41	
	4		64	3.0	38	0.14	
	7		40	0.0	13	0.22	

Table 22. Plasma enzyme changes following administration of carbon tetrachloride to sheep.

Sheep	Day	CCl_4 (ml)	GOT (i.u.)	GD (i.u.)	SD (i.u.)	OCT (i.u.)	Breed and Sex
34	-7		38	12	14	0.20	Scottish Blackface Castrated male
	-6		50	2.0	6.0	0.20	
	-5		42	2.0	10	0.25	
	-4		50	1.0	8.0	0.09	
	-3		50	4.0	12	0.14	
	0	3	32	0.0	6.0	0.08	
	½		38	1000	50	0.13	
	1		58	70	80	0.78	
	2		38	10	70	0.30	
	3		36	9.0	30	0.15	
	4		32	4.0	13	0.09	
	7		28	0.0	16	0.07	
35	-5		40	4.0	4.0	0.20	Cheviot Cross Female
	-2		34	2.0	4.0	0.20	
	-1		34	3.0	4.0	0.10	
	0	3	34	3.0	4.0	0.90	
	1		160	120	130	4.60	
	2		102	20	50	0.50	
	3		76	10	16	0.35	
	5		42	6.0	4.0	0.20	
	6		36	4.0	6.0	0.15	
	9		34	4.0	4.0	0.20	
36	-5		22	1.0	1.0	0.10	Cheviot Cross Female
	-2		28	1.0	16	0.40	
	-1		28	1.0	4.0	0.10	
	0	3	26	1.0	4.0	0.10	
	1		152	220	3400	6.70	
	2		94	40	220	2.30	
	3		68	16	56	0.38	
	5		36	3.0	10	0.23	
	6		28	2.0	14	0.10	
	9		28	2.0	12	0.10	

Table 23. Sheep bile and urine during administration of carbon tetrachloride (3ml).

	Vol ± s.d.	pH ± s.d.	Cl^- mM ± s.d.	SG ± s.d.
	Sheep 37 Urine			
predosing	365 ± 6	8.3 ± 0.1	288 ± 3	1045 ± 4
0 to 24 hr	1280	8.4	156	1022
1 to 2 day	490	8.1	113	1040
2 to 3 day	610	8.6	285	1042
3 to 4 day	420	8.9	211	1044
4 to 5 day	410	9.0	218	1046
5 to 11day	413 ± 39	9.0 ± 0.2	308 ± 78	1052 ± 5
	Sheep 38 Urine			
predosing	530 ± 20	8.8 ± 0.1	275 ± 26	1044 ± 7
0 to 24 hr	740	9.1	225	1030
1 to 2 day	660	8.7	151	1026
2 to 3 day	480	9.0	280	1034
3 to 4 day	550	9.0	210	1044
4 to 5 day	460	9.1	177	1042
5 to 11day	523 ±134	9.0 ± 0.1	241 ± 34	1041 ± 3
	Sheep 40 Bile			
predosing	1358 ± 69	7.2 ± 0.1	114 ± 1	1008 ± 1
0 to 6 hr	100	7.7	103	1015
0 to 24 hr	860	7.6	106	1008
1 to 2 day	1160	7.8	107	1008
2 to 3 day	1000	7.2	105	1008
3 to 4 day	1040	7.1	108	1010
4 to 5 day	1100	7.1	105	1008
5 to 11day	1112 ± 42	7.2 ± 0.1	102 ± 4	1008 ± 1
	Sheep 60 Bile			
predosing	1111 ± 35	7.3 ± 0.1	120 ± 1	1008 ± 1
0 to 6 hr	240	7.7	120	1012
0 to 24 hr	950	7.6	121	1012
1 to 2 day	920	7.8	122	1011
2 to 3 day	800	7.3	118	1010
3 to 4 day	525	7.5	120	1010
4 to 5 day	760	7.5	115	1009
5 to 11day	953 ± 52	7.3 ± 0.2	108 ± 5	1007 ±1

Table 24. Phenolsulphonephthalein clearance (PSP) in sheep which received carbon tetrachloride (3 ml).

	Percentage PSP cleared							μmoles
(minutes-)	0-15	15-30	30-45	45-60	60-75	75-90	0-90	0-90
Sheep 35								
Before CCl_4	40	26	14	7.6	4.2	3.2	95	202
28 hr after	35	25	14	8.5	5.6	3.7	92	195
48 hr after	42	25	13	7.5	4.7	3.0	93	198
11day after	41	26	14	8.0	4.4	3.6	97	206
Sheep 36								
Before CCl_4	39	28	13	7.4	3.8	2.8	94	230
24 hr after	36	27	13	7.5	3.2	2.8	90	219
48 hr after	40	27	12	7.9	3.6	2.8	93	229
11day after	41	28	13	8.3	3.2	2.6	96	235

Table 25. Motility of liver flukes incubated at 37° C in emulsions diluted with Hedon-Fleig solution

Composition of medium	No.	Bile (%)	50% lost motility in:
Hedon-Fleig solution	20	-	74 hr
Normal sheep bile	30	25	12 hr
Sheep bile 0 - 24 hr after CCl_4	10	25	26 hr
Sheep bile 24 - 48 hr after CCl_4	10	25	15 hr
25% control aqueous rabbit liver extract	10	-	11 hr
25% CCl_4 treated aq. rabbit liver extract	10	-	26 hr
1% control rabbit liver lipid	16	25	10 hr
1% CCl_4 treated rabbit liver lipid	16	25	2 hr
olive oil emulsion	10	25	28 hr
CCl_4 in olive oil emulsion	33	25	1 hr
Hedon-Fleig solution	12	-	86 hr
CCl_4 in olive oil emulsion	11	-	62 hr
1% oleic acid (methyl ester)	8	-	26 hr
Hedon-Fleig solution	28	-	63 hr
CCl_4 in olive oil emulsion	11	-	46 hr
½% oleic acid (methyl ester)	14	25	15 hr
½% oleic acid (methyl ester) CCl_4 treated	20	25	7 hr
Hedon-Fleig solution	10	-	76 hr
olive oil emulsion	10	25	32 hr
CCl_4 in olive oil emulsion	12	25	1 hr
1% oleic acid (methyl ester) CCl_4 treated	20	25	1 hr

Toxicity of hexachloroethane.

When hexachloroethane was administered to sheep a simultaneous increase in plasma GD, SD and OCT activity occurred which reached a maximum 48 hr after administration of the drug and returned to normal 4 to 5 days later. GOT activity increased slightly and was highest at 24 to 48 hr (Figures 11,12,14,15,16) or 48 to 72 hr (Figure 13); GD activity increased from 3 times (Figures 15,17) to over 6 times (Figure 12) although a very large increase of 55 times occurred in one sheep (Figure 11). SD activity increased from 3 times to 6 times (Figures 17,18). OCT activity increased from 2 times (Figure 18) to 10 times (Figure 17). When GPT activity was determined, the results were inconsistent (Figures 11,12,13, 14); the test was discarded and replaced by OCT determinations.

Sheep which received higher doses of hexachloroethane did not show consistently higher plasma enzyme activity (Figures 17,18).

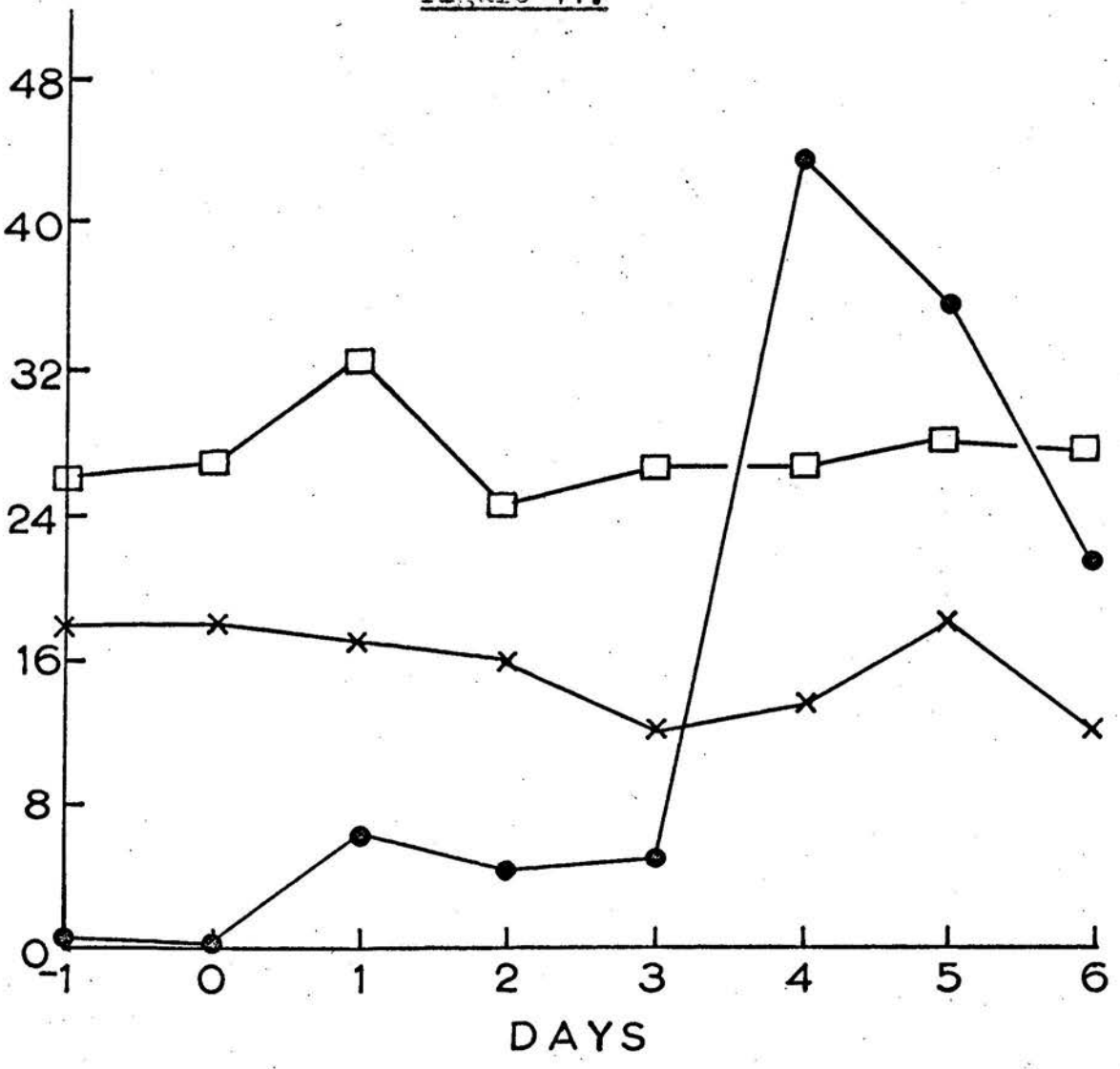
Administration of pentachloroethane and tetrachloroethylene, metabolites of hexachloroethane, also increased plasma enzyme activity. Pentachloroethane caused large increases in GD (200 times); in SD activity (20 to 30 times) and in OCT activity (15 to 19 times). Tetrachloroethylene administration did not increase plasma activity of OCT in sheep but did increase GD and SD activity (Figures 19,20,21,22).

Hexachloroethane did not increase the plasma activity of GD, SD or GOT in cockerels (Table 26).

Determination of BSP dye clearance in sheep which had received hexachloroethane indicated that hepatic damage had occurred and supported the evidence provided by plasma enzyme tests. Plasma disappearance of BSP occurred in two phases(as described by Cornelius, Holm and Jasper,1958).Determination of BSP dye transfer rates showed that hexachloroethane did not affect uptake from plasma by liver cells, but that a marked reduction of transfer rates from liver cells to bile occurred 72 hr after administration of the drug. BSP tests conducted on sheep which received olive oil showed that little variation in BSP transfer rates occurred in normal sheep within a 72 hr period (Table 11).

Liver flukes incubated in hexachloroethane emulsions <u>in vitro</u> did not lose motility during a 4 hr incubation period.

Figure 11.

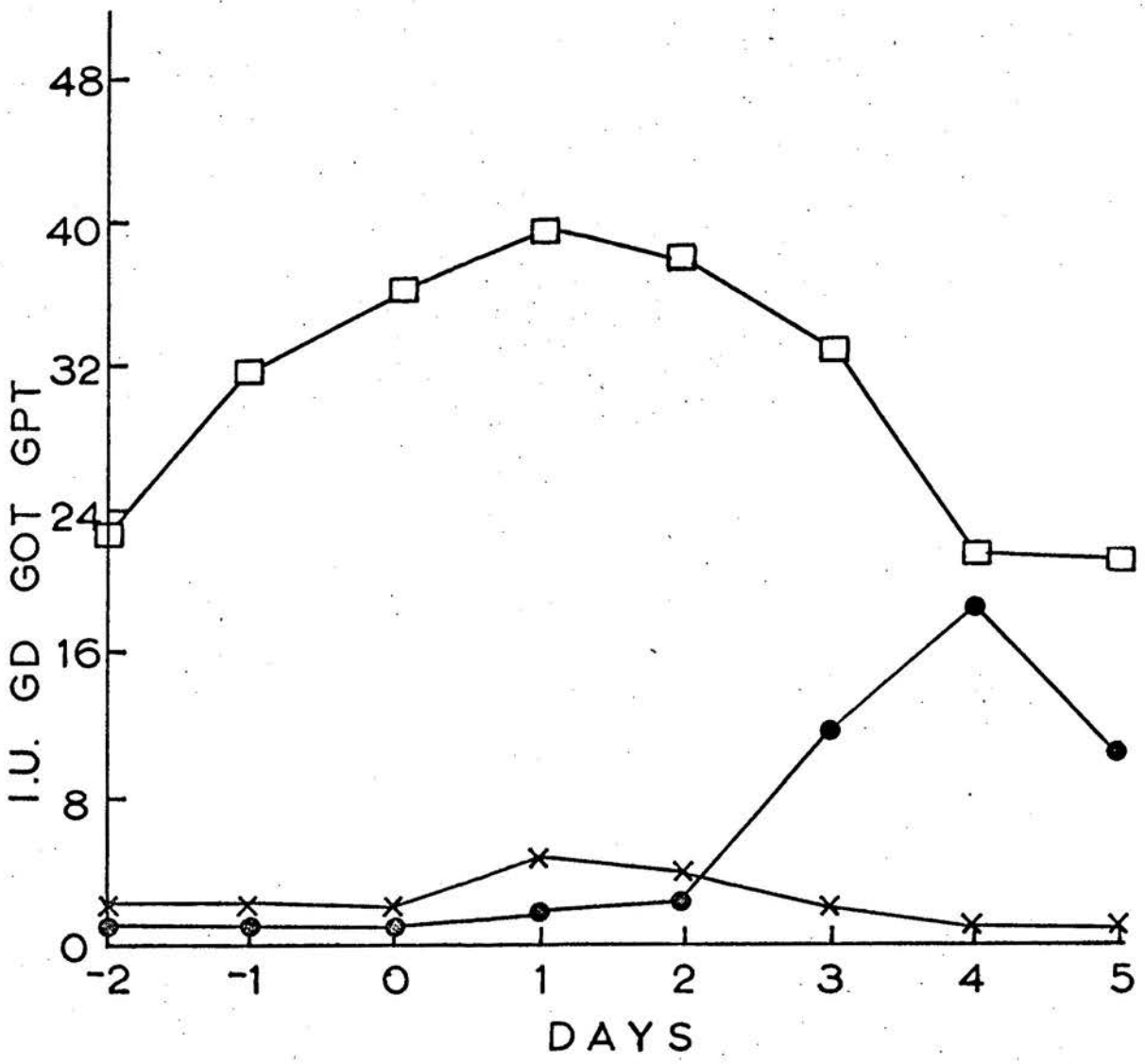


SHEEP EXPT 1

Symbols used in Figures 11 to 22:

Sorbitol dehydrogenase (SD)	(EC 1.1.1.14)	o----o
Glutamate dehydrogenase (GD)	(EC 1.4.1.3)	•----•
Ornithine carbamyl transferase (OCT)	(EC 2.1.3.3)	■----■
Aspartate aminotransferase (GOT)	(EC 2.6.1.1)	□----□
Alanine aminotransferase (GPT)	(EC 2.6.1.2)	x----x

Figure 12.



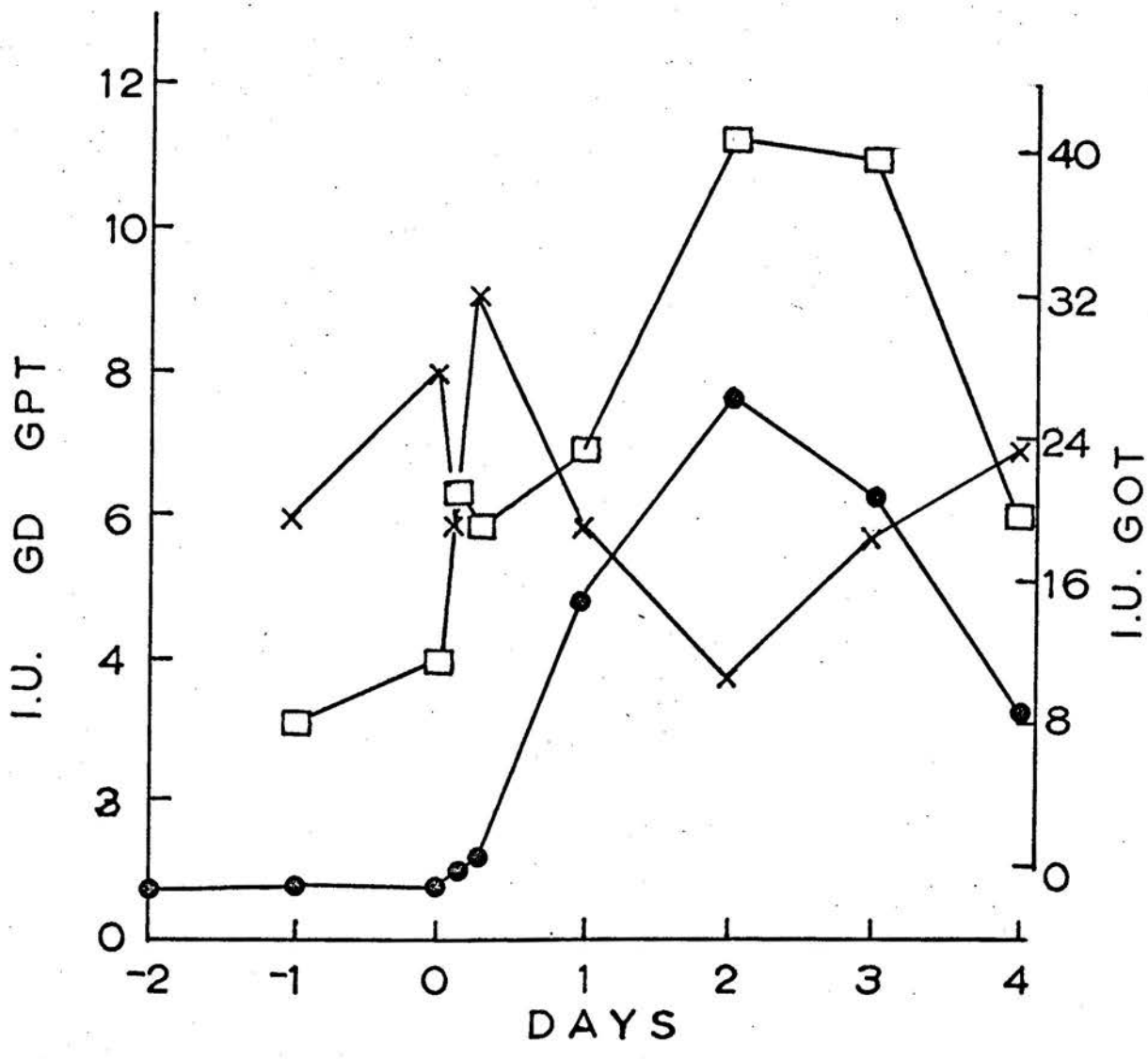
SHEEP EXPT 2

GOT

GPT

GD

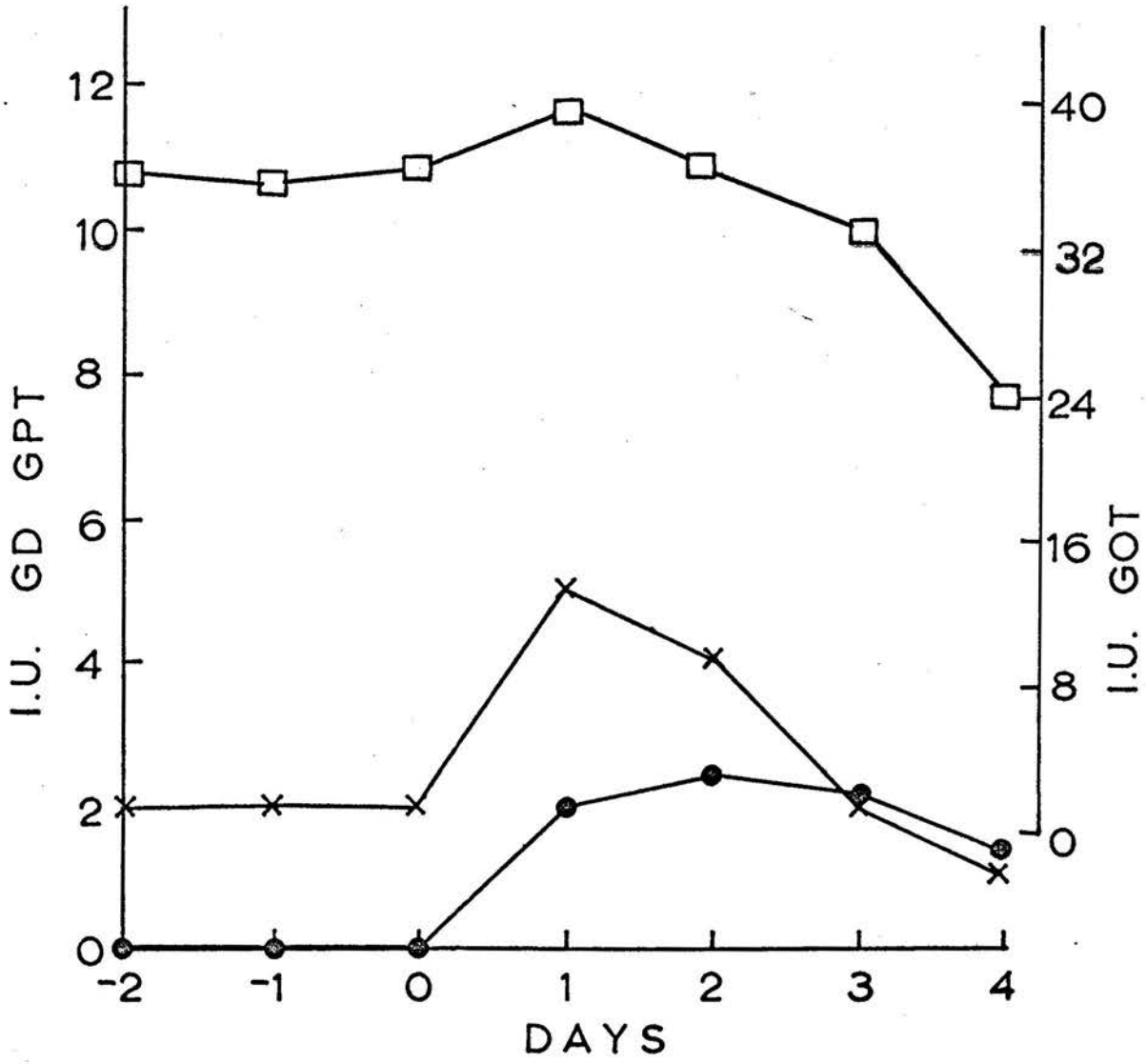
Figure 13.



SHEEP EXPT 3

GPT
GOT
GD

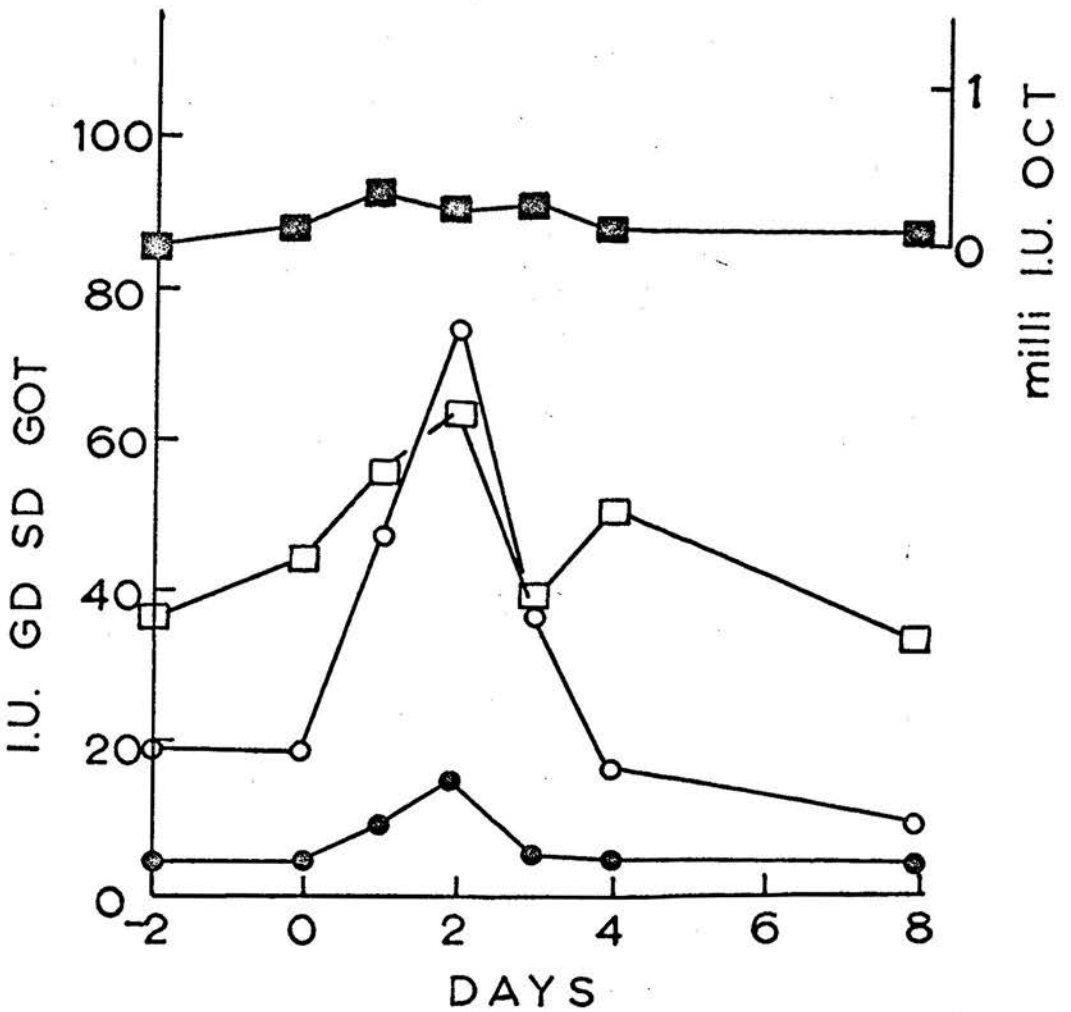
Figure 14.



SHEEP EXPT 4

GOT
GPT
GD

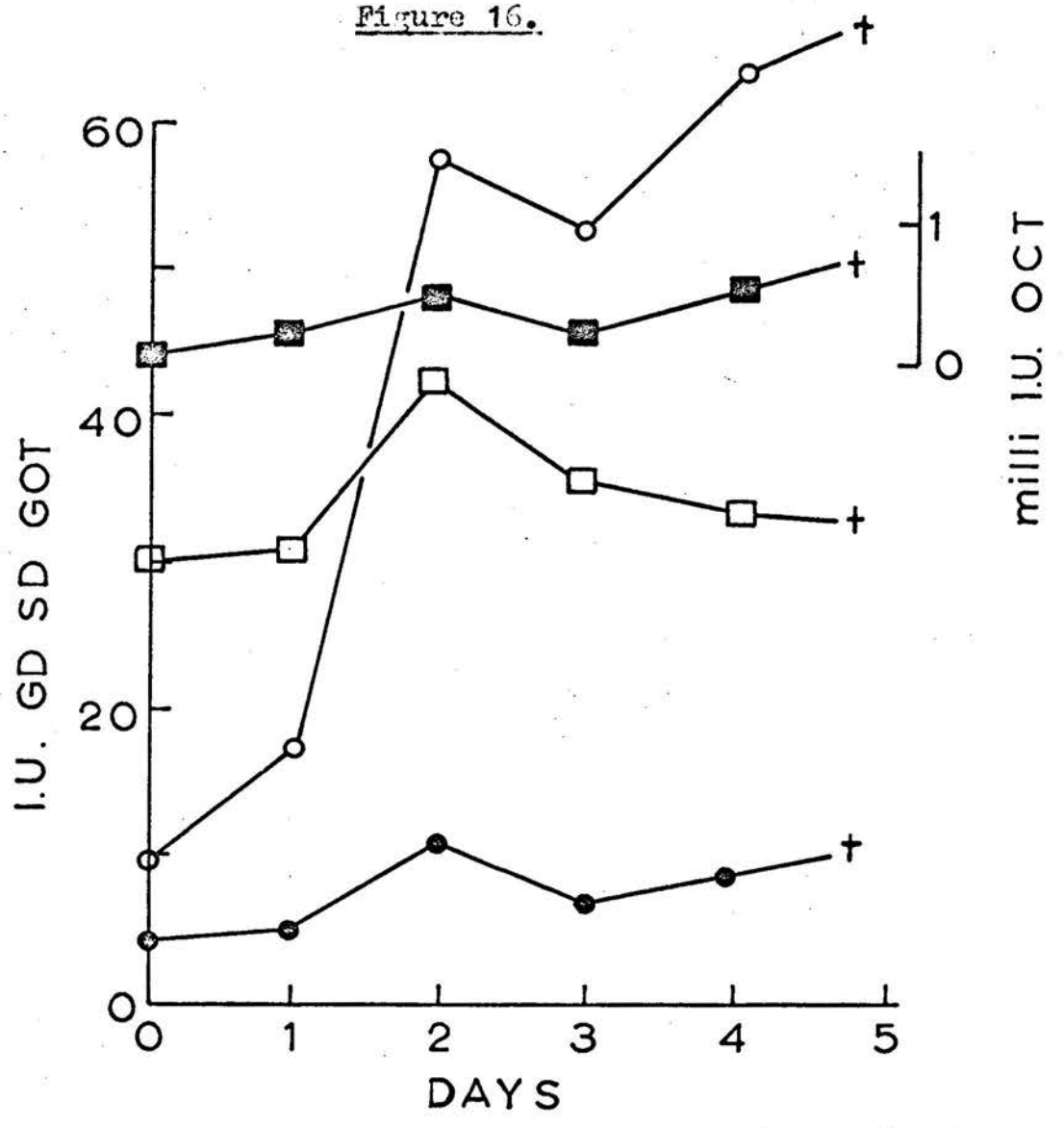
Figure 15.



SHEEP EXPT 5

OCT
GOT
SD
GD

Figure 16.



SHEEP EXPT 6

OCT
GOT
SD
GD

Figure 17.

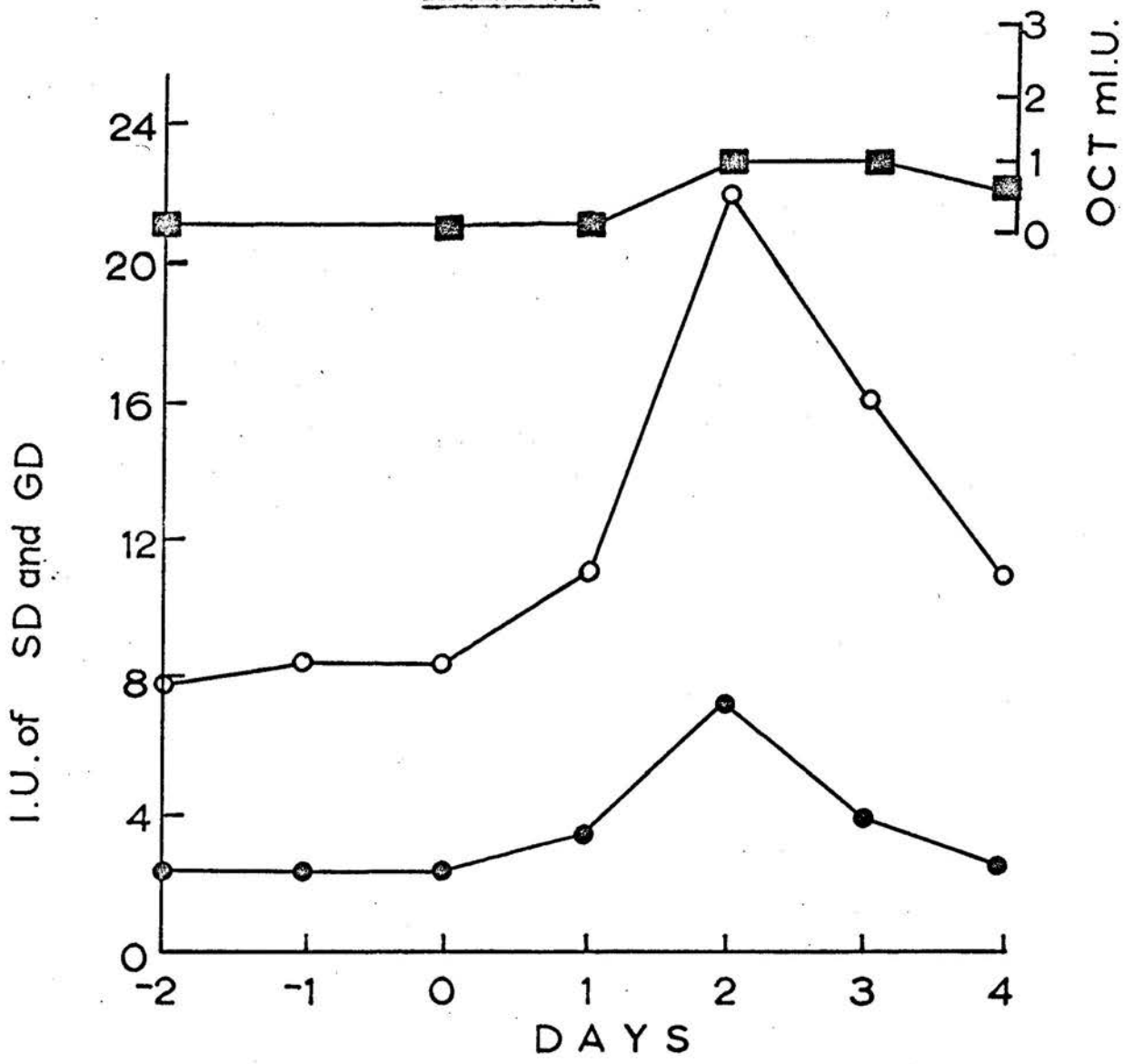
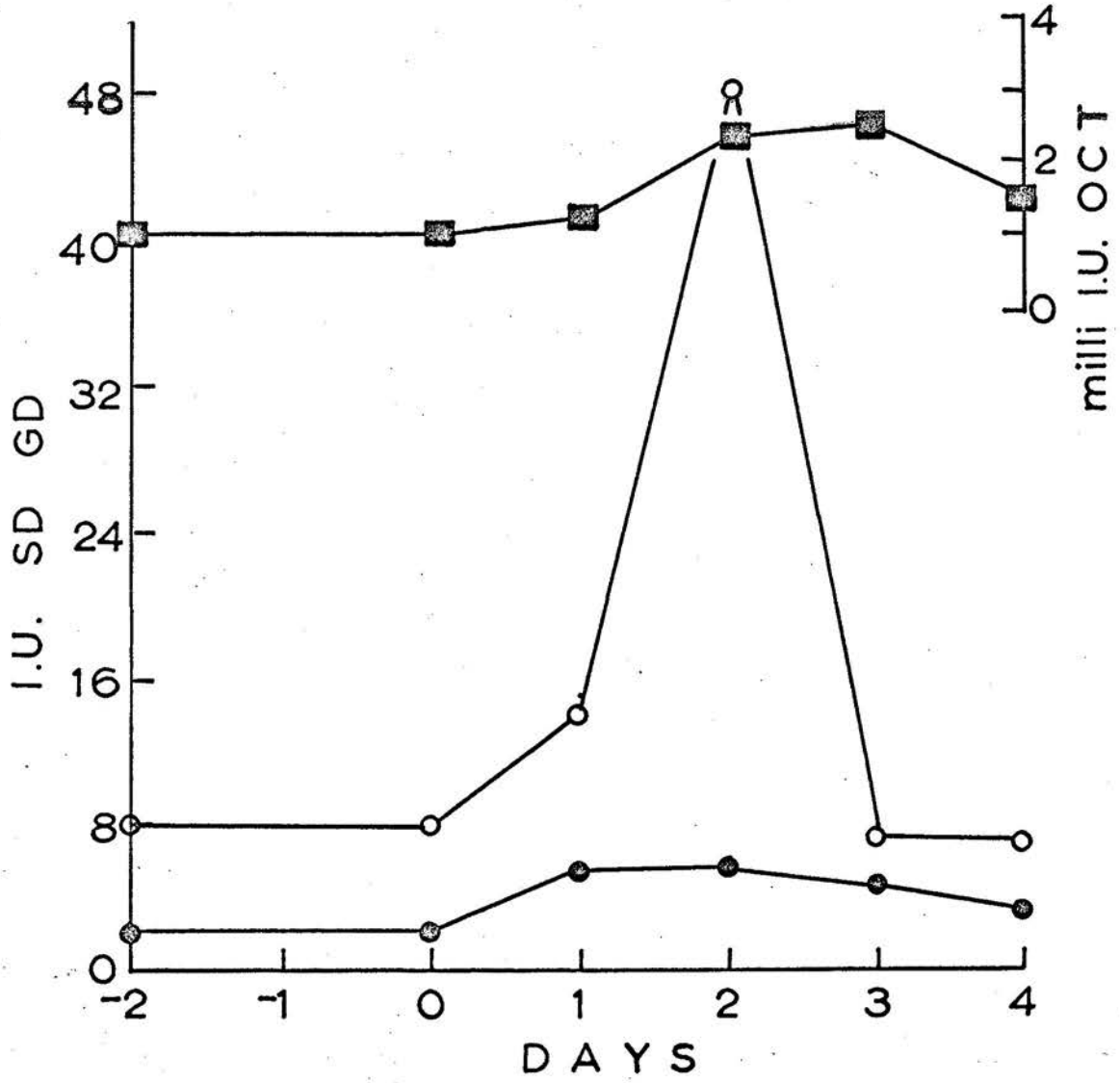


Figure 18.



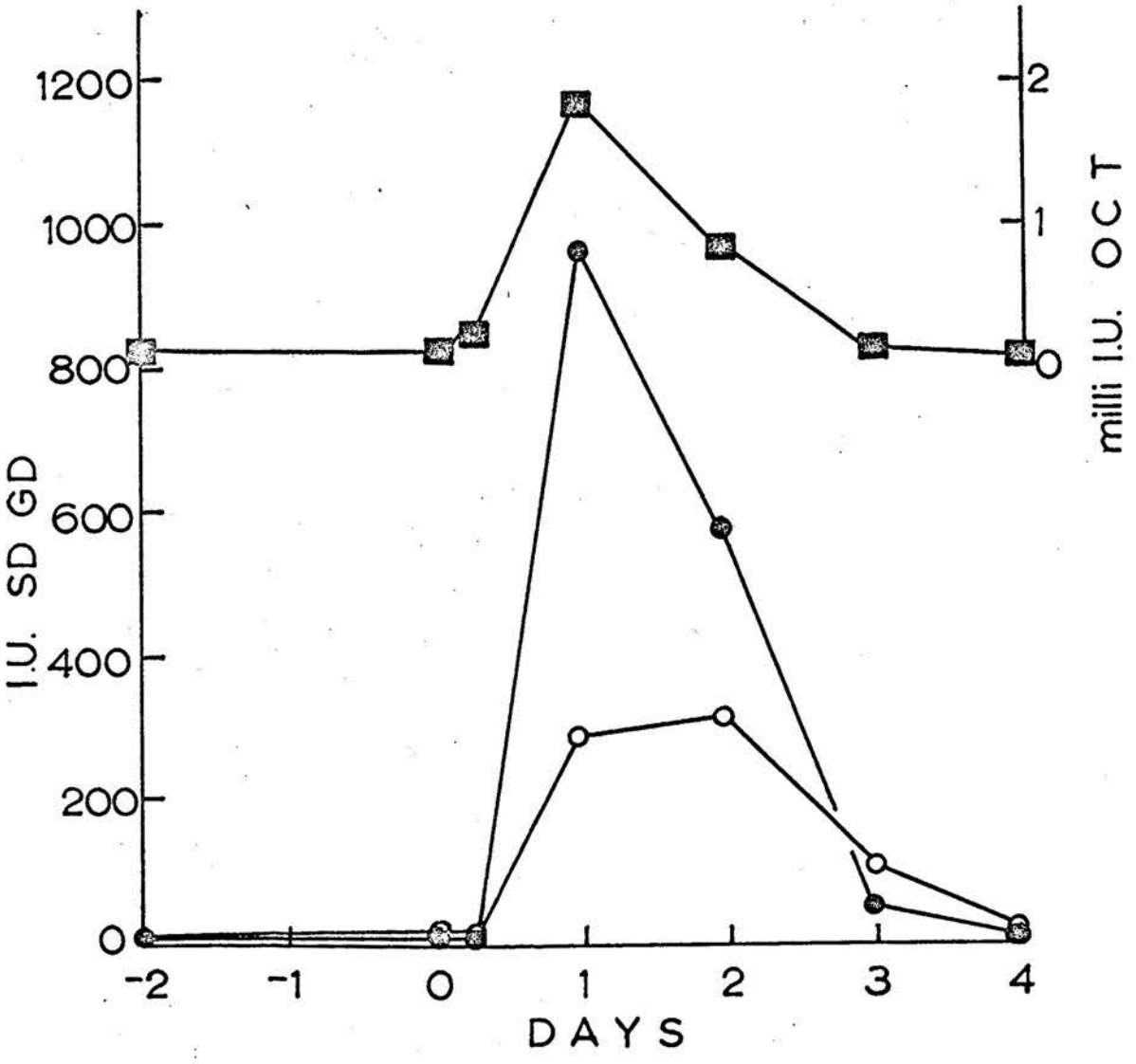
SHEEP EXPT 8

OCT

SD

GD

Figure 19.



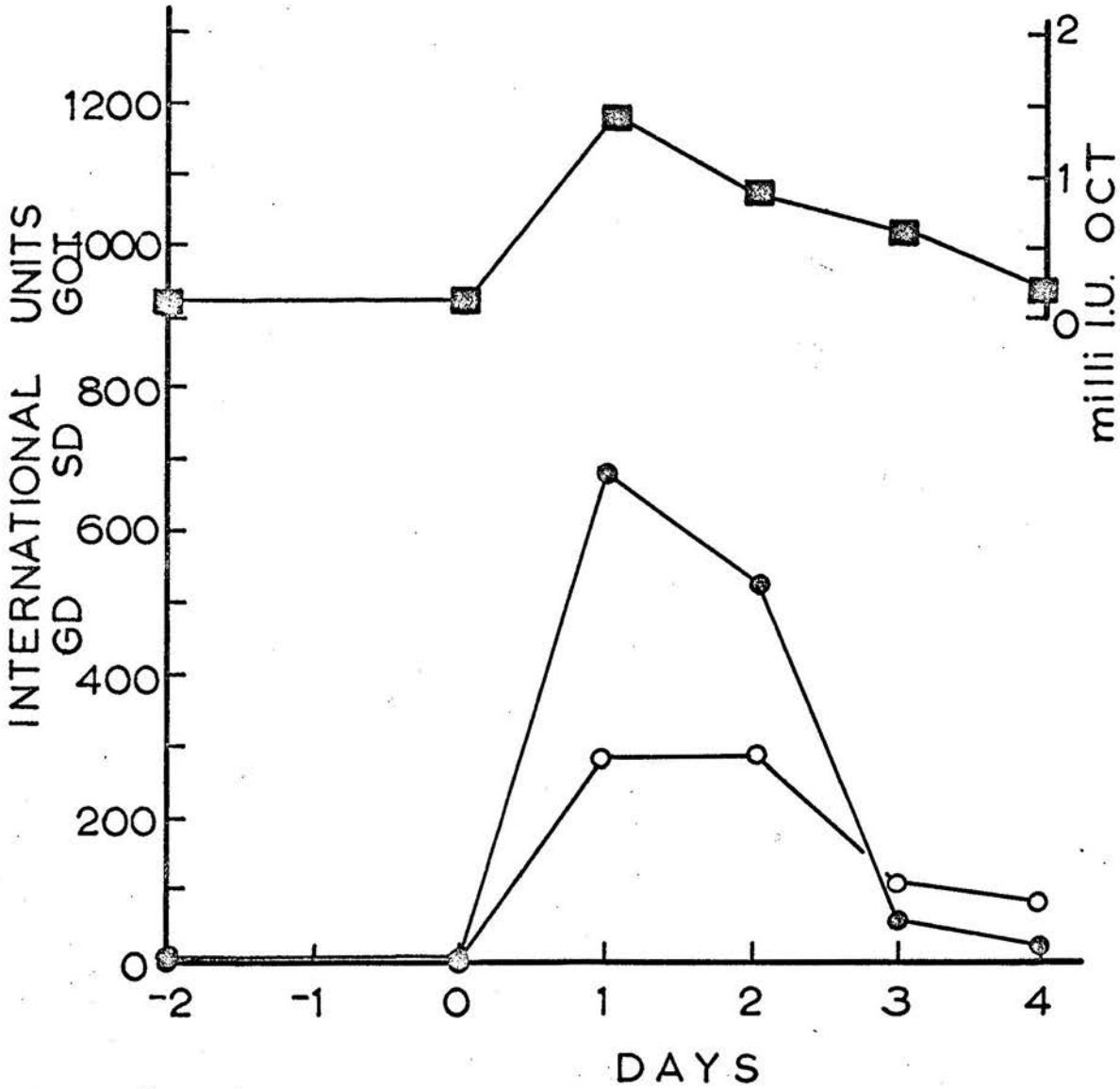
SHEEP EXPT 19

OCT

GD

SD

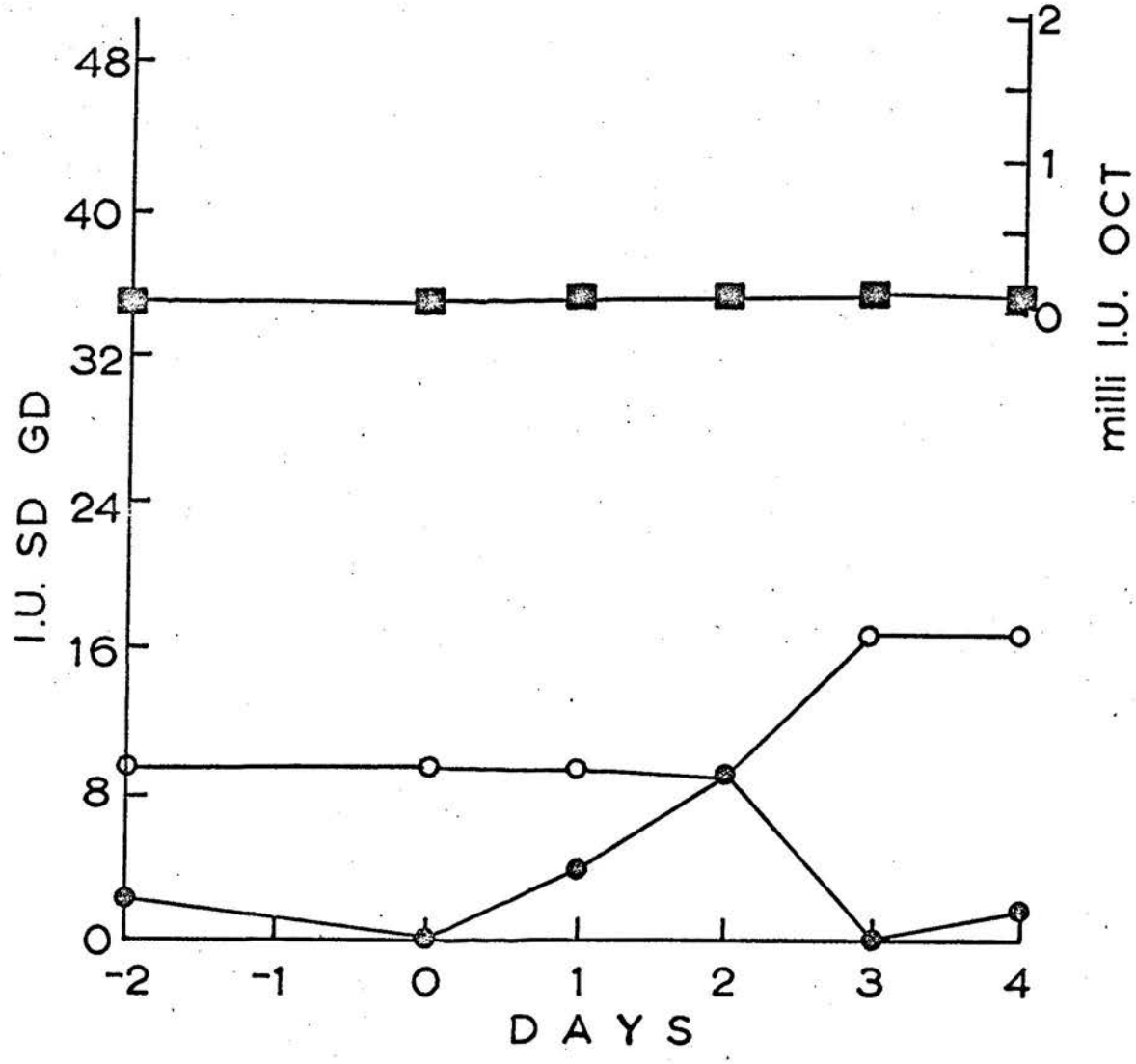
Figure 20.



SHEEP EXPT 25

OCT
GD
SD

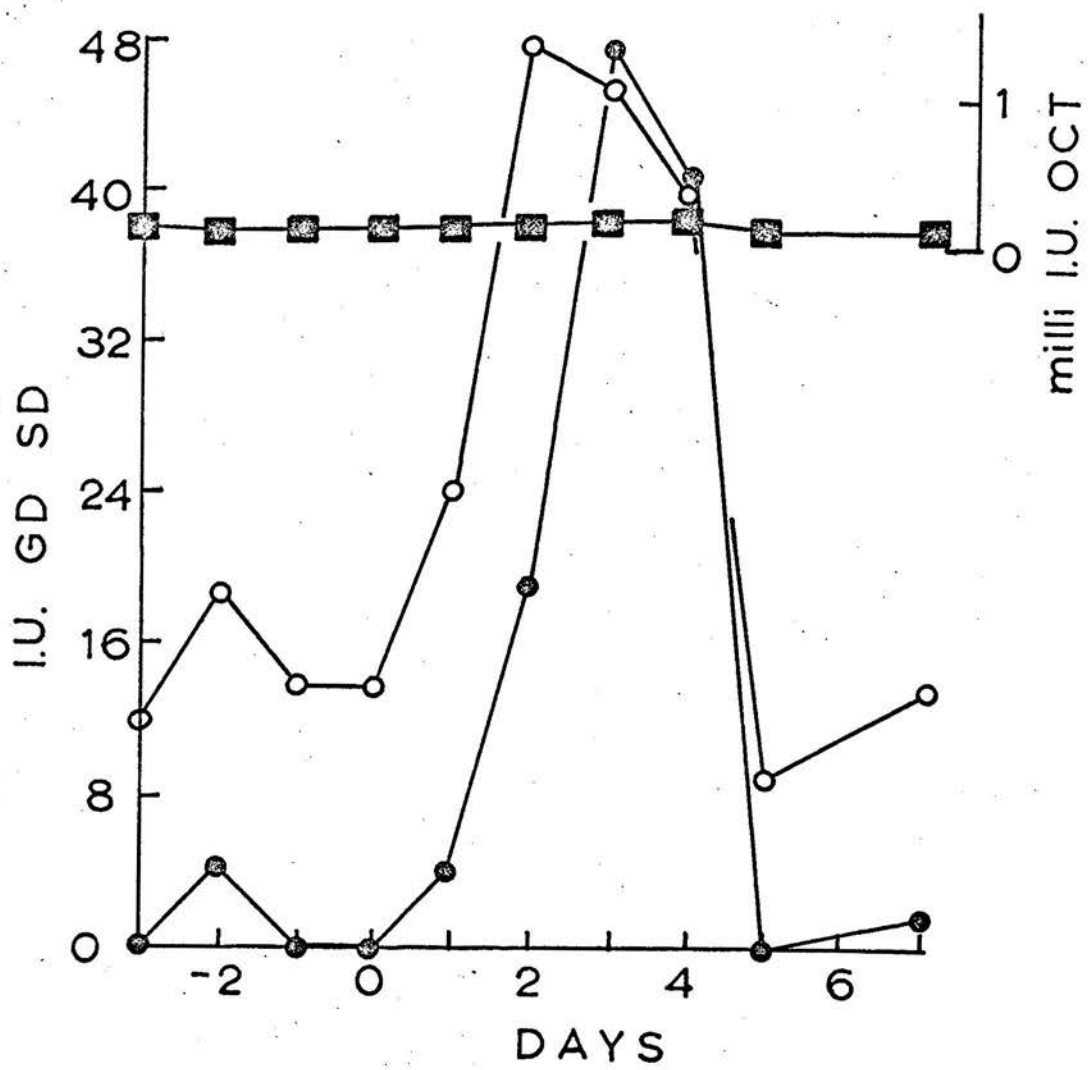
Figure 21.



SHEEP EXPT 20

OCT
SD
GD

Figure 22.



SHEEP EXPT 22

OCT

SD

GD

Table 26. Plasma activity of tissue enzymes (i.u.) in cockerels 10 and 11 which received hexachloroethane (4g) at 0 hr.

Time	GOT		GD		SD	
	10	11	10	11	10	11
0 hr	57	47	0.2	2.4	1.0	0.5
24 hr	62	47	2.4	1.5	1.0	0.2
42 hr	64	46	2.4	2.2	0.5	0.5
72 hr	48	60	1.7	2.4	1.0	0.7
5 day	58	70	2.4	2.4	0.2	0.5
9 day	50	52	1.5	0.2	0.5	0.2

Survival time of liver flukes in vitro.

Liver flukes retained activity for at least 2 to 3 days when incubated in Hedon Fleig solution in vitro. Other media were less suitable for maintenance of F.hepatica in vitro; emulsions of lipid from carbon tetrachloride treated rabbits, of carbon tetrachloride and of its addition product with methyl oleate were especially toxic in the presence of sheep bile (Table 25).

Normal sheep bile reduced the survival time of liver flukes in vitro whereas bile from carbon tetrachloride treated sheep was less toxic; an aqueous extract of liver from carbon tetrachloride treated rabbits was less toxic to liver flukes in vitro than control aqueous liver extract. This was reversed when rabbit liver lipid was added to liver flukes in vitro: liver lipid from carbon tetrachloride treated rabbits was much more toxic to flukes than control liver lipid was (Table 25).

Toxicity of 'Freons' and 'Hetol'.

Administration of 1,2-tetrachlorodifluoroethane to sheep was followed by an increase in plasma GOT activity, highest 3 days after administration of the drug, which returned to normal within 7 days (Table 27). There was no significant change in plasma activity of GD; OCT activity increased slightly in one sheep (Table 27).

1,1-tetrachlorodifluoroethane was also without a marked action on plasma activity of GOT, GD and SD although SD activity increased in one sheep and OCT activity in the other (Table 27).

Bistrichloromethylbenzene did not affect plasma activity of GD; SD activity increased in one sheep and GOT activity in the other; OCT activity increased in both sheep after administration of the drug (Table 28).

There was no visible clinical response to these drugs.

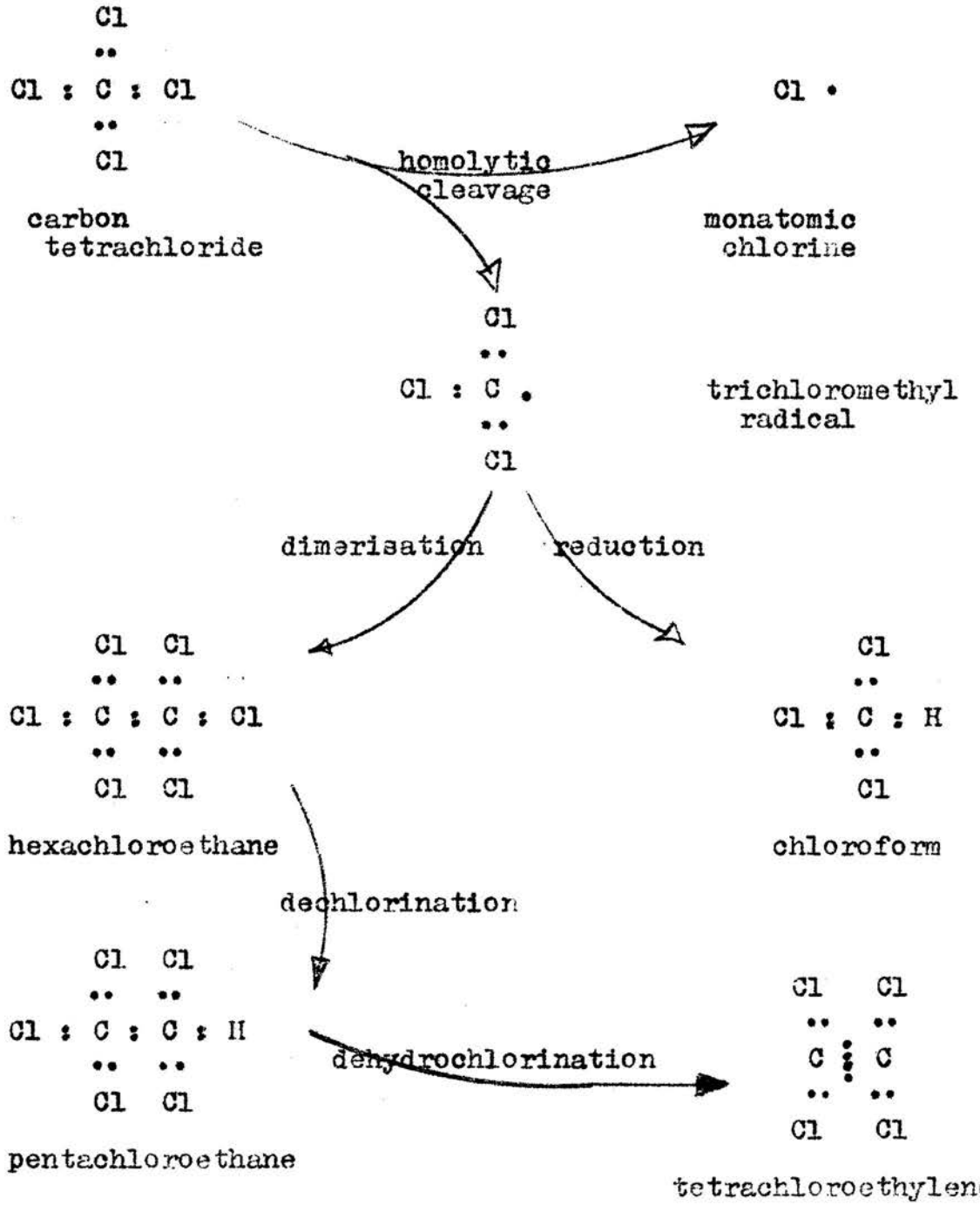
Table 27. Plasma activity of tissue enzymes following administration of tetrachlorodifluoroethanes (Freon 112 and 112a) to sheep. (i.u.)

Expt.	Day	Dose of:	GOT	GD	SD	OCT	Other details
29	-2	Freon 112 0.33 ml/kg	20	1.2	4.2	0.1	Scottish Blackface Castrated male 31.5 kg
	0		22	1.2	4.8	0.1	
	1		20	1.2	1.2	0.07	
	2		30	0.0	0.0	0.07	
	3		38	2.4	4.8	0.07	
	4		30	2.4	2.4	0.1	
	7		18	2.4	4.8	0.1	
30	-2	Freon 112 0.33 ml/kg	38	1.2	12	0.2	Scottish Blackface Castrated male 27.5 kg
	0		44	1.2	12	0.2	
	1		48	1.2	1.2	0.06	
	2		54	0.0	4.8	0.03	
	3		58	4.8	9.6	0.7	
	4		44	2.4	4.8	0.6	
	7		36	0.0	2.4	0.7	
31	-2	Freon 112a 0.33 ml/kg	34	1.2	2.4	0.0	Scottish Blackface Castrated male 23 kg
	0		28	1.2	4.8	0.0	
	1		42	1.2	4.8	0.0	
	2		52	0.0	4.8	0.0	
	3		50	4.8	14	0.0	
	4		48	4.8	12	0.0	
	7		50	4.8	9.6	0.0	
32	-2	Freon 112a 0.33 ml/kg	38	2.4	7.3	0.1	Scottish Blackface Castrated male 29 kg
	0		46	2.4	7.3	0.1	
	1		24	0.0	2.4	0.4	
	2		32	0.0	2.4	0.5	
	3		42	4.8	4.8	0.1	
	4		38	2.4	4.8	0.2	
	7		42	2.4	4.8	0.2	

Table 28. Plasma activity of tissue enzymes following administration of bistrichloromethylbenzene (Hetol) to sheep (150 mg per kg at day 0).

Sheep expt	Day	GOT (i.u.)	GD (i.u.)	SD (i.u.)	OCT (i.u.)
48	0	35	1.2	10	0.1
	1	47	1.2	7	0.4
	2	40	1.2	10	0.2
	3	37	1.2	7	0.2
	4	36	1.2	9	0.0
	6	36	1.2	9	0.0
49	0	62	1.2	9	0.1
	1	62	2.4	24	0.3
	2	72	1.2	29	0.2
	3	58	2.4	15	0.2
	4	66	1.2	10	0.1
	6	62	1.2	10	0.1

Discussion



Proposed metabolic pathways of carbon tetrachloride and hexachloroethane in sheep, rabbits and F. hepatica.

Discussion.

Study of the metabolism of carbon tetrachloride and hexachloroethane in sheep, rabbits, birds and liver flukes revealed only quantitative differences between species. However, such differences are of interest and may account for widely differing toxicity of these drugs in mammals, birds and liver flukes. In general, it appeared that carbon tetrachloride was dechlorinated in sheep, rabbits and liver flukes, but not in cockerels and ducks, to yield chloroform. This is not a new finding: metabolism of carbon tetrachloride ($^{14}CCl_4$) by monkeys was demonstrated in 1951 (76). Subsequent work on metabolism of the drug in dogs utilised gas chromatography (15); this technique, together with an electron capture detector, was found to be the most sensitive method for detection of chlorinated hydrocarbons in tissue extracts.

Metabolism of carbon tetrachloride.

After oral administration of carbon tetrachloride to sheep, absorption, as reflected by jugular venous blood and biliary concentrations of the drug, was rapid. Highest biliary concentrations were attained 1 to 2 hr after administration of the drug: this may explain the failure of other workers to

detect carbon tetrachloride in the bile, since by 6 hr after the dose concentrations had fallen to less than 1 p.p.m. Since gas chromatography was so suitable for detection of carbon tetrachloride in bile, conventional micro-diffusion techniques (Conway, 1957) are probably not sufficiently sensitive and have led to misleading results and failure to identify the drug in bile of sheep which received it in therapeutic doses (Alexander and Macdonald, 1960). Furthermore, a technique using ^{14}C labelled drug (Khalidi and Zaki, 1969) failed to demonstrate its excretion in bile; it is possible that loss of volatile biliary constituents or severe quenching effects may have occurred (Turner, 1967) and, almost certainly, a different breed of sheep was involved. A method that has been employed, involving heating of bile to drive off labelled carbon tetrachloride (65), may introduce a further inaccuracy: on repeating this method (with $^{12}CCl_4$) it was found that 29 to 64 per cent of carbon tetrachloride (determined by gas chromatography) was not displaced.

The demonstration of carbon tetrachloride in the bile of sheep may be of significance when considering the mechanism of the fasciocidal action of the drug, since adult F. hepatica reside in the bile duct and are bathed in the bile of the host.

Tissue distribution of carbon tetrachloride was not studied

in sheep. In rabbits, 6 hr after administration of carbon tetrachloride, the drug was widely distributed and was detected in fat,liver,kidney,muscle and gall-bladder bile; tissue concentrations diminished in the subsequent 42 hr. Also metabolites of carbon tetrachloride were detected in rabbits: chloroform was identified in tissues and in gall-bladder bile together with hexachloroethane.Hexachloroethane had not previously been described as a metabolite of carbon tetrachloride.The tissue distribution of hexachloroethane detected as a metabolite of carbon tetrachloride in rabbits was similar to that observed in sheep which received the drug hexachloroethane.The amount of hexachloroethane formed from carbon tetrachloride in rabbits probably represented only a fraction of the total since hexachloroethane was itself metabolised by the liver.

Concentrations of the metabolites chloroform and hexachloroethane were high in tissues of a rabbit which died 44 hr after receiving carbon tetrachloride; this is in accordance with the results of Garner and McLean (1969) which suggest that toxicity of carbon tetrachloride is related to the absolute amount metabolised.

In-vitro experiments with liver flukes showed that carbon tetrachloride may be taken up from aqueous emulsions by F. hepatica; this indicates that the flukes may be able to absorb the drug from bile in vivo.Carbon tetrachloride was

metabolised by flukes as ~~occurred in~~ it was by sheep and rabbits; chloroform and hexachloroethane were detected in flukes which had been incubated in carbon tetrachloride emulsions containing sheep bile.

The toxicity of carbon tetrachloride to liver flukes *in vitro* has not been observed by other workers (116)(19) and the success of the present experiments in reproducing a lethal action of the drug *in vitro* may be attributed to the presence of sheep bile in the incubating medium. The action of bile was not known; it seemed possible that it stabilised the drug emulsions or changed the distribution of toxic substances within the flukes. It is also possible that bile reacted with carbon tetrachloride to form a toxic product: if so, this was not detected by gas chromatography of extracts of the medium. However, it is known that exposure of helminth parasites to emulsions rather than to solutions of drugs may increase the activity of the drugs (Baldwin, 1943) and may more closely resemble the *in-vivo* situation. Emulsions used were of relatively small droplet size and may be likened to large micelles such as occur in mammalian bile (75).

It is perhaps important to note that metabolism of carbon tetrachloride, which is toxic to both rabbits and to liver flukes, yields identical products in each species.

Hexachloroethane is quantitatively a more important metabolite of carbon tetrachloride in liver flukes *in vitro*

than in rabbits; its formation may provide evidence for extensive 'activation' of carbon tetrachloride in liver flukes *in vivo*. This would support a 'direct toxic action' mechanism for the fasciocidal action of carbon tetrachloride and will be discussed later. Furthermore, these experiments provide evidence to support the 'activation theory' proposed by Slater (1966) since the production of a symmetrical 2-carbon metabolite from carbon tetrachloride may have involved dimerisation of active free radicals. Dimerisation is a common termination of free radical life (123) (2).

Further evidence was provided by studies of carbon tetrachloride metabolism in birds: no metabolites of carbon tetrachloride were detected in plasma samples from cockerels and ducks and it seemed possible that activation of the drug had not occurred in these species. Hence the low toxicity of the drug is correlated to the lack of evidence of metabolism of it in birds.

The significance of the metabolites.

Metabolism of carbon tetrachloride to halogen acids (43), urea or carbon dioxide (76) requires that dechlorination as described by Butler (1961) occurs.

Chloroform detected as a metabolite of carbon tetrachloride is a stable dechlorination product of the drug and may arise by reduction of trichloromethyl radicals which in turn may

be produced by homolytic cleavage of the molecule (15). Detection of hexachloroethane after carbon tetrachloride administration suggests that the drug undergoes homolytic cleavage to trichloromethyl radicals.

The significance of two further unidentified metabolites of carbon tetrachloride ('W' and 'Z') in rabbit tissues is unknown although it seemed that 'W' may have been a product of alkaline hydrolysis of 'Z'.

Gas chromatography (GLC) with an electron capture (EC) detector was the only method sufficiently sensitive to detect hexachloroethane, 'Z' and 'W' at the concentrations encountered. Ideally, confirmation of the identity of hexachloroethane should have been by a method other than GLC; however, the determination of retention times on three different types of GLC column complies with present criteria of peak identification in GLC of simple systems (Perry,1967).

The source of hexachloroethane was not known, however at least two routes for the formation of hexachloroethane from dehalogenated carbon tetrachloride are possible:one involving dimerisation of trichloromethyl radicals which have arisen by homolytic cleavage of carbon tetrachloride as envisaged by Butler (1961). A second may involve dimerisation of radicals arising from decomposition of intermediate trichloromethyl compounds such as trichloromethyl mercaptan, trichloromethanol, or trichloromethylated lipids (Gordis,1969).

In these cases it seems that trichloromethyl radicals may be involved in the formation of hexachloroethane.

Although hexachloroethane was detected in tissues after carbon tetrachloride administration and may have arisen by activation of the latter, it is also a fasciocidal drug and has been used in veterinary chemotherapy for many years (40).

Hexachloroethane ($^{14}C_2Cl_6$) metabolism was studied by Jondorf, Parke and Williams (1958) and, as Butler had found for carbon tetrachloride, gas chromatography was suitable for seperation of hexachloroethane and its metabolites.

Metabolism of hexachloroethane.

When compared with carbon tetrachloride, the absorption of orally administered hexachloroethane was slow, as reflected by jugular venous concentrations of the drug, especially in anaesthetised sheep. However, anaesthetised preparations demonstrated three points: hexachloroethane is excreted in the bile; biliary concentrations may markedly exceed blood concentrations and the drug is widely distributed in body tissues.

Excretion of hexachloroethane in sheep bile allows direct contact of the drug with mature liver flukes in the bile ducts. Moreover, contact time may be increased by entero-hepatic circulation which, if concentrations in bile are greater than those in systemic blood, may maintain portal

venous concentrations after systemic venous concentrations have fallen.

Metabolites of hexachloroethane may be detected in the general circulation. The rapid appearance of tetrachloroethylene may be contrasted with the apparent lag in the appearance of pentachloroethane; possibly, metabolism of pentachloroethane occurs rapidly in sheep. This reaction, which involves dehydrochlorination, is analogous to production of DDE from the insecticide DDT; however, the latter appears to proceed slowly in mammals and birds.

In cockerels, plasma concentrations of hexachloroethane and its metabolites were much lower than those in sheep although higher doses had been administered. The low concentrations of pentachloroethane may have been significant, since at least in mammals this is probably the most toxic of the three compounds hexachloroethane, pentachloroethane and tetrachloroethylene (25).

The low plasma concentrations of hexachloroethane and carbon tetrachloride after oral administration to cockerels may have been due to poor absorption of the drugs. Also, the relatively high body temperature may favour more rapid removal of the drugs through the lungs and airsacs than can occur in mammals. A similar situation may account for the low plasma concentrations of carbon tetrachloride encountered in ducks which received the drug.

In liver flukes, hexachloroethane was metabolised to pentachloroethane and tetrachloroethylene as had been observed in sheep and cockerels which received the drug. Since hexachloroethane is a fasciocidal drug, selective toxicity towards the parasite may be due to absolute differences in sensitivity, or to quantitative differences between rates of formation or elimination of toxic metabolites. It is possible that liver flukes *in vivo* are not able to eliminate non-polar compounds such as hexachloroethane or pentachloroethane rapidly. This may allow accumulation of such compounds to occur and may account for greater toxicity of them for the parasite than for the host.

Although low plasma concentrations of pentachloroethane were encountered in cockerels which received hexachloroethane, concentrations encountered in fluke tissues were relatively high with relation to the other metabolite, tetrachloroethylene. Pentachloroethane is apparently not fasciocidal *in vivo* (58), but may be toxic to flukes if formed within their tissues: it is very toxic to mammals (6). Pentachloroethane may give rise to free radicals during chemical reactions (126) but there is no evidence for their occurrence in biological systems.

Although metabolites of hexachloroethane were readily detectable the site of metabolism of the drug was not known; however the rapid appearance of hexachloroethane and tetrachloroethylene in bile of anaesthetised sheep indicated that

it had occurred either during absorption, or in the liver. Although metabolism of various chlorinated hydrocarbons by an enzyme present in the liver, kidney and spleen was demonstrated by Heppel and Porterfield (1948), Bray, Thorpe and Vallance (1952) had questioned these findings and described chloride liberating reactions of certain aliphatic 'chloro compounds with -SH groups of amino-acids. Since boiled tissue extracts liberated chloride from several compounds including hexachloroethane and pentachloroethane Bray et al. largely discounted enzyme reactions in favour of -SH conjugation systems.

However, in-vitro experiments with sheep liver slices and liver flukes supported the view of Heppel and Porterfield and did not confirm the findings of Bray et al..Although fresh liver slices and liver flukes produced pentachloroethane and tetrachloroethylene from hexachloroethane, boiled liver slices and liver flukes did not, which suggested that an enzymatic process may be involved in metabolism of the drug. Further support for this theory was afforded by the partial inactivation of liver slices which occurred at 70°C.. This affected metabolism of pentachloroethane more markedly than metabolism of hexachloroethane and indicated that probably at least two enzymes were involved in the degradation of hexachloroethane, both of which were present in the liver.

Toxicity of carbon tetrachloride and hexachloroethane.

Metabolism of carbon tetrachloride and hexachloroethane in the liver of mammals may be linked with their hepatotoxicity and a fasciocidal action may be correlated with their metabolic fate in liver flukes. This is possible since it has been shown that mature flukes may be exposed to these drugs in bile of treated sheep and that they can absorb them from aqueous emulsions *in vitro*.

In sheep, hepatotoxic lesions produced by carbon tetrachloride were most severe 24 hr after administration and those produced by hexachloroethane were most severe 48 hr after administration. It is probable that elevation of plasma enzyme activity was a result of increased cell membrane permeability and because GD is a mitochondrial enzyme (27)(28), permeability changes probably also occurred in intracellular membranes. Decreased BSP dye transfer rates from liver to bile after hexachloroethane administration and unchanged transfer rates from plasma to liver cell ~~implicated~~ Suggested a decreased excretory capacity of the liver. This supported the evidence provided by determinations of plasma enzyme activities that hepatic damage had occurred.

Although tests were chosen to show, in particular, hepatotoxic actions of drugs, examination of renal function in sheep showed that disturbances of urinary chloride ion concentration and urinary volumes had occurred after carbon

tetrachloride administration. Cell damage in the kidney may also have contributed to an increase in plasma GD and SD activity, but could not have given rise to the increased OCT activity. pH, specific gravity and phenolsulphonephthalein clearance in urine were unaffected by administration of therapeutic doses of carbon tetrachloride to sheep and renal damage was therefore considered slight. This is in contrast to the severe kidney lesions observed in sheep which received large doses (111) and in man after carbon tetrachloride administration (46)(85).

In sheep, it was found that carbon tetrachloride and pentachloroethane were markedly hepatotoxic and hexachloroethane was somewhat hepatotoxic. Although hepatotoxicity due to hexachloroethane had not prieviously been reported, toxicity of carbon tetrachloride for dogs (43)(78), sheep (4) and toxicity of pentachloroethane for sheep (25), dogs (61) and rabbits (6) had been well substantiated.

The high tolerance of birds to carbon tetrachloride (Hall and Shillinger, 1923) was of interest since it was correlated with the lack of metabolism of the drug in cockerels and ducks.

The plasma activity of tissue enzymes had not been widely used in the study of drug-induced toxic reactions in birds and so extractable GOT, GD, SD and OCT activity of several

normal tissues was determined. Although the distribution of enzyme activity was considerably different from that found in analogous mammalian tissues, the GOT, GD and SD activity detected in avian liver and kidney suggested that these tissues might have liberated enzyme into the plasma if cell membrane damage had occurred.

In fact, avian plasma GD and SD activity were almost unaffected by the administration of carbon tetrachloride or hexachloroethane although plasma GOT activity did rise significantly ($p = 0.01$). Since GOT activity was widely distributed in avian tissues elevation of plasma activity was inconclusive of hepatic damage and small increases in plasma GOT activity, such as were observed, could have been accounted for by mild cell damage in one or several tissues. BSP dye transfer rates were unaffected 48 hr after administration of carbon tetrachloride to cockerels or ducks and this, together with the observations on plasma enzymes , supported the view that hepatotoxicity was not a sequel to chlorinated hydrocarbon administration in these species.

There is no doubt that carbon tetrachloride and hexachloroethane are fasciocidal *in vivo*. However, *in vitro*, it has not been easy to show that carbon tetrachloride exerts a direct action on liver flukes. Thus Stephenson (1947) found no action and Chance and Mansour (1949) showed that high concentrations were required to modify normal fluke motility; the drug was

found to be toxic to flukes in the presence of liver slices (70). In the present experiments, these reports were largely confirmed and emulsions of carbon tetrachloride and hexachloroethane were not toxic to liver flukes *in vitro*. However, addition of 25 per cent sheep bile led to high mortality of flukes incubated with carbon tetrachloride emulsions and was associated with metabolism of the drug in flukes. It seemed likely that the composition of drug emulsions is most critical when considering the action of drugs on helminths *in vitro* (Baldwin,1943).

The mechanism of a toxic action.

It has been proposed that when metabolism of carbon tetrachloride has taken place, the drug may assume a transient 'active configuration' such as a free radical (15)(112). Such radicals may be expected to have severe disruptive effects on biological systems (2).

Metabolism of carbon tetrachloride in sheep, rabbits and liver flukes, but not in birds, has been established and the 'activation hypothesis' may be tested by application of it to known facts. If free radicals arise during metabolism of carbon tetrachloride, they might be expected to react with hydrogen (reduction); to react with unsaturated compounds (for example: addition to unsaturated lipids (Gordis,1969)); or to react with each other (dimerisation: a common termination of free radical life (Albert,1968)).

If carbon tetrachloride underwent homolytic cleavage to trichloromethyl radicals and monatomic chlorine, as suggested by Butler (1961), reaction of trichloromethyl radicals with hydrogen would yield chloroform. Chloroform was found as a metabolite of the drug in sheep, rabbits and liver flukes. Furthermore, reaction of such radicals with unsaturated fatty acids of triglycerides and phospholipids may result in structural damage to cellular membranes which in turn may allow escape of tissue enzymes. An increase in plasma activity of GOT, GD, SD and OCT was observed in sheep following administration of carbon tetrachloride. Finally, if carbon tetrachloride underwent homolytic cleavage to trichloromethyl radicals such radicals might be expected to react together or 'dimerise'. The dimer of trichloromethyl radicals would be hexachloroethane. Hexachloroethane was detected in tissues of rabbits which received carbon tetrachloride and in liver flukes which were incubated in emulsions of the drug with sheep bile.

Thus these results provide evidence in support of the 'activation theory' as an explanation of the toxic action of carbon tetrachloride in mammals and in liver flukes.

From the results of <u>in-vitro</u> studies on liver flukes three hypotheses may be advanced to account for the fasciocidal action of carbon tetrachloride.

The first and second are based on the suggestion of

Alexander and Macdonald (1960) that 'the anthelmintic action of carbon tetrachloride on liver fluke was due to the liver damage produced by the drug and not to a direct effect on the fluke in the bile duct'. A third hypothesis involving a direct action of carbon tetrachloride is also presented.

Direct and indirect mechanisms for a fasciocidal action of carbon tetrachloride are not mutually exclusive. They may provide an additive effect which amounts to a useful therapeutic response.

(i) An indirect action through release of products of liver damage.

The anthelmintic action of carbon tetrachloride on liver flukes may be due to liver damage produced by the drug and not through a direct action on the flukes in the bile duct (4). This hypothesis was further developed by Khalidi and Zaki (1969) who suggested that damaged liver cells may release products lethal to liver flukes. Such products might occur in the aqueous or lipid fraction of the bile. In support of this hypothesis, ether extractable material from carbon tetrachloride treated rabbits' livers was toxic to liver flukes in vitro.

Toxic compounds could arise from the interaction of carbon tetrachloride with liver lipids. Trichloromethylated oleate (Gordis, 1969) was also toxic to liver flukes in vitro and

may resemble the lipids excreted after carbon tetrachloride administration. Carbon tetrachloride or its metabolites have been detected in the lipid fraction of rats' bile; the aqueous fraction contained ^{36}Cl as chloride ion and ^{14}C as organic anions (or possibly bicarbonate: Gordis, personal communication). The bile of sheep contains phospholipid and about 8 to 15 g reach the intestine daily; the associated fatty acids include unsaturated fatty acids such as oleic (30 per cent)(1)(56) and may be susceptible to attack by carbon tetrachloride. However, bile from sheep which had received carbon tetrachloride was not toxic to liver flukes in vitro; it is possible that a toxic agent was volatile or unstable or that it may have been lost during freezing, storage or thawing of bile. Furthermore, alteration of physical characteristics of bile, for example, micelle ~~structure~~ stability, could alter toxic properties of the bile. In-vitro results may not be relevant to the in-vivo situation.

It seems that liver damage alone is unlikely to account for the fasciocidal activity of the halogenated aliphatic hydrocarbons as a group since the tetrachlorodifluoroethanes and bistrichloromethylbenzene had little hepatotoxic activity.

(ii) An indirect action through changing the environment.

The observed reduction in bile flow after carbon tetrachloride administration may have the effect of lowering amounts of substrates available to flukes or allowing

excretory products to reach toxic concentrations. Moreover, the pH of the bile increased in these experiments and may contribute to a fasciocidal action. The liver fluke is not a strict anaerobe (Thorsell, 1963) and a high pCO_2 due to biliary stasis may prove toxic. Biliary flow rates found in the non-medicated sheep were comparable with those reported by Mortimer and Stanbridge (83)(with enterohepatic circulation maintained) but were much greater than those reported by Khalidi and Zaki (1969).

(iii) A direct fasciocidal action of carbon tetrachloride.

The liver fluke in vitro was able to metabolise carbon tetrachloride in the presence of bile and this was correlated with a lethal action of the drug. Hexachloroethane, which may arise by dimerisation of free trichloromethyl radicals, was detected in extracts of liver flukes and may provide evidence of activation of carbon tetrachloride. Activation of the drug within fluke tissues may provide a direct fasciocidal mechanism ; hexachloroethane, although also metabolised by flukes, was not markedly fasciocidal in vitro and it seems unlikely that the fasciocidal action of carbon tetrachloride can be attributed to synthesis of this substance by the flukes.

Since it has been established that carbon tetrachloride is present in bile for at least 6 hr following dosage it seems likely that adult flukes have direct access to the drug, although in much lower concentrations than those reported lethal

to liver flukes *in vitro* (Chance and Mansour,1949).Mature flukes *in vivo* ingest blood and tissue cells (122)(26) and these may provide a further source of carbon tetrachloride.

Immature *F. hepatica* are resistant during the 4 to 6 post-invasive weeks in sheep (8) and during this time they are undergoing maturation in the liver tissue. If metabolism of carbon tetrachloride contributes to its fasciocidal action resistance of such flukes may be attributed to a reduced capacity to metabolise or activate the drug; they are deficient in certain enzymes. Glutamate dehydrogenase and alkaline phosphatase, which are present in mature liver flukes,are less active in immature flukes (120) and may reflect a similar situation in drug-metabolising enzymes (comparable to the enzyme status of new-born rats (95)).

In conclusion, although a direct fasciocidal action of carbon tetrachloride has been discounted by other workers (Alexander and Macdonald,1960; Khalidi and Zaki,1969) there are several attractive features of the 'direct action' hypothesis and the extent of correlation with the situation in mammals is thought to be of interest.

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Some hepatotoxic actions of hexachloroethane and its metabolites in sheep

J. S. L. FOWLER

Department of Veterinary Pharmacology, Royal (Dick) School of Veterinary Studies, Edinburgh 9

1. Pentachloroethane and tetrachloroethylene were major metabolites of hexachloroethane in sheep.

2. Concentrations of hexachloroethane, pentachloroethane and tetrachloroethylene were determined by gas-liquid chromatography in blood, bile, faeces, urine and tissues after oral administration of hexachloroethane emulsions to sheep.

3. Increased blood concentrations of sorbitol dehydrogenase, glutamate dehydrogenase, and ornithine carbamoyl transferase were found to follow oral administration of hexachloroethane or pentachloroethane.

4. The rate of bromsulphthalein transfer from liver cells to bile was found to decrease after oral administration of hexachloroethane.

Hexachloroethane has been widely used in cattle and sheep as an anthelmintic (Thienal, 1926 ; Olsen, 1944 ; Olsen, 1946). It is effective against mature liver flukes *Fasciola hepatica* which are found in the bile ducts of sheep and cattle, but has little activity against immature flukes migrating through the liver parenchyma.

Hexachloroethane is a volatile, fat soluble, solid. It is given orally in the form of a suspension or bolus (Olsen, 1946 ; Olsen, 1947) ; parenteral administration has not been favoured because it leads to carcase taint in treated animals.

Large numbers of animals were treated without reports of marked toxicity until Olsen published an account of poisoning in sheep (Olsen, 1946). Adverse reactions have included intoxication, inco-ordination, muscle tremors and death (Olsen, 1947 ; Southcott, 1951 ; and Byewater, 1955). As with carbon tetrachloride, the toxicity of hexachloroethane is enhanced by a high protein diet and withdrawal of such feed-stuffs at the time of dosage has been advocated (Gibson, 1962). The present work on hexachloroethane comprised an investigation of the metabolism and excretion of the drug, and its hepatotoxicity. Previously hexachloroethane metabolism has been studied by ^{14}C-labelling techniques (Jondorf, Parke & Williams, 1957) and *in vitro*, by the action of tissue homogenates with particular reference to sulphydryl group conjugation (Bray, Thorpe & Vallance, 1952).

Methods

Experimental animals

Scottish Blackface (1, 2, 3, 4, 27, 28) or Cheviot cross (5, 6, 7, 8, 9, 10, 11, 12, 17, 18, 19, 20, 25, 26) male (6, 8) or castrated male (others) sheep weighing 15–23 kg each were used. Animals were housed with hay and water available *ad lib.*

Administration of drugs

Hexachloroethane was dissolved in olive oil (15% w/v) then emulsified with water using gum acacia (12.5 %w/v) and gum tragacanth (0.7% w/v) as emulsifying agents to produce a final volume of about 300 ml. This was orally administered by drenching bottle.

Pentachloroethane and tetrachloroethylene were dissolved in olive oil (33% v/v) and administered orally by a syringe with a four inch tubular extension.

Dosage rates: Hexachloroethane, 0.5 g/kg, sheep 1, 2, 3, 4, 5, 6, 11, 12, 27, 28. Hexachloroethane, 0.75 g/kg, sheep 7. Hexachloroethane, 1.0 g/kg, sheep 8. Pentachloroethane, 0.3 ml/kg, sheep 19, 25. Tetrachloroethylene, 0.3 ml/kg, sheep 20, 26. Olive oil (control), 0.6 ml/kg, sheep 9, 10, 17, 18.

Sample collection

1. Blood samples

Blood samples (5 ml.) were taken from the jugular vein with evacuated glass tubes containing sodium heparin as anticoagulant. Plasma and erythrocytes were separated by centrifugation for 20 min at RCF 1600. Plasma was used immediately for the various analyses, or stored frozen at $-20°$ C for subsequent use. Erythrocytes were washed three times with 0.15 M sodium chloride solution before use or storage.

2. Urine and faeces samples

Urine and faeces samples were collected from sheep 11 and 12 while they were confined in metabolism cages. Hay and water were available *ad lib.* throughout the collection period of 4 days. Faeces were collected on polyethylene sheeting and recovered at 24, 48, 72 and 96 hr for examination. Urine was collected by the method of Warwick (1966) into a receiver maintained at low temperature by ice/water jacket.

3. Bile and tissue samples

Sheep 27 and 28 were fasted for 24 hr and then anaesthetized with pentobarbitone sodium. The hepatic duct was cannulated with 4 mm (outside diameter) polyvinyl tubing and the cystic duct ligated. The common duct was also ligated and the duodenum occluded above and below the sphincter of Oddi with bowel clamps to avoid any chance of samples becoming directly contaminated with hexachloroethane. After collection of a sample of bile, hexachloroethane solution (15% w/v in olive oil) was injected at a dose of 0.5 g/kg directly into the rumen and lower duodenum (divided dose). Bile was collected continuously and 2 ml. was retained each 30 min for analysis ; any remaining bile was returned to the lower duodenum after recording its volume.

The preparation was artificially respired with a mixture of 95% oxygen and 5% carbon dioxide for 1 min every 30 min to supplement normal respiration and to reduce any tendency to hypoxia. Anaesthesia was maintained by intravenous infusion of pentobarbitone sodium and the experiments were terminated 8.5 hr after administration of hexachloroethane.

Samples (3–4 g) of the following tissues were taken within 10 min of death and stored frozen at −20° C until required: brain; liver (diaphragmatic and visceral surfaces); kidney (cortex and pelvis); muscle (masseter, peroneus tertius, infraspinatus); perinephric, subcutaneous and inguinal fat.

4. Storage of biological samples

Urine and faeces samples were used as procured. Aliquots of plasma, erythrocytes, bile or tissues were stored frozen in glass tubes closely covered with metal foil secured by an elastic ring. Before use, bile, blood and erythrocyte samples were stood at room temperature until liquid; tissue samples were ground from the frozen state.

Study of the metabolism of hexachloroethane

1. Hexane extraction of biological samples

Samples (1 ml.) of bile or plasma, or weighed samples of erythrocytes, were extracted in closed tubes with hexane (2 ml.) containing hexachlorobut-1, 3-diene as internal standard. 2.5 M ammonium sulphate solution (1 ml.) was included to reduce the extent of emulsion formation, and saponin (2 ml. 0.01% w/v solution) was added to erythrocyte extraction tubes to aid haemolysis. The tubes were tightly covered with metal foil, shaken for 40 min and centrifuged to break down any emulsion. Rapid cooling to −20° C followed, which enabled the hexane to be decanted from the frozen aqueous portion. The hexane extracts were examined within 24 hr by gas-liquid chromatography. When prolonged storage was required, samples were sealed into ampoules.

Urine samples were extracted by a similar technique. 100 ml. of urine was extracted by a single partition with 20 ml. of hexane which was then washed to remove interfering substances. (Successive washings with equal volumes of water, 1 N sodium hydroxide, 1 N hydrochloric acid and finally water, followed by drying with anhydrous sodium sulphate.) Faecal samples were macerated under warm hexane, and washed in a similar manner.

These methods reduced losses by volatilization and recovery rates (determined by addition of standard solutions to control samples) were greater than 90%.

Tissue samples were weighed and ground with silver sand using a glass pestle-and-mortar. Extraction was by three partitions with hexane containing internal standard. In order to avoid contamination of the columns used in the gas-liquid chromatographic analysis, the fat content of samples was reduced by chilling (3 hr at −20° C) and a Pyrex glass trap was incorporated in the injector port of the gas chromatograph.

2. Analysis 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 hexane extracts were injected with a Hamilton syringe.

Retention times of extracted materials were compared on three columns with those due to reference solutions of hexachloroethane, pentachloroethane and tetrachloroethylene. Retention times for 1,2-dichloroethane and 1,1,2,2-tetrachloroethane were also determined. Aliquots of compounds administered to sheep, of extracts of samples from sheep and of solvents used in making extractions were analysed on the following columns:

Column 1. 2.12m × 3 mm stainless steel tubing packed with acid-washed Celite 60/72 mesh, coated with 5% (w/w) SE-30 grease.

Column 2. 2.00 m × 3 mm stainless steel tubing packed with firebrick, coated with 1.5% (w/w) SE-30 grease and 2% (w/w) polyethylene glycol 20 M.

Column 3. 1.25 m × 3 mm stainless steel tubing packed with Chromosorb 'G' 60/80 mesh, coated with 3% (w/w) dinonylphthalate.

3. *Interpretation of gas-liquid chromatographic results*

In the gas-liquid chromatographic analysis of extracts of samples from sheep which received hexachloroethane 0.5 g/kg. two unidentified peaks (referred to as "X" and "Y") were detected in hexane extracts of blood, bile, urine and faeces. The "X" and "Y" peaks were identical on three columns and in all conditions tested with peaks produced by standard solutions of tetrachloroethylene and pentachloroethane in hexane. The evidence afforded by the retention times of the unknown compounds "X" and "Y" present in the extracts was taken as circumstantial evidence for identifying these as the same as the reference compounds tetrachloroethylene and pentachloroethane. This is in accordance with present criteria of peak identification in gas-liquid chromatography (Perry, 1967).

The areas of peaks "X" and "Y" were calculated by triangulation and their ratio to the internal standard peak area computed. The concentrations of "X" and "Y" were estimated by comparison of these peak ratios with ratios due to several concentrations of tetrachloroethylene and pentachloroethane dissolved in hexane containing hexachlorobut-1, 3-diene as internal standard.

Samples were diluted where necessary to obtain a linear response from the electron capture detector (utilizing less than 30% of the available standing current). Standard mixtures containing reference compounds in similar concentrations to the compounds in extracts were analysed frequently during analysis of samples from sheep experiments.

4. In vitro *metabolism of hexachloroethane*

(a) 1–1.5 mm slices of fresh liver in olive oil emulsion (10 ml.); (b) the same as (a) with hexachloroethane (18 mg or 52 mg/l.); (c) as (b) but liver slices previously boiled (5 min at 100° C); (d) as (b) but liver slices previously heated (5 min at 70° C); (e) olive oil emulsion (10 ml.) with hexachloroethane, tissue omitted. The emulsion contained olive oil (3 ml.); powdered acacia (1 g) per l. of Hedon-Fleig solution (0.1% glucose). Hexachloroethane was dissolved in the olive oil before emulsification.

The liver slices were incubated at 37° C for 4 hr in a water bath with continuous agitation, before hexane extraction. Hexane extracts were examined by gas-liquid chromatography.

Reagents

Analytical grade reagents were used where available. Commercial hexachloroethane was purified by sublimation; technical grade pentachloroethane and tetrachloroethylene by two fractional glass distillations.

Fractions distilling at 158° C (uncorr.) (pentachloroethane) and 121° C (uncorr.) (tetrachloroethylene) were collected. After purification, hexachloroethane and tetrachloroethylene each produced a single response from the gas chromatograph; however, pentachloroethane was still contaminated with tetrachloroethylene at a level of 7.6 p.p.m.

Hexane fraction of petroleum b.p. 67°–70° C was purified in 200 ml. aliquots as follows: successive washes with concentrated sulphuric acid (20 ml.) (specific gravity 1.84); water (100 ml., twice) sodium bicarbonate solution (100 ml.) (1.1 M); water (100 ml., twice), and finally dried with anhydrous sodium sulphate (50 g).

Olive oil was B.P. grade, hexane extracts of which gave no response when analysed by gas-liquid chromatography.

Study of the hepatotoxicity of hexachloroethane

1. Plasma enzymes

Glutamate dehydrogenase E.C.1.4.1.3 (GD) was determined by the method of Ford & Boyd (1962); sorbitol dehydrogenase E.C.1.1.1.14 (SD) by the method of Ford (1967) and ornithine carbamoyl transferase E.C.2.1.3.3. (OCT) by the micromethod of Moore (1967).

Aspartate aminotransferase E.C.2.6.1.1 (GOT) was determined colorimetrically by the method of Reitman & Frankel (1957), but with modifications as suggested by Wootton (1964).

Plasma enzyme concentrations were expressed in International Units (i.u.) or, in the case of OCT, in m-i.u.

Fresh or recently unfrozen plasma was used throughout for enzyme determinations; activities of the above enzymes were stable for at least a week at −20° C (storage temperature).

2. Bromsulphthalein dye clearance tests

Bromsulphthalein (BSP) dye clearance tests were carried out on sheep 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10. BSP sodium solution (50 mg/ml. distilled water) was injected intravenously at a dose rate of 5 mg/kg. Six blood samples were collected at accurately timed intervals between 2 and 30 min after injection of BSP. Optical density (O.D.) was determined at 575 nm after dilution of 1 ml. aliquots of plasma. The control reading was obtained by dilution of plasma with 2 ml. of 0.05 N hydrochloric acid; the test reading by dilution of plasma with 2 ml. of 0.5 N ammonium hydroxide. The concentration of BSP in each sample of plasma was calculated by reference to a standard curve relating O.D. at 575 nm to concentration of several standard solutions of BSP. A water blank and a standard solution of BSP were read with each diluted plasma sample.

In the sheep a dose of BSP 5 mg/kg disappears in a biphasic manner as described by Cornelius, Holm & Jasper (1958); this allows calculation to be made of two

transfer rates for BSP as described by Richards, Tindall & Young (1959). Resolution of the plasma disappearance curve into two components was by the graphical " trial-and-error " method described by Clarkson & Richards (1967).

3. Assessment of hepatotoxicity

Plasma enzyme concentrations were determined daily until stable. After drug administration, plasma enzymes were estimated at least daily until predosage concentrations were present ; this was usually for 7 days. A BSP dye clearance test was conducted 1 hr before and 72 hr after drug administration. BSP transfer rates were calculated and results were expressed as " percentage change in BSP transfer rate from liver to bile," any reduction at 72 hr being attributed to hepatic dysfunction. BSP clearance tests were discontinued when it became apparent that simultaneous increase of concentrations of OCT, GD, SD, and GOT accompany hepatic damage in the sheep, thus confirming the findings of Ford (1967).

Results

1. Clinical response to drug administration

Hexachloroethane (0.5–1.0 g/kg), was well tolerated, animals continuing to eat after administration. Slight tremors of the facial muscles occurred in three sheep between 1 and 4 hr after dosage.

Pentachloroethane (0.3 ml./kg) produced narcosis ; after 30 min sheep 19 became recumbent and only regained normal posture 9 hr after dosage. During this period, the limbs were flaccid and normal reflexes depressed ; defaecation and urination did not occur but labial tremors were noticed. The heart rate was slightly increased. Twenty-four hours after dosage sheep 19 was taking some hay and 72 hr after dosage appeared normal. Sheep 25 became recumbent 20 min after dosage and remained so for 40 min. Labial tremors were noted during this period. Feeding recommenced 1.5 hr after pentachloroethane and the animal appeared normal.

Tetrachloroethylene (0.3 ml./kg) and olive oil (0.6 ml./kg) produced no adverse reaction.

2. Metabolism and excretion of hexachloroethane

Blood concentrations of hexachloroethane. Blood concentrations in unanaesthetized sheep were in every case maximal at the 24 hr sample (Fig. 1) and approximately 100 times greater than in anaesthetized sheep, where the maximum concentrations occurred 6 hr after administration.

Concentrations of hexachloroethane, pentachloroethane and tetrachloroethylene in the plasma of sheep 5 and 6 24 hr after hexachloroethane administration were 2.3–2.6 times greater than the corresponding concentrations in erythrocytes.

Urine and faeces concentrations of hexachloroethane. More than 80% of the total faecal hexachloroethane (1–2 mg) was excreted in the 24 hr following drug administration and little hexachloroethane was detected in the urine (Table 2).

Bile and tissue concentrations of hexachloroethane. Hexachloroethane was first detected in bile 15 min after administration, but was not detected in venous blood

until after the 27th minute (sheep 28). The maximum concentrations were 8–10 times greater in bile than in blood (Table 3). Fat concentrations were highest and muscle lowest (sheep 27, Table 3). Muscle and fat from different sites did not show significant variation in hexachloroethane concentrations.

Metabolites of hexachloroethane. Two metabolites were detected in sheep which had received hexachloroethane and shown to be tetrachloroethylene and pentachloroethane.

Pentachloroethane was detected in venous blood 24 hr after hexachloroethane administration and was still present after 96 hr (Table 1, Fig. 1). The urine content was relatively high (Table 2); however, in anaesthetized sheep, blood, bile and tissue levels were very low (Table 3).

TABLE 1. *Blood concentrations of hexachloroethane (HCE) pentachloroethane (PCE) and tetrachloroethylene (TCE) as μg/ml. whole blood and change in the excretory capacity of the liver following administration of hexachloroethane*

Sheep No.	HCE dose g/kg	24 hr blood conc. HCE	TCE	PCE	72 hr BSP transfer rate (as % of 0 hr. transfer rate)
1	0·5	27	0·6	0·15	25
2	0·5	—	—	—	12
3	0·5	28	0·7	0·30	66
4	0·5	10	0·8	0·05	47
5	0·5	27	1·0	0·50	79
6	0·5	23	1·1	0·15	35
7	0·75	—	—	—	8
8	1·0	—	—	—	36
9	Control	—	—	—	94
10	Control	Nil	Nil	Nil	105

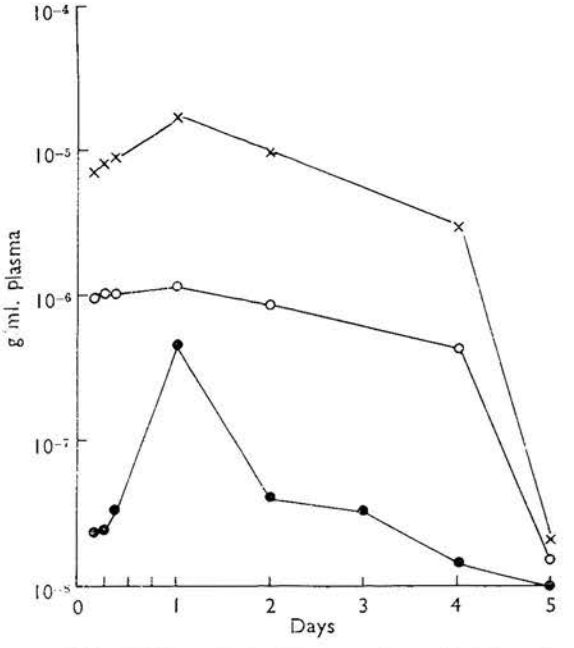

FIG. 1. Concentrations of hexachloroethane (×----×), pentachloroethane (●---●) and tetrachloroethylene (○---○) in venous blood samples of sheep 5 which received an oral dose of hexachloroethane 0.5 g/kg at day 0.

Sheep 19 and 25, which received pentachloroethane 0.3 ml./kg showed blood concentrations of pentachloroethane of the same order as the concentrations of hexachloroethane following administration of hexachloroethane. The second metabolite of hexachloroethane, tetrachloroethylene, was also present. This was the only metabolite of pentachloroethane detected (Fig. 2).

Tetrachloroethylene was always found in the blood of sheep given hexachloroethane and the maximum concentration was usually reached at 24 hr (Table 1, Fig. 1). After pentachloroethane, blood concentrations of tetrachloroethylene reached the maximum at 3 hr and remained at similar concentrations to pentachloroethane for at least 3 days (Fig. 2).

TABLE 2. *Total (μg) hexachloroethane (HCE), pentachloroethane (PCE) and tetrachloroethylene (TCE) in the urine and faeces of two sheep that received hexachloroethane*

	Sheep 11			Sheep 12		
Faeces	HCE	TCE	PCE	HCE	TCE	PCE
0–24 hr	780	854	Trace	1260	1300	468
24–48 hr	Trace	22	Trace	280	440	Trace
48–72 hr	Trace	Trace	Trace	Trace	15	Trace
72–96 hr	Nil	Trace	Nil	Trace	Trace	Trace
	Sheep 11			Sheep 12		
Urine	HCE	TCE	PCE	HCE	TCE	PCE
0–24 hr	50	25	20	70	29	25
24–48 hr	4·4	8·8	1·3	10·8	8·9	1·0
48–72 hr	0·4	6·5	0·4	Trace	5·4	0·5
72–96 hr	Nil	Nil	Nil	Trace	5·4	Trace

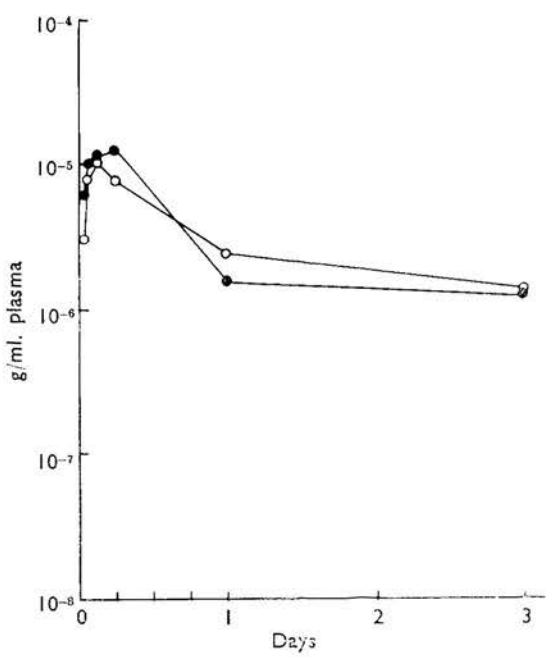

FIG. 2. Concentrations of pentachloroethane (●- - -●) and tetrachloroethylene (○- - -○) in venous blood samples of sheep 19 which received an oral dose of pentachloroethane 0.3 ml./kg at day 0.

Concentrations of tetrachloroethylene were high in the urine and faeces and could be detected when hexachloroethane was absent (Table 2); they were also high in the anaesthetized sheep whilst hexachloroethane concentrations were low, indicating that although absorption was slower, metabolism was not as markedly affected by anaesthetics (Table 3). Brain and muscle contained least of all the tissues, whereas liver showed the highest content in sheep 28 and fat in sheep 27.

Blood from sheep which had received tetrachloroethylene 0.3 ml./kg showed only one peak on gas-liquid chromatography, that due to tetrachloroethylene.

Other metabolites of hexachloroethane. These are presumed to be water soluble, and were not detected in hexane extracts. There were no unidentified peaks, and none corresponding to those produced by reference solutions of 1,2-dichloroethane or 1,1,2,2-tetrachloroethane, the most toxic of the series of chlorinated ethanes (Williams, 1959).

Controls. Blood samples from sheep which received olive oil 0.6 ml./kg produced no response on gas-liquid chromatography.

In vitro *experiments.* Fresh liver slices liberated pentachloroethane and tetrachloroethylene from hexachloroethane emulsions over a period of 4 hr at 37° C (Table 4).

Boiled liver slices took up hexachloroethane but pentachloroethane and tetrachloroethylene were not detected (Table 4).

TABLE 3. *Concentrations (μg/g) of hexachloroethane (HCE), pentachloroethane (PCE) and tetrachloroethylene (TCE) in two anaesthetized sheep which received hexachloroethane. Post-mortem tissue samples (8·5 hr)*

	Sheep 27			Sheep 28		
Tissue	HCE	TCE	PCE	HCE	TCE	PCE
Bile (4 hr)	1·7	0·3	Trace	2·2	0·5	Nil
Blood (6 hr)	0·2	0·4	Trace	0·2	0·2	Nil
Brain	0·2	0·9	0·02	Trace	Trace	Trace
Fat	1·1	2·1	0·02	Trace	0·6	Nil
Kidney	0·1	1·2	Trace	Trace	0·6	Trace
Liver	0·2	0·9	0·01	Trace	2·8	Trace
Muscle	0·04	0·5	0·01	Trace	Trace	Trace

TABLE 4. *Liberation of tetrachloroethylene (TCE) and pentachloroethane (PCE) from hexachloroethane (HCE) by sheep liver as fresh, heated (70° C for 5 min) or boiled (100° C for 5 min) slices incubated at 37° C for 4 hr*

Tissue	No. of expts	HCE μg/g (±S.D.)	TCE μg/g (±S.D.)	PCE μg/g (±S.D.)
(1) Olive oil emulsified in Hedon–Fleig solution (0·1% glucose)				
Fresh	10	Nil	Nil	Nil
Boiled	10	Nil	Nil	Nil
(2) Emulsion+hexachloroethane, 18 μg/ml.				
Fresh	10	13·3±0·5	9·1±0·1	0·76±0·01
Heated	10	50·8±0·1	2·4±0·8	1·74±0·66
Boiled	10	58·4±0·9	Nil	Nil
None	4	15·9±0·8	Nil	Nil
(3) Emulsion+hexachloroethane, 54 μg/g				
Fresh	10	56·4±3·0	56·4±8·6	0·95±0·35
Heated	16	20·2±1·5	0·36±0·01	0·12±0·01
None	4	50·3±1·5	Nil	Nil

Heated liver slices liberated pentachloroethane and tetrachloroethylene from hexachloroethane but considerably less tetrachloroethylene was detected (Table 4).

3. *Hepatotoxicity of hexachloroethane and its metabolites*

Plasma enzyme determinations. Hexachloroethane administration was followed by simultaneous increases in the concentrations of GD, SD and OCT, reaching a maximum at 48 hr and returning to normal 4–5 days later (Fig. 3). GOT concentrations increased slightly and were maximal at 24–48 hr (sheep 2, 4 and 5) or 48–72 hr (sheep 3, 6, 7 and 8); no increase occurred in sheep 1.

GD was increased fifty-five times in sheep 1, but a typical increase was three to six times (sheep 3, 5, 6, 7 and 8). SD always increased by three to six times. OCT increased from two to ten times after hexachloroethane. Administration of pentachloroethane resulted in large increases in the plasma concentrations of GD, SD and OCT; GD increased 200 times in both sheep; SD increased 30 fold (sheep 19) and 20 fold (sheep 25). OCT showed a 15-fold increase in sheep 19 and a 19-fold increase in sheep 25.

Tetrachloroethylene administration (two sheep) caused some plasma enzyme increases. OCT did not change in either sheep; GD increased eight-times in one and three times in the other (48 hr maxima); SD increased two fold (sheep 20, 24 hr maximum; sheep 26, 48 hr maximum).

BSP dye clearance tests. Plasma disappearance of BSP (5 mg/kg) occurred in two phases as described by Cornelius *et al.* (1958). Transfer rates for BSP showed that hexachloroethane did not affect uptake from plasma by liver cells, but that

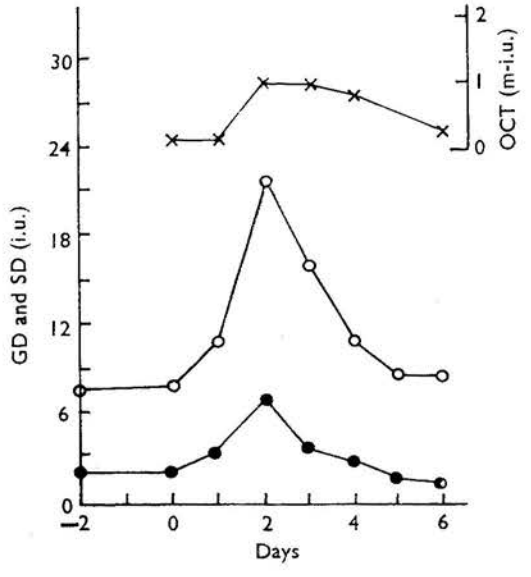

FIG. 3. Concentrations of GD (●- - -●) and SD (○- - -○) expressed in i.u. and of OCT (×- - - -×) m-i.u. in plasma of sheep 6, which received an oral dose of hexachloroethane 0.5 g/kg at day 0.

72 hr after administration a marked reduction of transfer from liver cells to bile occurred. BSP dye clearance tests conducted on sheep 9 and 10, which received olive oil, showed little variation within the 72 hr test period (Table 1).

Discussion

Metabolism of hexachloroethane

The metabolism of hexachloroethane in the rabbit was studied by Jondorf *et al.*, (1957), using ^{14}C-labelled drug together with isotopic dilution techniques. Gas-liquid chromatography with electron capture detection provided a sensitive and easily quantitated method for separation and detection of hexachloroethane and its hexane soluble metabolites in sheep.

The absorption of orally administered hexachloroethane as reflected by jugular venous blood concentrations of the drug was slow, especially in anaesthetized sheep ; pentobarbitone seemed to depress intestinal motility ; also release of bile and pancreatic lipase were interrupted by the duct cannulation and duodenal clamping. Despite these limitations, three important points were demonstrated by the anaesthetized preparations: firstly, hexachloroethane excretion in the bile was established ; secondly, biliary concentrations may markedly exceed blood concentrations and, finally, hexachloroethane is widely distributed.

Excretion of hexachloroethane in bile allows direct contact with parasites such as the mature liver fluke. Moreover, contact time may be increased by enterohepatic circulation which, if concentrations in bile are greater than those in systemic blood, may maintain portal venous concentrations after systemic venous concentrations have fallen.

The metabolism of various chlorinated hydrocarbons by an enzyme present in the liver, kidney and spleen, was demonstrated by Heppel & Porterfield (1948). Bray *et al.* (1952), however, questioned these findings and described the reactions of certain aliphatic chloro' compounds with the sulphydryl groups of amino-acids, liberating chloride. Boiled tissue extracts were capable of liberating chloride from several compounds including hexachloroethane and pentachloroethane, so enzymatic systems were discounted, in favour of $-SH$ conjugation systems (Bray *et al.*, 1952).

The *in vitro* experiments with liver slices did not confirm the findings of Bray *et al.* (1952) ; although fresh liver slices produced pentachloroethane and tetrachloroethylene from hexachloroethane, boiled liver slices did not, which suggested that an enzymatic process might be involved in the metabolism of the drug. Further support for this was afforded by the partial inactivation which occurred at 70° C ; this affected metabolism of pentachloroethane more markedly than metabolism of hexachloroethane and indicated that probably at least two enzymes were involved in the degradation of hexachloroethane, both of which were present in liver.

The rapid appearance of tetrachloroethylene in the systemic circulation may be contrasted with the apparent lag in the appearance of pentachloroethane, possibly the metabolism of pentachloroethane was rapid in sheep. The removal of H–Cl and introduction of the double bond of tetrachloroethylene is analogous to the metabolism of DDT which yields DDE ; however, this reaction appears to proceed slowly in mammals.

Toxicity of hexachloroethane

It is interesting to note that the metabolism of hexachloroethane, which probably occurred to a large extent in the liver, was closely linked with hepatotoxicity. This is similar to the situation with carbon tetrachloride (Slater, 1966).

The results of plasma enzyme determinations and BSP dye clearance tests indicated that a degree of hepatic dysfunction followed the oral administration of hexachloroethane.

BSP dye clearance tests and plasma enzyme concentrations have been widely used as indices of hepatic function in sheep (Cornelius *et al.*, 1958 ; Alexander & MacDonald, 1960 ; Ford & Boyd, 1962 ; Ford & Lawrence, 1965 ; Ford, 1967). The elevations of plasma concentrations of GD, SD, OCT and GOT which followed hexachloroethane administration were greatest at 48 hr ; this compares with 24 hr for carbon tetrachloride and 144 hr for sporidesmin (Ford, 1967).

Elevation of plasma enzyme concentrations after drug administration suggested that cell membrane permeability changes occurred and because GD is a mitochondrial enzyme (de Duve, Wattiaux & Baudhuin, 1962), permeability changes probably occurred in intracellular membranes. The decreased BSP dye clearance 72 hr after hexachloroethane administration and unchanged hepatic uptake rates for BSP implicated a decreased excretory capacity of the liver, not merely an increased albumin-BSP binding effect (Crawford & Hooi, 1968), even though it was shown that plasma albumin concentrations fell in sheep which received chlorinated hydrocarbons (Alexander & MacDonald, 1960).

The causes of hepatic dysfunction following hexachloroethane administration are not known, but Alexander & MacDonald (1960) suggested that the anthelmintic actions of carbon tetrachloride might depend on the release of products of liver damage rather than direct action on intrabiliary flukes.

The excretion of hexachloroethane in the bile allows direct contact of the drug with adult liver flukes in addition to contact with products of liver damage. Hexachloroethane and/or pentachloroethane may have a direct action on the liver and liver fluke, or their metabolism may yield active radicals as suspected with carbon tetrachloride (Slater, 1966). The presence of dechlorinated radicals with a high electron affinity such as the $CCl_3^{\bullet}$, which may result from carbon tetrachloride metabolism, would be expected to produce severe, if only very localized damage to the endoplasmic reticulum and possibly to the mitochondria (Albert, 1968). Assuming that the liver fluke also metabolizes hexachloroethane and pentachloroethane, this provides a basis for an hypothesis on the nature of the hepatotoxic and anthelmintic action of hexachloroethane.

Apart from metabolism of these drugs which probably occurs in liver cells, the nervous symptoms and narcosis which followed the administration of hexachloroethane and pentachloroethane indicated that the drugs also entered the cells of the central nervous system. It is not known if metabolism occurred in this tissue ; if the dechlorination of these drugs is related to their toxicity, damage will occur in tissues which have enzyme systems available for the metabolism of hexachloro- and pentachloroethane.

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A new metabolite of carbon tetrachloride

J. S. L. FOWLER (introduced by F. ALEXANDER), *Department of Veterinary Pharmacology, Royal (Dick) School of Veterinary Studies, Edinburgh*

The possibility of trichloromethyl radicals arising from homolytic cleavage of carbon tetrachloride *in vivo* was put forward by Butler (1961), and a role in hepatotoxicity for such radicals has been discussed (Slater, 1966). Gas-liquid chromatography with electron capture detection has provided a sensitive means of detecting $Cl_3C.CCl_3$ (Fowler, 1969). Traces of this dimer, which caused liver damage in sheep (Fowler, 1969) were detected in tissues of rabbits to which carbon tetrachloride had been administered.

Samples of liver, kidney, fat, muscle and bile were taken from rabbits 0, 6, 24 and 48 hr after administration of carbon tetrachloride (1 ml./kg) by stomach tube (20% v/v in olive oil). After extraction by heptane partition, carbon tetrachloride, chloroform and $Cl_3C.CCl_3$ were separated on an SE–30/Celite column. Identification of $Cl_3C.CCl_3$ was supported by comparison of retention times with standards on three columns: SE–30/Celite; SE–30/PEG 20M/firebrick and di(2-ethylhexyl) sebacate/chromosorb G.

Maximum concentrations (with standard deviations) of CCl_4 were in fat (6 hr: 787 ± 289 μg/g); of $CHCl_3$, in liver (6 hr: $4{\cdot}9 \pm 1{\cdot}5$ μg/g); and of the dimer, $Cl_3C.CCl_3$, in fat (24 hr: $16{\cdot}5 \pm 1{\cdot}6$ ng/g).

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Carbon tetrachloride metabolism in the rabbit

J. S. L. FOWLER

Department of Veterinary Pharmacology, Royal (Dick) School of Veterinary Studies, Edinburgh 9

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 ($CCl_3^{\bullet}$). 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 libφ*, 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~~ 1969). 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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CHLORINATED HYDROCARBON TOXICITY IN THE FOWL AND DUCK

By

J. S. L. Fowler

Department of Veterinary Pharmacology, Royal (Dick) School of Veterinary Studies, University of Edinburgh

INTRODUCTION

The resistance of the domestic fowl to carbon tetrachloride poisoning is well documented (Hall and Schillinger, 1923; Slater, 1966). There is evidence that toxicity of carbon tetrachloride to mammals depends on metabolism of the drug (Butler, 1961; Slater, 1966; Garner and MacLean, 1969) and that toxicity of hexachloroethane may be related to blood concentrations of pentachloroethane, an intermediate metabolite toxic to sheep (Fowler, 1969a). The fate of carbon tetrachloride and hexachloroethane was studied by gas-liquid chromatography (GLC) of avian blood at intervals after oral administration of the drugs.

Liver damage in ruminants caused by carbon tetrachloride increased serum activities of aspartate aminotransferase (GOT) (EC 2.6.1.1); glutamate dehydrogenase (GD) (EC 1.4.1.3); iditol dehydrogenase (SD) (EC 1.1.1.14) and ornithine carbamyl transferase (OCT) (EC 2.1.3.3) (Ford, 1967). Hexachloroethane decreased bromsulphthalein (BSP) dye clearance in sheep (Fowler, 1969a).

These parameters were applied to cockerels and ducks which received carbon tetrachloride and hexachloroethane.

METHODS

Experimental animals. White Leghorn cockerels (Nos. 1 to 13) weighing 1·4 to 1·6 kg. were maintained in battery cages (40 cm. × 35 cm. × 35 cm.) on layer's mash (16 per cent. protein) and water ad lib. Khaki Campbell ducklings of both sexes (Nos. 14 to 24) weighing 1·6 to 1·9 kg. were maintained indoors on straw with layer's mash and water available ad lib.

Liver Function Tests

Normal enzyme activity in tissue. Cockerels 6, 7, 8, 9 and ducks 19, 20, 21, 22 were killed by cervical dislocation. Fresh samples of liver, kidney, spleen, cardiac muscle, skeletal muscle, gizzard wall, section of small intestine, brain, lung and pancreas were homogenised with distilled water by 25 strokes of an all-glass hand homogeniser. Extracts (10 per cent.) were centrifuged at RCF 1600 for 20 min. to remove particulate matter and the supernatant fluid was stored at −20°C.

GOT activity was determined on 1 per cent. extracts by the method of Reitman and Frankel (1957), but with modifications suggested by Wootton (1964); GD and SD activity on 1 per cent. or 10 per cent. extracts as described by Ford and Boyd (1962) and Ford (1967); OCT activity by the micro-method of Moore (1967).

Blood sample procedure. Blood samples (2 ml.) were taken from the median vein with a heparinised disposable syringe and 26G needle. Plasma was separated by

centrifugation for 20 min. at RCF 1600 and used immediately or frozen at −20°C. for subsequent use. Plasma enzyme activity was determined by methods described above for tissue enzyme activities.

BSP dye clearance tests. Tests were conducted on cockerels 12, 13 and ducks 23, 24 immediately before and after 48 hr. after carbon tetrachloride administration. BSP sodium solution (10 mg./ml. distilled water) was injected intravenously at a dose of 5 mg. BSP/kg. Six blood samples were taken at accurately timed intervals 2 to 30 min. after injection of BSP. Plasma concentrations were determined as described previously (Fowler, 1969a) using 0·5 ml. aliquots of plasma.

In the fowl and duck a dose of 5 mg. BSP/kg. disappears in a biphasic manner; this allows calculations to be made of two transfer rates for BSP as described by Richards, Tindall and Young (1959). Resolution of the plasma disappearance curve into two components was determined by the graphical "trial-and-error" method of Clarkson and Richards (1967).

Administration of drugs. Carbon tetrachloride was diluted with olive oil (33 per cent. v/v) and administered by a 3 mm. (outer diameter) silicone rubber stomach tube, from an all-glass syringe. Cockerels 1, 2, 3, 4, 5, 12, 13 and ducks 14, 15, 16, 17, 18, 23, 24 each received 6 ml. carbon tetrachloride. Cockerels 10 and 11 received 4 g. hexachloroethane by stomach tube as a warm solution in olive oil (15 per cent. w/v). Cockerel 6 and duck 12 received olive oil (12 ml.) by stomach tube. Cockerels 7, 8, 9 and ducks 20, 21, 22 did not receive drugs.

Blood concentrations of drugs. Concentrations of chlorinated hydrocarbons in blood were determined on an Aerograph Hi-Fi 600-C model gas chromatograph with a concentric tube electron capture detection system. Fresh whole blood samples were 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 suspensions and freeze the aqueous layer. The heptane extract was decanted from the frozen aqueous layer and examined by gas liquid chromatography for carbon tetrachloride (Fowler, 1969b). Samples from birds which received hexachloroethane were extracted by a single hexane partition, as above, and were examined by gas-liquid chromatography for hexachloroethane and metabolites (Fowler, 1969a).

Reagents. Analytical grade reagents were used where available. Carbon tetrachloride B.P.C. (1959) was distilled twice in glass apparatus, collected at 86°C. (uncorr.) and washed with sulphuric acid (SG 1·84) (Fowler, 1969b). Commercial hexachloroethane was purified by sublimation in a current of air. Hexane fraction of petroleum b.p. 67° to 70°C. and n-heptane (British Drug Houses) were washed or redistilled (Fowler, 1969a, b). Olive oil was B.P. grade and direct injection to the gas chromatograph gave no response.

RESULTS

Normal Tissue Enzyme Activity

Distributions of enzyme activity in extracts of avian tissue differed from those found in analogous mammalian tissues (Table 1) (Boyd, 1962; Ford, 1967).

In cockerels, although GOT activity was highest in extracts from liver and kidney, all tissue extracts contained appreciable activity, with the exception of lung in which the activity was low (Table 1). In duck tissue extracts, GOT activity was more evenly distributed throughout the tissues (Table 1). GD activity in cockerel tissue extracts was highest in liver and kidney extracts. There was also some actvity in other tissue extracts, particularly brain (Table 1). Liver and kidney extracts contained almost all the total activity detected in duck tissue extracts (Table 1). SD activity was highest in extracts of cockerel liver and

kidney and was present in several other tissues. In duck tissue SD activity was low (Table 1). OCT activity was not detected in plasma or tissue extracts from cockerels or ducks.

TABLE 1

ENZYME ACTIVITY (IU/LITRE) IN 1 PER CENT. EXTRACTS

Tissues from cockerels (6, 7, 8, 9) and ducks (19, 20, 21, 22)

Cockerel	*GOT ± s. d.*	*GD ± s. d.*	*SD ± s. d.*
Liver	126 ± 42	67 ± 1·6	36 ± 3·3
Kidney	113 ± 2·9	50 ± 11	38 ± 2·5
Spleen	28 ± 8·2	10 ± 3·5	3·6 ± 1·2
Cardiac muscle	93 ± 15	7·2 ± 2·0	2·4 ± 2·0
Skeletal muscle	83 ± 4·4	0·5 ± 0·5	1·5 ± 3·0
Small intestine	42 ± 9·5	4·9 ± 6·1	2·9 ± 3·1
Gizzard	35 ± 15	1·8 ± 1·2	2·6 ± 0·6
Pancreas	82 ± 4·2	7·2 ± 5·9	6·0 ± 3·1
Lung	14 ± 9·1	4·8 ± 3·7	0·0 ± 0·0
Brain	99 ± 8·2	31 ± 5·9	2·2 ± 2·5
Duck			
Liver	95 ± 19	84 ± 7·4	5·5 ± 0·5
Kidney	107 ± 8·7	115 ± 6·9	2·6 ± 0·1
Spleen	38 ± 2·9	4·8 ± 2·2	0·0 ± 0·0
Cardiac muscle	118 ± 28	5·5 ± 1·5	1·1 ± 0·1
Skeletal muscle	119 ± 9·9	1·7 ± 0·7	0·7 ± 0·3
Small intestine	19 ± 6·3	1·2 ± 0·7	0·0 ± 0·0
Gizzard	52 ± 2·7	0·0 ± 0·0	0·4 ± 0·2
Pancreas	64 ± 18	5·8 ± 5·0	2·4 ± 1·1
Lung	26 ± 5·0	1·9 ± 0·5	1·2 ± 0·4
Brain	99 ± 4·1	15 ± 1·7	1·2 ± 0·1

Clinical Signs following Drug Administration

Administration of carbon tetrachloride to cockerels led to light narcosis, anorexia and reduced water intake lasting 48 to 72 hr. Hexachloroethane was also narcotic to cockerels. Carbon tetrachloride produced no adverse reaction when administered to ducks. Olive oil produced no reaction in cockerels or ducks.

Plasma Enzyme Activity

Carbon tetrachloride administration to five cockerels was followed by an increase in plasma activity of GOT reaching a maximum at 24 to 48 hr. and returning to normal at 6 days. GD activity did not increase in the plasma of cockerels after carbon tetrachloride administration. SD activity was not detected in cockerel plasma at any time (Table 2).

Carbon tetrachloride administration to five ducks was followed by simultaneous increases in plasma activities of GD, SD and GOT. Plasma GD and SD activities were greatest at the 72 hr. sample. Plasma GOT activity was greatest at 5 days after carbon tetrachloride administration (Table 3). These figures were significant as shown in the Tables. Cockerels 10 and 11 which received 4 g. hexachloroethane showed increased plasma GOT activity, but no significant changes in plasma GD or SD activities.

TABLE 2

VARIATION OF PLASMA ACTIVITIES

Tissue enzymes (i.u.) and plasma concentrations (μg./ml.) of CCl_4 in cockerels (5) which received carbon tetrachloride (6ml.) at 0 hr. (all ± S.D.)

Time	*GOT*	*GD*	*SD*	*CCl⁴*
−48 hr.	55·6 ± 11·8	3·0 ± 3·5	nil	nil
−24 hr.	54·6 ± 5·0	1·0 ± 2·2	nil	nil
0 hr.	55·8 ± 8·4	1·0 ± 1·0	nil	0·000 ± 0·000
6 hr.	119·4 ± 21·6 (p = < 0·01)	1·8 ± 1·1 (p = < 0·4)	nil	0·590 ± 0·203
24 hr.	145·4 ± 23·1 (p = < 0·01)	0·4 ± 0·9 (p = < 0·5)	nil	1·706 ± 0·695
48 hr.	143·8 ± 36·0 (p = < 0·01)	1·6 ± 0·9 (p = < 0·5)	nil	1·651 ± 0·858
6 day	49·8 ± 13·3 (p = < 0·5)	2·3 ± 1·0 (p = < 0·2)	nil	0·158 ± 0·472
9 day	—	—	—	0·034 ± 0·025

TABLE 3

VARIATION OF PLASMA ACTIVITY

Tissue enzymes (i.u.) and, plasma concentrations (μg./ml.) of CCl_4 in ducks (5) which received carbon tetrachloride (6ml.) at 0 hr (all ± S.D.)

Time	*GOT*	*GD*	*SD*	*CCl_4*
0 hr.	4·3 ± 1·8	2·9 ± 2·0	3·1 ± 2·3	0·000 ± 0·000
24 hr.	—	—	—	1·302 ± 0·292
42 hr.	7·4 ± 9·9 (p = < 0·6)	5·0 ± 5·6 (p = < 0·6)	4·6 ± 2·7 (p = < 0·5)	0·888 ± 0·076
72 hr.	11·2 ± 9·0 (p = < 0·3)	5·3 ± 5·6 (p = < 0·3)	5·3 ± 2·5 (p = < 0·3)	0·672 ± 0·135
5 day	12·4 ± 5·0 (p = < 0·05)	4·3 ± 5·6 (p = < 0·7)	2·4 ± 2·7 (p = < 0·8)	0·206 ± 0.344
9 day	2·4 ± 2·9 (p = < 0·8)	1·4 ± 2·2 (p = < 0·4)	1·4 ± 2·2 (p = < 0·4)	0·009 ± 0·018

TABLE 4

PLASMA CONCENTRATIONS (μG/ML.)

Hexachloroethane (HCE), tetrachloroethylene (TCE) and pentachloroethane (PCE, ng/ml.) in cockerels 10 and 11 which received hexachloroethane (4g.) at 0 hr

	HCE		*TCE*		*PCE*	
Time	*10*	*11*	*10*	*11*	*10*	*11*
0 hr.	nil	nil	nil	nil	nil	nil
24 hr.	1·432	1·380	1·40	2·32	10·1	8·2
42 hr.	1·807	1·847	1·70	2·70	14·6	11·5
72 hr.	1·657	0·735	0·63	0·85	15·2	7·0
5 day	0·064	0·027	0·07	0·04	trace	trace
9 day	0·000	0·000	0·00	0·00	0·0	0·0

Bromsulphthalein Dye Clearance Tests

Plasma disappearance of BSP (5 mg./kg.) occurred in two phases. Cockerels 12 and 13 and ducks 23 and 24 showed no significant change in BSP transfer rates 48 hr. after receiving carbon tetrachloride.

Gas-liquid Chromatography of Plasma Extracts

Plasma concentrations of carbon tetrachloride. Plasma concentrations were maximal at the 24 hr. blood sample and declined to one-tenth of the peak concentration 5 to 6 days after carbon tetrachloride administration (Tables 2 and 3). No metabolites of carbon tetrachloride were detected in plasma extracts from cockerels or ducks.

Plasma concentrations of hexachloroethane. Plasma concentrations were maximal at the 42 hr. blood sample and declined to less than one-tenth of the peak concentration 5 days after hexachloroethane administration.

Metabolites of hexachloroethane. Two metabolites were detected in extracts of plasma from cockerels 10 and 11 which received 4 g. of hexachloroethane and were shown to be pentachloroethane and tetrachloroethylene (Fowler, 1969a), the former being detected in low concentrations and the latter in high concentrations (Table 4).

DISCUSSION

Carbon tetrachloride is hepatotoxic to mammals with the possible exception of newborn animals (Slater, 1966). Toxicity is enhanced by a high protein intake (Gibson, 1962; McLean and McLean, 1966) and by prior administration of phenobarbitone or DDT (Seawright, McLean and Forrest, 1968) all of which stimulate the microsomal hydroxylating enzyme system (McLean and McLean, 1969). Carbon tetrachloride may be activated within the mammalian liver cell by the loss of a chlorine atom leaving a free trichloromethyl radical (Butler, 1961), and such 'free radicals' would have severe disruptive effects on membranous structures within the cell (Judah, 1969). Such radicals have not been demonstrated, although a possible interaction product, the dimer $Cl_3C.CCl_3$, has been identified in liver, fat, kidney and muscle of rabbits which received carbon tetrachloride (Fowler, 1969b).

The apparent resistance of birds to carbon tetrachloride poisoning is of interest and the failure to demonstrate metabolites such as chloroform or $Cl_3C.CCl_3$ supports the hypothesis that "activation" precedes the toxic actions of carbon tetrachloride in the mammal and did not occur in cockerels and ducks which received the drug. Plasma GD and SD activity and BSP dye clearance were little affected by carbon tetrachloride. Plasma GOT activity increased after carbon tetrachloride administration ($p = <0{\cdot}01$), but may not have been evidence for hepatic damage in the absence of a significant rise ($p = \not< 0{\cdot}3$) in plasma GD or SD activity or a change in BSP transfer rates. An increase in plasma GOT activity may equally indicate damage to another tissue, such as skeletal or cardiac muscle, since several tissue extracts contained marked GOT activity.

These results are in contrast to the situation in mammals where carbon tetrachloride causes a marked increase in plasma GOT, GD, SD and OCT activities (Ford, 1967) and is commonly used to produce experimental liver damage. This suggested that carbon tetrachloride is less toxic to birds than to mammals since considerable GOT, GD and SD activity was demonstrated in avian tissues, particularly in liver and kidney; moreover the plasma activities were comparable to those found in the serum and plasma of sheep (Ford, 1967; Fowler, 1969a). Nevertheless, in view of the relative tissue activities of SD, it was surprising to find higher plasma activities of this enzyme in duck than in cockerel plasma in which activity was not detected at any stage.

Although in contrast to mammals there is no evidence of activation of carbon tetrachloride by cockerels or ducks, hexachloroethane was metabolised to pentachloroethane and tetrachloroethylene as demonstrated in sheep (Fowler, 1969a). Concentrations of pentachloroethane in avian blood were much lower than those in sheep which received hexachloroethane. Since pentachloroethane was particularly toxic to sheep, whereas tetrachloroethylene was of low toxicity, it may be possible to relate the rate of accumulation of pentachloroethane, the active metabolite, to the toxicity of the parent compound, hexachloroethane, when this is administered to an animal.

The effects of administration of carbon tetrachloride and hexachloroethane to birds support the hypothesis that the toxicity of these drugs is closely connected with their breakdown in the body.

SUMMARY

Carbon tetrachloride was administered orally to cockerels and ducks. Blood concentrations were determined at intervals.

Metabolites of carbon tetrachloride were not detected in cockerels or ducks. In contrast hexachloroethane was metabolised to pentachloroethane and tetrachloroethylene as occurs in mammals.

Aspartate aminotransferase, iditol dehydrogenase and glutamate dehydrogenase activities were determined in extracts of liver, kidney, spleen, in cardiac, skeletal and gizzard muscle, and in pancreas, small intestine, lung, brain and plasma from cockerels and ducks.

A moderate increase in plasma aspartate aminotransferase activity ($p = < 0{\cdot}01$) occurred after administration of carbon tetrachloride, but was not accompanied by significant increases in plasma activities of other enzymes ($p = \not< 0{\cdot}3$) or by changes in BSP transfer rates.

These findings are consistent with the hypothesis that the toxicity of carbon tetrachloride in the mammal is due to an active metabolite.

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CARBON TETRACHLORIDE METABOLISM IN SHEEP AND IN *FASCIOLA HEPATICA*

BY

J. S. L. FOWLER

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Carbon tetrachloride metabolism in sheep and in *Fasciola hepatica*

J. S. L. FOWLER

Department of Veterinary Pharmacology, University of Edinburgh

Summary

1. The excretion of carbon tetrachloride and its metabolites in bile and urine were studied.

2. Liver flukes *in vitro* metabolized carbon tetrachloride and hexachloroethane by dechlorination.

3. Carbon tetrachloride, liver lipid from rabbits which received carbon tetrachloride and a carbon tetrachloride methyl oleate complex were toxic to liver flukes *in vitro*, in the presence of sheep bile.

4. A direct fasciocidal action of carbon tetrachloride may contribute to the therapeutic effect of the drug.

Introduction

Orally administered carbon tetrachloride and hexachloroethane are effective against mature liver flukes (*Fasciola hepatica*) which reside in the bile ducts of sheep and cattle (Gibson, 1962).

Recent evidence has suggested that metabolism or activation precedes the toxic actions of carbon tetrachloride (Slater, 1966). Direct fasciocidal action could occur if the flukes themselves metabolized the drug. Carbon tetrachloride is well tolerated by newborn rats (Cameron & Karunaratne, 1936), by cockerels and by ducks (Fowler, 1970), and metabolites have not been detected in the latter.

Normally liver flukes absorb nutrients and foreign substances through the tegument or intestinal caecae (Pantelouris, 1965); some blood is utilized (Todd & Ross, 1966) and bile duct epithelial cells may be ingested (Dawes, 1963). Drugs may thus enter liver flukes *in vivo* from bile, blood or tissue cells. Alexander & Macdonald (1960) were unable to detect carbon tetrachloride in sheep bile using a microdiffusion technique sensitive to concentrations greater than 5 μg/ml (Conway, 1957) and suggested that the fasciocidal action may be due to the liver damage produced by the drug. Khalidi & Zaki (1969), using $^{14}CCl_4$, confirmed that sheep do not excrete volatile radioactivity in the bile and proposed a similar hypothesis for the mode of fasciocidal action of carbon tetrachloride.

The formation of toxic derivatives of carbon tetrachloride by liver flukes, for example, trichloromethylated lipids (Gordis, 1969), could also explain fasciocidal action. Most attention has been focused on water-soluble fasciocidal metabolites (Alexander & Macdonald, 1960; Khalidi & Zaki, 1969). In rats, water soluble radioactivity appeared as organic anions containing labelled carbon; ^{36}Cl appeared as chloride ion (Gordis, personal communication).

In order to study the fate of carbon tetrachloride in liver flukes and the action of biological fluids and extracts, particularly with regard to lipid and water soluble material, the parasites were incubated *in vitro* in several media, with and without sheep bile.

Gas liquid chromatography (GLC) was utilized as a sensitive method for detection and estimation of hexachloroethane (Fowler, 1969a), carbon tetrachloride (Fowler, 1969b) and their metabolites in extracts of liver flukes. GLC was also used to investigate excretion of carbon tetrachloride in sheep bile and urine ; with an electron capture detector, the method was suitable for estimation of fractions of a picogram of the drug (less than 1 ng/ml of extract).

Methods

Collection of sheep bile

A 3·2 mm (outer diameter) silicone rubber cannula was inserted in the bile duct of sheep 40 (Cheviot cross ; female ; 25 kg) and sheep 60 (Blackface cross ; castrated male ; 20 kg). In sheep 60 the gall-bladder and cystic duct were functionally obliterated by a 5 mm (O.D.) silicone rubber cannula introduced through the gall-bladder into the cystic duct, which enabled flushing out of the system. Bile was returned to the duodenum by a second 3·2 mm (O.D.) cannula. Bile duct and duodenal cannulae were exteriorized to the right sub-lumbar fossa and connected by a polyethylene non-return valve (Griffin S 42-630) with adaptors. This obviated blockage of bile cannulae by aspiration of duodenal contents and reduced risk of ascending biliary infection by preventing reversal of bile flow during duodenal activity. A ruminal cannula with fenestrated flange was also inserted (Alexander & Chowdhury, 1958). Bile was collected in sterile 2 litre polyethylene bags supported by a non-crushable harness on the sheep.

The operation was conducted under thiopentone sodium and cyclopropane anaesthesia ; 2 weeks' post-operative recovery were allowed before collection of bile.

Collection of rabbit bile

The contents of the gall-bladders of five rabbits were taken 6 h, 24 h and 48 h after carbon tetrachloride administration.

Collection of sheep urine

Urine was collected by the method of Warwick (1969) from castrated male sheep for determination of volume, pH, specific gravity (SG), Cl^- concentration and chlorinated hydrocarbons. The urine receiver was cooled by an ice-water bath.

Urine was collected from female sheep with a silicone rubber retention catheter (Folatex 60:8 FG) for determination of phenolsulphonephthalein (PSP) clearance after carbon tetrachloride administration.

Phenolsulphonephthalein (PSP) clearance

PSP clearance was determined 24 h, 48 h and 11 days after carbon tetrachloride administration and compared with normal clearance rates. 4 mg PSP/kg was

injected into the jugular vein of two female Cheviot cross sheep at zero time ; urine was collected for 90 min in 15 min aliquots. Each sheep received a 4 litre water load 1 h before injection of PSP. Samples were diluted with 0·5 N sodium hydroxide and optical density determined at 550 nm on an EEL spectrophotometer. The proportion of PSP excreted was determined for each 15 min period and compared with control values.

Storage of biological samples

Bile and urine were used immediately or stored at −20° C. Unfrozen bile was used in fluke incubation experiments.

Analysis of bile and urine samples from sheep

Specific gravity was determined with a hydrometer at laboratory temperature. Chloride concentration was determined by an EEL Chloridemeter and pH by a Marconi TF 1093 pH meter.

Bile and urine were extracted as described previously (Fowler, 1969a), but with heptane as extracting solvent ; extracts were examined by GLC.

Extraction of rabbit gall-bladder bile

The contents of rabbit gall-bladders were extracted as described elsewhere for rabbit tissues (Fowler, 1969b). Extracts were examined by GLC.

Gas chromatography of extracts from bile and urine

Heptane extracts of sheep bile and urine and of rabbit gall-bladder bile were examined for carbon tetrachloride and metabolites by GLC on four columns (Fowler, 1969b).

Maintenance of experimental animals and drug administration

Sheep were maintained indoors with hay and water freely available. Urine and short interval bile collections were made with animals confined to metabolism crates.

Rabbits received British Pelleted Diet and water *ad lib.*

Carbon tetrachloride was administered to rumen fistulated sheep from an all-glass syringe as a 20% (v/v) solution in olive oil, direct to the rumen. Carbon tetrachloride was administered to non-fistulated sheep and to rabbits by stomach tube as a 20% solution in olive oil. Sheep 40 and 60 received 3 ml carbon tetrachloride (0·12 and 0·15 ml/kg respectively) and rabbits received 1 ml/kg.

Liver fluke in vitro *studies*

Viable liver flukes obtained from sheep bile ducts within 1 h of slaughter were washed with Hedon-Fleig solution (Gatenby, 1937) at room temperature. Hedon-Fleig solution also contained glucose (0·005 M), procaine penicillin (5×10^5 units/l.) and streptomycin sulphate (0·6 g/l.). Entire, motile flukes were incubated one per tube at 37° C with emulsions of drugs or various media. Flukes were examined frequently during incubation and always 3, 5, 10, 21 and 26 h after the start of

incubation for signs of movement. Non-motile flukes were stimulated with a 15 V d.c. current at 60 Hz for 3 s to test their ability to respond.

Inactivated flukes were obtained by heating viable flukes for 5 min in saline (0·15 M) at 100° C.

Preparation of emulsions

A primary emulsion was prepared in a pestle-and-mortar with olive oil, acacia and water in the ratio 4:2:1. Drugs were dissolved in olive oil before emulsification. Primary emulsions were diluted with Hedon-Fleig solution with shaking and contained droplets 1·4–5·0 μm in diameter (95%) when measured by microscope stage micrometer.

Methyl oleate/carbon tetrachloride addition product

The addition product ("trichloromethylated oleate") was prepared as described by Gordis (1969) and was purged with nitrogen for 18 h at 100° C to remove volatile contaminants.

Preparation of rabbit liver extracts

Of four New Zealand White rabbits (1·5–1·8 kg), two received carbon tetrachloride by stomach tube (2 ml/kg). Two hours later, control and treated rabbits were stunned, bled, the livers removed and ground with acid-washed silversand in glass pestles and mortars under nitrogen. The macerated livers were extracted three times with ether (total 300 ml), the extracts combined and the ether evaporated off at room temperature under a reduced pressure of nitrogen.

The aqueous mass was centrifuged for 30 min at RCF 1,600 to remove tissue debris and the supernatant was stored frozen at −20° C. 25 ml of aqueous liver extract was equivalent to about 12·5 g ether extracted liver tissue. The ether extracted material was also stored at −20° C under nitrogen to protect unsaturated fatty acids from oxidation.

Gas chromatography of fluke extracts

After incubation, liver flukes were blotted dry, weighed, homogenized, extracted with heptane and examined by GLC as described for rabbit tissue (Fowler, 1969b). In experiments with hexachloroethane emulsion, fluke homogenates were extracted with hexane before GLC analysis (Fowler, 1969a).

Reagents

Analar grade reagents were used where available. Hexane fraction of petroleum and *n*-heptane (British Drug Houses), carbon tetrachloride and hexachloroethane were purified as described elsewhere (Fowler, 1969a, b).

Olive oil and powdered acacia were B.P. grade.

Methyl oleate was oleic acid methyl ester (Sigma).

Results

Gas chromatography showed that carbon tetrachloride, chloroform and hexachloroethane were present in gall-bladder bile of rabbits which had received carbon

tetrachloride (Table 1). Carbon tetrachloride was also detected in the bile of sheep within 3 min of intra-ruminal administration. The highest biliary concentration of the drug (4–5 μg/ml) occurred 1–3 h after administration and fell to less than 1 μg/ml 6 h after dosage.

Chloroform was also excreted in the bile of sheep which received carbon tetrachloride and traces of carbon tetrachloride and chloroform were found in the urine of sheep which received the drug (Table 2).

The pH of sheep bile increased after carbon tetrachloride administration and returned to normal 6–7 days later ; biliary volume decreased sharply and remained depressed for 12–14 days. pH of sheep urine did not change markedly although an initial, transient diuresis was observed ; no change in specific gravity of urine was seen but that of bile increased after administration of the drug. The concentration of chloride ion decreased in bile and in urine.

Phenolsulphonephthalein clearance was unaffected by administration of carbon tetrachloride.

In vitro *studies of liver fluke motility*

Liver flukes retained motility for 2–3 days *in vitro* when incubated in Hedon-Fleig solution (Table 3). Other media were less suitable for maintenance of *F. hepatica in vitro* (Table 3). Emulsions of liver lipid from carbon tetrachloride treated rabbits, of carbon tetrachloride and of its addition product with methyl oleate were especially toxic in the presence of sheep bile.

Gas chromatography of liver fluke extracts

Neither carbon tetrachloride nor its metabolites were detected in normal flukes. Flukes incubated in carbon tetrachloride emulsions took up the drug. Chloroform and hexachloroethane were also detected in the flukes when 25% sheep bile was added to carbon tetrachloride emulsions (Table 4).

TABLE 1. *Volatile chlorinated constituents of gall-bladder bile from rabbits which received carbon tetrachloride (1 ml/kg)*

Time after dose	CCl_4 μg/g $\pm$S.D.	$CHCl_3$ μg/g$\pm$S.D.	$Cl_3C.CCl_3$ ng/g$\pm$S.D.
6 h	37$\pm$7	0·50$\pm$0·12	Trace
24 h	7·8$\pm$1·5	0·14$\pm$0·02	5·5$\pm$1·8
48 h	1·1$\pm$1·3	0·45$\pm$0·21	Trace

TABLE 2. *Comparison of total amounts of carbon tetrachloride and chloroform in sheep bile and urine (μg) following administration of carbon tetrachloride*

Dose	Sheep 37 0·1 ml/kg Urine		Sheep 38 0·12 ml/kg Urine		Sheep 40 0·12 ml/kg Bile		Sheep 60 0·15 ml/kg Bile	
	CCl_4	$CHCl_3$	CCl_4	$CHCl_3$	CCl_4	$CHCl_3$	CCl_4	$CHCl_3$
0–6 h	—	—	—	—	398	—	438	—
0–1 day	19·2	3·7	1·2	6·6	433	241	543	210
1–2 days	5·9	2·0	1·0	3·3	7	122	9	126
2–3 days	4·6	1·8	0·7	2·2	6	95	8	120
3–4 days	1·3	0·8	0·7	2·0	5	50	2	20
4–5 days	0·6	0·2	0·5	0·2	5	Nil	1	Nil
5–6 days	Trace	Trace	Trace	Trace	Trace	Nil	Nil	Nil
6–7 days	Trace	Trace	Trace	Trace	Trace	—	Nil	Nil

Viable and inactivated flukes incubated in hexachloroethane emulsions took up the drug; viable flukes also contained pentachloroethane and tetrachloroethylene but did not lose motility during 4 h incubation. Inactivated flukes did not contain metabolites of hexachloroethane (Table 5).

Discussion

Gas chromatography was found to be a most sensitive method for detection of carbon tetrachloride in bile. Conventional micro-diffusion techniques (Conway, 1957) are not sufficiently sensitive, have led to misleading results and failure to identify carbon tetrachloride in the bile of sheep which received therapeutic doses of the drug (Alexander & Macdonald, 1960). Furthermore, a technique usine ^{14}C labelled drug (Khalidi & Zaki, 1969) failed to demonstrate its excretion in the bile; it is possible that loss of volatile biliary constituents or severe quenching effects may

TABLE 3. *Motility of liver flukes incubated at 37° C*

Composition of medium	No. of flukes	Sheep bile	50% lost motility in:
Hedon-Fleig solution	20	Nil	74 h
Normal sheep bile	30	25%	12 h
Bile 0–24 h after CCl_4	10	25%	26 h
Bile 24–48 h after CCl_4	10	25%	15 h
Control aqueous rabbit liver extract 25%	10	Nil	11 h
CCl_4 treated aqueous rabbit liver extract 25%	10	Nil	26 h
Control rabbit liver lipid 1%	16	25%	10 h
CCl_4 treated rabbit liver lipid 1%	16	25%	2 h
Olive oil emulsion	10	25%	28 h
CCl_4 in olive oil emulsion	33	25%	1 h
Hedon-Fleig solution	12	Nil	86 h
CCl_4 in olive oil emulsion	11	Nil	63 h
Oleic acid (methyl ester) 1%	8	Nil	26 h
Hedon-Fleig solution	28	Nil	63 h
CCl_4 in olive oil emulsion	11	Nil	46 h
Oleic acid (methyl ester) ½%	14	25%	15 h
Oleic acid (methyl ester) CCl_4 treated ½%	20	25%	7 h
Hedon-Fleig solution	10	Nil	76 h
Olive oil emulsion	10	25%	32 h
CCl_4 in olive oil emulsion	12	25%	1 h
Oleic acid (methyl ester) CCl_4 treated 1%	20	25%	1 h

Media emulsified with Hedon-Fleig solution.

TABLE 4. *Concentration of carbon tetrachloride, chloroform and hexachloroethane (μg/g±S.D.) in fluke tissues after incubation in carbon tetrachloride emulsion*

CCl_4 in medium (μg/ml)	Flukes: Weight (mg±S.D)	Flukes: No.	Tissue extracts: CCl_4	Tissue extracts: $CHCl_3$	Tissue extracts: $Cl_3C.CCl_3$
50± 5	102±12	17	76± 8	0·38±0·04	0·19±0·01
93±14	161±22	10	141± 5	0·57±0·06	0·22±0·02
126±19	160±15	18	223±71	1·91±0·21	0·38±0·06

TABLE 5. *Concentrations of hexachloroethane, pentachloroethane amd tetrachloroethylene (μg/g ±S.D.) in fluke tissues after incubation in hexachloroethane emulsion*

C_2Cl_6 in medium (μg/ml)	Flukes: Weight (mg±S.D)	Flukes: No.	Tissue extracts: C_2Cl_6	Tissue extracts: C_2HCl_5	Tissue extracts: C_2Cl_4
14·2±0·3	290±58	19	8·51±0·40	0·19±0·06	0·55±0·14
16·0±2·0	263±82	10*	13·8±0·90	Nil	Nil
21·8±0·3	257±57	16	17·49±2·45	0·16±0·04	0·64±0·17

*Flukes inactivated by boiling.

have occurred (Turner, 1967) and almost certainly a different breed of sheep was involved. A method involving heating of bile to drive off labelled carbon tetrachloride (Khalidi & Zaki, 1969) may introduce a further inaccuracy: on repeating this method (with $^{12}CCl_4$) it was found that 29–64% of carbon tetrachloride (determined by gas chromatography) was not displaced.

The toxicity of carbon tetrachloride to liver flukes *in vitro* is not in agreement with results of some other workers. A toxic action was observed in the presence of bile; bile may have stabilized the drug emulsions, changed the distribution of toxic substances within the flukes or reacted with carbon tetrachloride to form a toxic product. Although the mechanism is not known, exposure of parasites to emulsions rather than solutions of drugs may increase the activity of the drugs (Baldwin, 1943) and may more closely resemble the *in vivo* situation. Emulsions used were of relatively small droplet size and may be likened to large micelles such as occur in mammalian bile (Masoro, 1968).

Several mechanisms may be postulated for a fasciocidal action of carbon tetrachloride *in vivo*:

(1) *An indirect action through release of products of liver damage.* The anthelmintic action of carbon tetrachloride on liver flukes may be due to liver damage produced by the drug and not through a direct action on the fluke in the bile duct (Alexander & Macdonald, 1960). This hypothesis was further developed by Khalidi & Zaki (1969), who suggested that damaged liver cells may release products lethal to liver flukes. Such products might occur in the aqueous or in the lipid fraction. In support of this hypothesis, ether extractable material from carbon tetrachloride treated rabbits' livers was found to be toxic to liver flukes *in vitro*.

Toxic compounds could arise from the interaction of carbon tetrachloride with liver lipids. Trichloromethylated oleate (Gordis, 1969) was also toxic to liver flukes *in vitro* and may resemble the lipids excreted after carbon tetrachloride administration. Bile from sheep which had received carbon tetrachloride, however, was not toxic to liver flukes in the present experiments. It is possible that the toxic agent is volatile or unstable and was lost during freezing, storage or thawing of bile. Furthermore, alteration of physical characteristics of bile, for example, micelle structure, could alter toxic properties of the bile. *In vitro* results may not, perhaps, be relevant to the *in vivo* situation.

(2) *An indirect action through changing the environment.* The reduction in bile flow after carbon tetrachloride administration may lower amount of substrates available to flukes or may allow excretory products to reach toxic levels.

Moreover, the pH of the bile was seen to rise in these experiments and may have contributed to a fasciocidal action. The liver fluke is not a strict anaerobe (Thorsell, 1963) and a high P_{CO_2} due to biliary stasis may prove toxic. Biliary flow rates found in the non-medicated sheep were similar to those described by Mortimer & Stanbridge (1969) with enterohepatic circulation maintained, but much greater than those reported by Khalidi & Zaki (1969).

(3) *A direct fasciocidal action of carbon tetrachloride.* The liver fluke *in vitro* was able to metabolize carbon tetrachloride. Hexachloroethane, which may arise by dimerization of free trichloromethyl radicals (Fowler, 1969b), was detected in extracts of liver flukes and its presence may provide evidence of activation (Slater,

1966). Activation of the drug within fluke tissues may provide a direct fasciocidal mechanism.

Hexachloroethane, although metabolized, was not markedly toxic to liver flukes *in vitro* and it seems unlikely that the fasciocidal actions of carbon tetrachloride can be attributed to synthesis of this substance by the flukes.

Carbon tetrachloride is toxic to flukes in the presence of liver slices (Kondos & McClymont, 1965) and it has now been established that the drug is present in bile for at least 6 h following dosage. It seems likely, therefore, that adult liver flukes have direct access to carbon tetrachloride, although in much lower concentrations than those reported active *in vitro*. Since mature liver flukes *in vivo* ingest blood and tissue cells (Todd & Ross, 1966 ; Dawes, 1963) these may provide a further source of carbon tetrachloride.

Immature flukes are resistant during the 4–6 post-invasive weeks in sheep (Boray & Happich, 1968), during which period they are undergoing maturation in the liver tissues. If metabolism of carbon tetrachloride contributes to its fasciocidal action such flukes may have a reduced capacity to metabolize or activate the drug ; they are deficient in certain enzymes. Glutamate dehydrogenase and alkaline phosphatase, which are present in mature liver flukes, are less active in immature flukes (Thorpe, 1968) and may reflect a similar situation in drug metabolizing enzymes.

Direct and indirect mechanisms for the fasciocidal action of carbon tetrachloride are not mutually exclusive and both may play a part in the therapeutic action of the drug.

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