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ACTIONS OF CARBON TETRACHLORIDE, HEXACHLOROETHANE AND THE PRODUCTS OF THEIR METABOLISM IN SHEEP ON *Fasciola hepatica*

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1 An *in vitro* preparation of *Fasciola hepatica* is described which responded to electrical stimulation with tetanic spasms. Both carbon tetrachloride (20–500 nl/ml), and its metabolite chloroform (50–1000 nl/ml), produced contractions in the preparation which extinguished the responses to electrical stimulation. It is suggested that the spasmogenic action of carbon tetrachloride and its metabolite may contribute to the fasciolifugal action of the drug.

2 Hexachloroethane, another fasciolifuge, had very little effect in the preparation. However, pentachloroethane and tetrachloroethylene, the main products of the metabolism of hexachloroethane in sheep, were potent spasmogens in preparations of *Fasciola hepatica*. Pentachloroethane was about twice as potent as carbon tetrachloride.

3 Tetrodotoxin (2 μg/ml) did not antagonize the responses of the preparation to electrical stimulation or carbon tetrachloride.

Introduction

It has been shown that carbon tetrachloride is without effect on the survival time of *Fasciola hepatica in vitro* (Stephenson, 1947; Thorpe, 1967). The mode of the fasciolifugal action of carbon tetrachloride is unknown, and may be an indirect effect mediated by metabolic products of the drug or materials produced by the liver when it is affected by the drug (Alexander & MacDonald, 1960; Kondos & McClymont, 1965; Khalidi & Zaki, 1969). In sheep considerable amounts of carbon tetrachloride are metabolized to chloroform, and the main products in the metabolism of hexachloroethane, another fasciolifuge, are pentachloroethane and tetrachloroethylene (Fowler, 1969; 1970).

According to Chance & Mansour (1949) both carbon tetrachloride and hexachloroethane contract an *in vitro* preparation of *Fasciola hepatica*. This observation is of interest as spasm of the mature liver flukes might cause them to leave their location in the bile ducts. This would be in accord with the effect of therapeutic doses of carbon tetrachloride and hexachloroethane which eliminate the liver flukes in bile ducts and not those sequestered in liver parenchyma (Boray & Happich, 1968). In the present experiments the effects of carbon tetrachloride and hexachloroethane on *Fasciola hepatica* have been re-examined and compared with those of chloroform, pentachloroethane and tetrachloroethylene.

Methods

Preparations of Fasciola hepatica

Fasciola hepatica was obtained from the bile ducts of infested liver from cattle or sheep before the organ had cooled to room temperature. Each liver fluke was washed and subsequently placed in a conical flask (25 ml) containing Ringer solution at 32°C. Viable liver flukes were motile for at least 24 h, and preparations were made from them within 2 h of collection.

Fine thread was passed through the body wall posterior to the ventral sucker and close to the caudal end of the animal. The liver fluke was attached, oral sucker downwards, to the electrode assembly (Figure 1), which was placed in an organ bath (5 ml) filled with Ringer solution maintained at 37°C and gassed with 5% CO_2 in O_2. The preparation relaxed and started rhythmic activity within 10 min, the activity being recorded on smoked paper with an isotonic lever, load 500 mg, which magnified responses twenty times. In most experiments the preparation was subsequently impaled with the platinum electrode between the oral and ventral suckers. These preparations were quiescent. In some experiments the preparations were stimulated with square wave pulses (5 ms, 30 Hz,

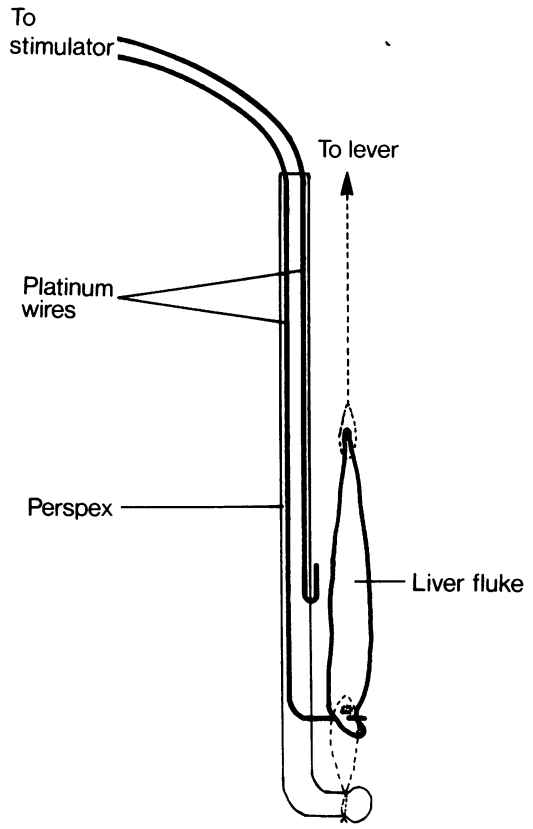

Figure 1 Electrode assembly for stimulation of *Fasciola hepatica.*

25 V) delivered from a Bell (1968) stimulator for 20 s, the interval between trains of stimuli being 16 minutes. Drugs were washed from the organ bath by overflow.

Chemicals

A Ringer solution of the composition described by Beretta & Locatelli (1968) was made with Analar salts and de-ionized water (composition (mM): NaCl 154.00, KCl 5.36, $CaCl_2$ 0.54, $NaHCO_3$ 5.95 and glucose 2.78). Hexachloroethane (Technical, BDH) was recrystallized from ethanol to constant melting point, 186°C uncorrected (sealed tube). Tetrachloroethylene and pentachloroethane (Technical, BDH) were purified by distillation, collecting the fractions at 119°C and 156°C (uncorrected), respectively. Other drugs were carbon tetrachloride (Analar, BDH), chloroform (Analar, BDH) and tetrodotoxin (Sigma). *n*-Heptane (Laboratory Reagent grade, BDH) was washed with 0.1 M NaOH, 0.1 M HCl and three times with distilled water.

The halogenated hydrocarbons were dissolved in olive oil B.P. (Evans Medical Ltd) and the solutions emulsified by trituration with acacia powder (BDH) and de-ionized water in a pestle and mortar. The primary emulsion contained oil, water and gum in the proportions 3:2:1, and was diluted with Ringer solution before testing on preparations of *Fasciola hepatica.* The droplet size (mean ± s.e. mean) in six emulsions as measured by microscope stage micrometer was 15.5 ± 0.7 μm.

Chloroform and carbon tetrachloride were estimated in the emulsions by gas/liquid chromatography as a significant amount of chloroform was lost in emulsification. Emulsion (1 ml) and heptane (4 ml) were shaken by hand in a glass stoppered centrifuge tube for 90 s, and the layers separated by centrifugation at 900 *g* for 10 min in an MSE Mistral 2L centrifuge at −10°C. The heptane extract was poured off the frozen residue into another tube and the residue re-extracted with heptane (4 ml).

Gas/liquid chromatography

The chromatographic column was prepared according to a modification of the method described by Street (1969), who kindly supplied the packing materials. A 6 ft length of stainless steel tubing (i.d. 2.2 mm) was coiled into a helix and conditioned by heating in a Gallenkamp muffle furnace at 600°C for 16 hours. After cooling, chloroform (50 ml) and air were pulled through the tubing. A sintered steel plug was pressed into the column exit and the column packed with a preparation of Chromosorb G coated with silicone gum rubber (SE 52), tapping the tubing whilst sucking in the preparation at a water pump. The column was heated at 420°C for 3 h to make the inner metal surface unreactive. It was then emptied and repacked with a preparation of silanised Chromosorb G coated with PEG (Carbowax 400). A sintered Teflon plug was pressed into the column entrance and swagelock ferrules and compression nuts fitted on the steel tubing for attachment to a Perkin-Elmer Model F11 chromatograph.

The column temperature was adjusted to 85°C, the injector and detector temperatures being about 50° higher. The flow of carrier gas (N_2) was 45 ml/minute. Heptane extracts and solutions (1 to 5 μl) were injected with a micro-syringe fitted with a 3-inch needle and the signal from the electron capture detector recorded on a Servoscribe recorder (5 mV full scale deflection). The electron capture detector was sensitive to pl of choroform and carbon tetrachloride and a linear dose-response relationship was obtained with response expressed as peak height times width at half-height.

Results

Preparations of *Fasciola hepatica* which were not impaled with an electrode exhibited irregular rhythmic

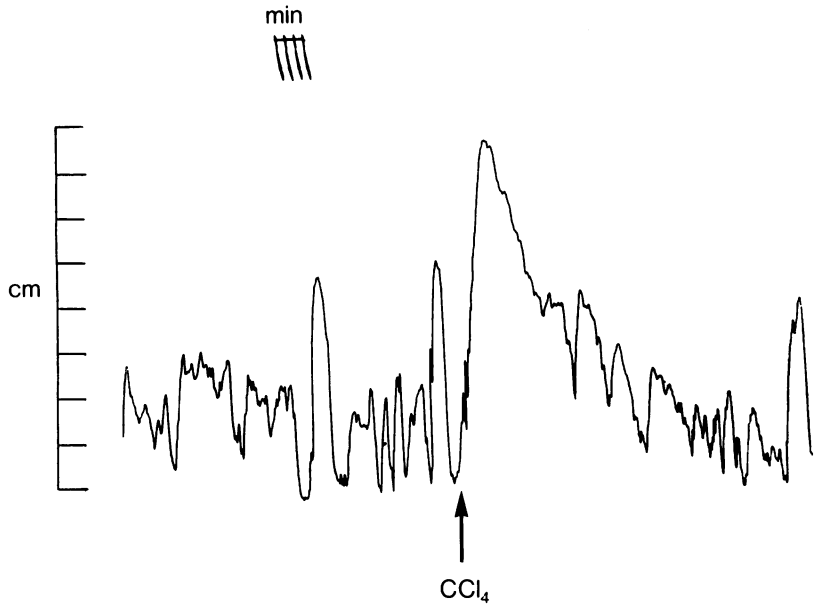

Figure 2 *Fasciola hepatica* preparation. At the arrow carbon tetrachloride was added to the organ bath to produce a concentration of 100 nl/ml bathing solution. Although the preparation was not washed with fresh Ringer solution it resumed spontaneous rhythmic movements when the response to the drug subsided.

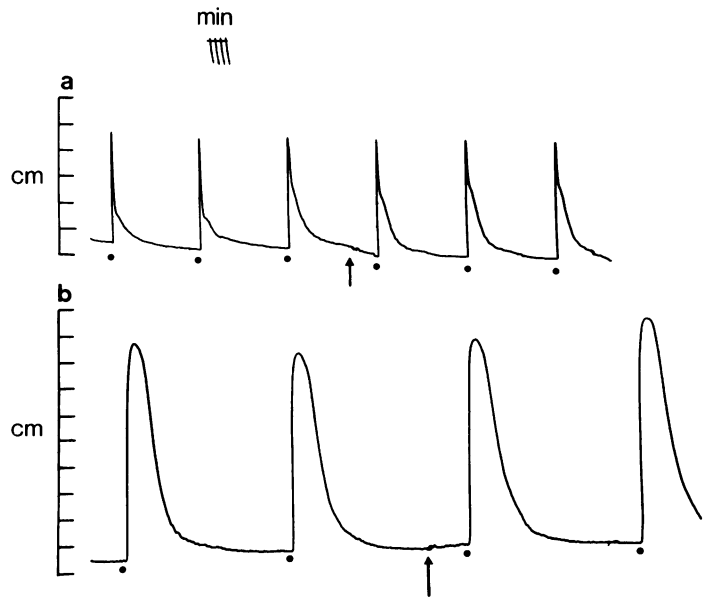

Figure 3 Two preparations of *Fasciola hepatica* impaled with electrodes. In (a) the dots mark stimulation (5 ms, 30 Hz, 25 V) for 20 s and in (b) exposure to carbon tetrachloride (100 nl/ml) for 3 minutes. In both experiments tetrodotoxin (2 μg/ml) was present in the bathing solution from the arrow to the end of the trace. Tetrodotoxin did not affect the responses to electrical stimulation or carbon tetrachloride.

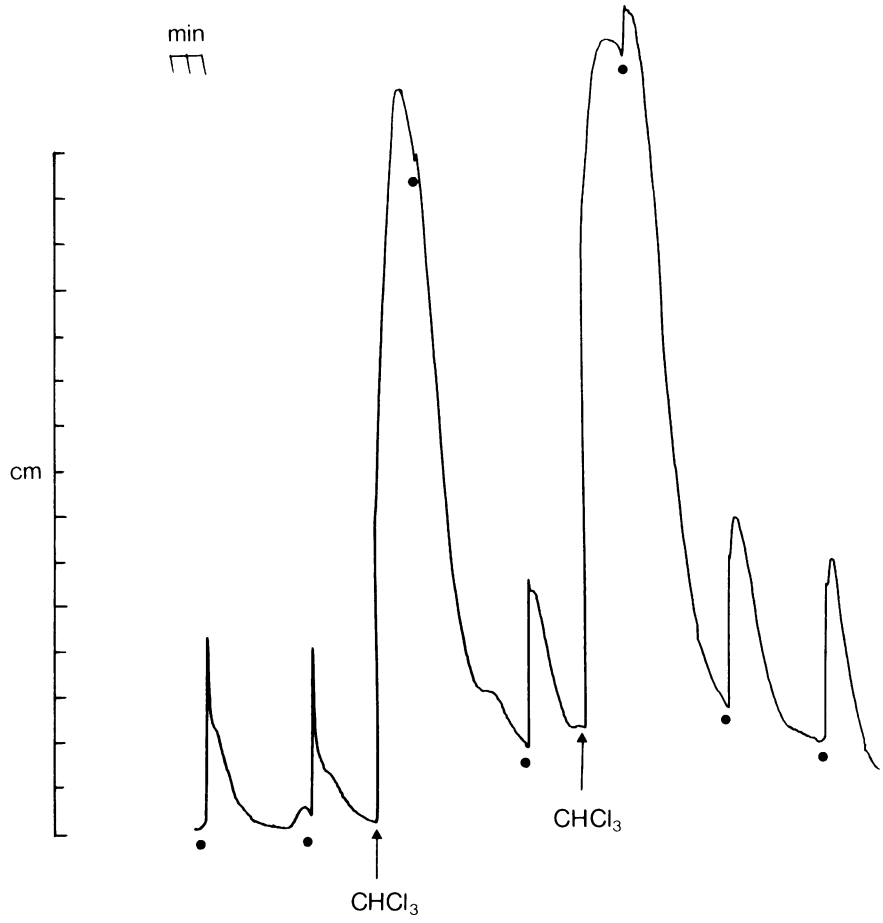

Figure 4 *Fasciola hepatica* preparation. The dots mark stimulation (5 ms, 30 Hz, 25 V) of the preparation for 20 s and the arrows the addition of chloroform (200 nl/ml) to the bathing solution for 4 minutes. Chloroform produced large contractions which extinguished responses to electrical stimulation.

movements for several hours (Figure 2). On exposure to carbon tetrachloride (100 nl/ml) a slow contraction started after a delay of 1 to 2 min reaching a maximum in about 4 minutes. The response to the drug subsided and the rhythmic movements in the preparation resumed without washing the drug out of the organ bath. However, responses to drugs were irregular in preparations exhibiting spontaneous activity the height of the responses sometimes being ill-defined due to the spontaneous movements in the preparation.

In four experiments preparations were exposed to carbon tetrachloride (1000 nl/ml) for 30 min, the bubbling with 5% CO_2 in O_2 being stopped whilst the drug was present in the organ bath. This treatment produced a contracture. After removal of the drug from the bathing solution the preparations contracted normally to carbon tetrachloride (30 nl/ml) or electrical stimulation although spontaneous rhythmic activity did not recover. The treatment did not produce irreversible paralysis in the preparation as described by Chance & Mansour (1949).

The spontaneous rhythmic movements usually ceased when a preparation was impaled with an electrode, and this preparation responded regularly to electrical stimulation and carbon tetrachloride (Figure 3). Responses to single pulses were not obtained and trains of pulses (30 Hz, 25 V) were effective only when the pulse width was 1 ms or more. In the present experiments 20 s stimulations (5 ms, 30 Hz, 25 V) were delivered to the preparation which responded with slow contractions that continued to develop for several seconds after stimulation had stopped. Contractions to carbon tetrachloride at concentrations

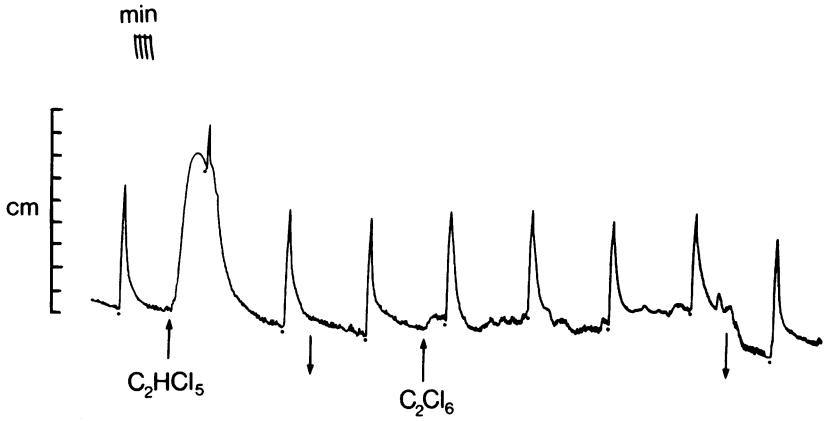

Figure 5 *Fasciola hepatica* preparation. The dots mark stimulation (5 ms, 30 Hz, 25 V) of the preparation for 20 seconds. At the first and third arrows pentachloroethane (C_2HCl_5, 20 nl/ml) and hexachloroethane (C_2Cl_6, 1000 μg/ml) were added to the bathing solution, respectively, and at the second and fourth drugs were washed out of the organ bath. Pentachloroethane produced a large contraction which partly extinguished the response to electrical stimulation; hexachloroethane had little effect on the preparation.

of 20 to 500 nl/ml started within 10 s of exposure to the drug, and were reproducible when the preparation was exposed to the drug for 3 min at intervals of 20 minutes. Addition of tetrodotoxin, 2 μg/ml, to the bathing solution did not affect the responses to electrical stimulation or carbon tetrachloride (Figure 3).

Chloroform produced a contraction in preparations of *Fasciola hepatica* which started within 5 s of exposure to the drug (Figure 4), the response to chloroform being brisker than that to carbon tetrachloride. Both carbon tetrachloride and chloroform produced large contractions in the preparations which extinguished responses to electrical stimulation (Figure 4).

Six emulsions of chloroform and carbon tetrachloride were assayed by gas/liquid chromatography and tested on preparations of *Fasciola hepatica*. In these experiments the preparations were exposed to drugs for 3 min at intervals of 20 min and were not stimulated electrically. Emulsification produced a significant loss of chloroform ($P < 0.0001$) but not carbon tetrachloride ($P > 0.3$), the emulsions containing $70 \pm 2\%$ (mean ± s.e. mean) (6 expts) of the chloroform employed and $92 \pm 8\%$ (6) of the carbon tetrachloride. The spasmogenic activity of 1 μl carbon tetrachloride was found to be equivalent to that of 2.5 ± 0.6 μl chloroform (6 expts).

According to Chance & Mansour (1949) hexachloroethane, 100 μg/ml, stimulated preparations of *Fasciola hepatica*, and at higher concentration, 1000 μg/ml, produced paralysis. However, in the present experiments hexachloroethane (20 to 1000 μg/ml) had little effect in preparations responding to electrical stimulation, the effect of the drug (1000 μg/ml) shown in Figure 5 being typical of that in four preparations. Both pentachloroethane and tetrachloroethylene contracted preparations of *Fasciola hepatica*, the effect of pentachloroethane (20 nl/ml) being shown in Figure 5. The time course of the response to pentachloroethane was almost identical to that to carbon tetrachloride; that to tetrachloroethylene was somewhat slower. The spasmogenic activity of 1 μl carbon tetrachloride was matched by that of 0.56 ± 0.09 μl pentachloroethane and 3.46 ± 1.14 μl tetrachloroethylene (6 expts in each instance).

Discussion

In the liver fluke preparation, tetrodotoxin did not block the contractions produced by electrical stimulation and carbon tetrachloride. Thus the component in the preparation which responded to these stimuli was qualitatively different from vertebrate nerve and striped muscle which are highly sensitive to tetrodotoxin. Spike generation in the musculature of the body wall of *Fasciola hepatica* may resemble that in plain muscle which is also insensitive to tetrodotoxin (Kuriyama, Osa & Toida, 1966). It is equally possible however, that the affinity of tetrodotoxin for Na channels in the membranes of nerve and muscle may be considerably less in *Fasciola hepatica* than in vertebrates.

The present experiments confirm that carbon tetrachloride contracts an *in vitro* preparation of *Fasciola hepatica*, the threshold concentration of the drug in

an organ bath being 20–40 nl/ml. Furthermore chloroform contracted the preparation, being about a third as potent as carbon tetrachloride in this respect. Thus in infested ruminants the direct effect of carbon tetrachloride on *Fasciola hepatica* would summate with that of chloroform, its major metabolite.

In sheep carbon tetrachloride is rapidly absorbed from the rumen, the concentration in venous blood rising to 15–20 μg/ml following administration of 8 ml of the drug (Kondos & McClymont, 1961). The concentration of carbon tetrachloride in portal blood does not seem to have been estimated, but it is likely to be higher than that in the jugular vein. Kondos & McClymont (1965) found peak concentrations of 36 and 65 μg carbon tetrachloride per ml of sheep bile following oral administration of 2 and 4 ml of the drug, respectively, and Fowler (1970) detected 4–5 μg/ml after giving 3 ml of the drug.

Mature liver flukes ingest blood and produce extensive haemorrhages in the biliary tract (Sewell, Hammond & Dinning, 1968). Thus it seems probable that *Fasciola hepatica* is exposed to carbon tetrachloride at concentrations which are pharmacologically active following administration of the drug to sheep.

In the present experiments hexachloroethane had little effect in preparations of *Fasciola hepatica*, but pentachloroethane and tetrachloroethylene, the main products of the metabolism of hexachloroethane in sheep (Fowler, 1969), possessed marked pharmacological activity. Pentachloroethane was about twice as potent as carbon tetrachloride as a spasmogen in preparations of *Fasciola hepatica*. These observations suggest that spasmogenic activity may contribute to the fasciolifugal action of the chlorinated hydrocarbons.

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