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TOXIC EFFECTS OF CHLORFENVINPHOS IN DOGS AND RATS

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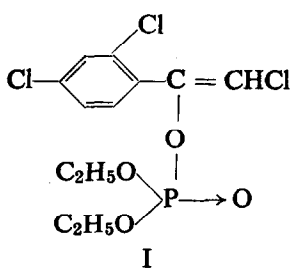
Abstract—A pronounced difference in acute oral toxicity shown by dogs and rats towards chlorfenvinphos is commensurate with a concerted intereaction of the relatively small differences observed in the rates of absorption and metabolism, the availability in blood, the rate of brain uptake and the sensitivity of brain acetylcholinesterase towards the insecticide.

IN PREVIOUS work,[1] 90 per cent of a single oral dose of 2-chloro-1-(2′,4′-dichlorophenyl) vinyl diethyl phosphate (chlorfenvinphos) (I) (also known as compound 4072 and SD 7859) was found to be metabolized and excreted in the urine of animals, and although dogs and rats showed differences in the rate of elimination via the kidneys and in the proportions of the metabolites excreted by this route, these differences do not account *per se* for the pronounced difference in acute oral toxicity shown by the two species towards this insecticide (Table 1). Dogs and rats exhibit a much smaller

TABLE 1. ACUTE TOXICITY DATA FOR CHLORFENVINPHOS

Route of administration	LD_{50} in mg/kg body wt.					Vehicle
	Dog	Guinea-pig	Mouse	Rabbit	Rat	
Oral	>5000	125–250		500–1000		Technical Material
			150–200		10–15	Dimethyl sulphoxide
Intraperitoneal			37		8·5	Polyethylene glycol
Intravenous	50·4				6·6	Cotton seed oil emulsion

difference in toxicity, when the insecticide is administered parenterally (Table 1); this is independent of the nature of the carrier. A parallel investigation of the distribution and action of chlorfenvinphos was therefore required to put the toxic effects of this insecticide into perspective.



I

After oral administration of chlorfenvinphos to dogs and rats, we have measured particularly the concentration of unchanged insecticide in circulating blood, the brain uptake of ^{14}C-labelled insecticide and its effect on cerebral acetylcholinesterase (AChE). The distribution of (^{14}C)chlorfenvinphos between erythrocytes and plasma lipoprotein and proteins has also been measured *in vitro*. We have found: (1) that the concentration of insecticide in portal blood exceeds that in peripheral blood; (2) that at comparable dose levels, the concentration of chlorfenvinphos in the blood of both species is roughly of the same order of magnitude, but in dogs lower than in rats; (3) that dogs withstand blood concentrations of the insecticide many times higher than those that are fatal to rats; (4) that brain uptake of chlorfenvinphos is greater for rats than dogs at similar blood levels of insecticide; (5) that at levels of insecticide in the brain, at which the activity of rat-brain AChE is reduced to one-tenth of the normal value, the activity of dog brain AChE is unaffected; (6) that chlorfenvinphos partitions differently between the erythrocytes and plasma in the two species.

The present paper describes the results obtained and their possible interpretation.

METHODS

Chlorfenvinphos

Technical chlorfenvinphos (2-chloro-1-(2′,4′-dichlorophenyl)vinyl diethyl phosphate) was obtained from Shell Development Company, New York and was purified as described by Hutson *et al.*[1] We are indebted to Shell Development Company, Modesto, California, for synthesizing the radioactive insecticide labelled in both vinyl carbon atoms with ^{14}C.

Experiments with animals

Young adult rats (150–200 g body wt., Porton strain, maintained as a specific pathogen free colony in this laboratory) were used. For the studies on uptake of chlorfenvinphos by blood and brain, the rats were treated by stomach tube with 1 ml of olive oil containing 5 mg of insecticide (dose rate 25–30 mg/kg). This dose, which represented about three times the LD_{50} to rats, caused visible symptoms of organophosphate poisoning. Defaecation, urination, salivation, lachrymation and fasciculation, followed by muscular weakness and prostration began to develop at approximately 25 min, and became progressively worse, culminating in death at 70–85 min. At 25, 50 and 70 min after dosing, samples of blood (2 ml) were withdrawn from each rat by cardiac puncture under ether anaesthesia, and stored at 4° for analysis within a few hours. For the brain studies, female animals were dosed as above but using [^{14}C]chlorfenvinphos (5 mg, 6·05 μc or 5·48 μc), and killed by cervical dislocation at 25, 50 and 70–85 min after dosing. The brains were excised, weighed, and homogenized with 10 ml of ice-cold water using an Ultra-Turrax TP 18 homogenizer. Part of the homogenate was assayed for acetylcholinesterase activity, and the remainder was poured into acetone, to store prior to extraction of the radioactivity.

A beagle bitch (11·5 kg) in which a hepatic portal cannula had been inserted[2] was dosed orally with a capsule containing 1g of non-radioactive chlorfenvinphos (88 mg/kg). Samples of blood were withdrawn via the cannula and from a front leg vein at intervals between 0 and 6 hr and analysed for chlorfenvinphos. During this time the animal showed no signs of intoxication or discomfort. A second dog, a beagle hound (11 kg) was dosed orally with 35 g (3180 mg/kg) of chlorfenvinphos by stomach

tube. Blood was withdrawn from a front leg vein at intervals between 0 and 24 hr and analysed for chlorfenvinphos. The animal showed no visible symptoms of organophosphate intoxication.

A beagle hound was treated orally with 1 g of [^{14}C]chlorfenvinphos (82 μc) in 20 ml of olive oil by stomach tube. Blood samples were taken at intervals from 0 to 1 hr, and assayed for radioactivity and chlorfenvinphos. After 60 min the dog was anaesthetized with sodium pentobarbitone (60 mg/kg) and exsanguinated via the jugular vein. The brain (81·2 g) was removed and a transverse slice (8·0 g) containing the optic chiasma was cut and homogenized with water for AChE assay. This procedure has been used with six animals of the same age and sex in this laboratory and normal levels of acetylcholinesterase have been found not to vary greatly. This average value was used as a control value for this experiment.

*Measurement of chlorfenvinphos in blood**

Blood (1 ml) was pipetted into 2 ml of acetone above a column of silicic acid (2 × 1 cm) (M.F.C., Hopkins and Williams). After stirring, the fluid was allowed to flow from the column, which was then eluted with a further 5 ml of acetone. A further 2 ml of acetone was added with stirring, and eluted, followed by 5 ml more acetone. Distilled *n*-hexane (2 ml) and 2% aq. Na_2SO_4 (to 50 ml) were added to the acetone eluent and the mixture shaken. The hexane layer was analysed directly, or after dilution as necessary, by gas–liquid chromatography. This procedure afforded a 94 per cent recovery of chlorfenvinphos from a solution (5×10^{-6} M) in dog blood, and 80 per cent from rat blood. However, only 30 per cent of the insecticide could be recovered from a 10^{-6} M solution in rat blood. Gas–liquid chromatographic estimations were carried out on a 2 ft column packed with 100–120 mesh Celite coated with 2% Apiezon and 0·2% Epikote 1001 as stationary phase. The running conditions were: temperature, 184°; inlet pressure, 20 lb/in^2 (O_2-free nitrogen). Electron capture was used for detection. The appearance of a large late peak on the chromatograms was traced to the use of olive oil as the dosing vehicle. The accuracy of the procedure was not affected but it was necessary to allow a considerable time lapse between analyses. When the ^{14}C concentration of blood was required, part of the hexane extract was mixed with liquid scintillator,[3] and assayed using a Tri-Carb Spectrometer (Packard Instrument Co. La Grange, Ill., U.S.A.).

Recovery of [^{14}C]chlorfenvinphos from plasma

Experiments were designed to measure the efficiency of extraction of chlorfenvinphos directly from whole blood, erythrocytes, or plasma, by Brays scintillator. The test solution or suspension (1 ml) was added to 5 ml of scintillator. The mixture was shaken for 1 min and centrifuged at 1000 rpm for 5 min. The supernatant was poured into the counting bottles and the residue thoroughly shaken with a further 5 ml of scintillator, which was then added to the original extract. This technique gave virtually quantitative recovery from rat and dog erythrocytes and from dog serum or plasma at 10^{-5} or 10^{-6} M chlorfenvinphos. However, there was some retention of the insecticide by rat serum and plasma. Recoveries at various concentrations were as follows:

* This method was developed by our colleagues, A. Richardson, J. Robinson, B. Bush, and J. M. Davies (*Archs expl Hlth.* in press) for the measurement of pesticides in blood.

99% at 10^{-4} M, 75% at 10^{-5} M, and 13% at 10^{-6} M. An identical experiment using dog plasma gave recoveries of 97–100 per cent from each solution. Quantitative recoveries were also obtained from bovine serum albumin solution. Recoveries from rat plasma at 10^{-6} M were also very inefficient with acetone and methanol, indicating the possibility of co-valent binding to protein of most of the ^{14}C.

Thus each sample of blood containing insecticide detectable by the g.l.c. assay method contains at least $0{\cdot}5 \times 10^{-6}$ M unmeasured chlorfenvinphos. Appropriate allowance should therefore be made when the total amount of unchanged insecticide in rat blood is considered in relation to toxic symptoms. These considerations do not apply to analysis of dog blood since recoveries from 10^{-6} M chlorfenvinphos were quantitative.

Extraction and identification of radioactivity in brain

Homogenized brain samples were stirred with 10 ml of acetone as soon as possible after preparation and then extracted in a soxhlet apparatus for 18 hr with chloroform–methanol (1:1, v/v). The solution was evaporated and made to a known volume with chloroform and assayed for radioactivity in NE 221 gel scintillator [Nuclear Enterprises (G.B.) Ltd, Sighthill, Edinburgh, 11]. The extracts from rat brain were then combined, evaporated, and partitioned between hexane and water. Dog brain was treated similarly. The hexane layer contained 88 per cent of the rat brain radioactivity and 90 per cent of that from dog brain. Rat brain extract in hexane was applied to a silicic acid column (2·5 cm × 30 cm) and eluted with hexane–ether mixtures. Most of the radioactivity (90 per cent) was eluted with ether–ethanol (1:1, v/v). This fraction was evaporated, mixed with 50 mg of chlorfenvinphos and analysed by thin-layer chromatography on Kieselgel G (E. Merck, Darmstadt, Germany) using hexane–acetone (4:1, v/v) as solvent. Chlorfenvinphos and 2,4-dichlorophenacyl chloride, an intermediate metabolite of the insecticide,[1] were used as standards. The chromatogram was analysed by spraying with phenoxyethanol-$AgNO_3$[4] and then by scintillation counting of the zones scraped from the chromatoplates. The chlorfenvinphos zone, (R_f 0·4) contained 93 per cent of the recovered radioactivity, and the ketones zone 0·8 per cent. Dog brain extract in hexane was applied to a similar column, when 91 per cent of the radioactivity appeared in the first fraction eluted with 200 ml of hexane. This solution was examined by thin-layer chromatography as above. The radioactivity appeared at R_f 0·1–0·4, near chlorfenvinphos (0·4). but the low number of counts (25 per cent above background) made further characterization impossible. There is thus some doubt as to the identity of the radioactivity in dog brain because of its rapid elution from the column. It may in fact be chlorfenvinphos rapidly eluted by contaminating brain lipids.

Measurement of brain acetylcholinesterase activities

Δ pH *method* The method used was similar to that of Michel.[5] Rat brain homogenate (1·5 g of tissue in 10 ml of water) from the earlier experiment (0·25 ml) was incubated at 37° with 1·75 ml of water, and 2·00 ml. of Michel[5] buffer (sodium barbital, potassium phosphate, and potassium chloride, pH 8·1). At zero time, 0·4 ml of 2·5 (w/v) acetylcholine bromide was added. The pH was noted at 2·5 min and 62·5 min to give a Δ pH/hr value of enzyme activity. Appropriate controls were run and the AChE values expressed as percentages of the normal value (0·84), obtained from duplicate

determinations on six rats of the same age and sex as those used in the experiments. A transverse section of dog brain containing the optic chiasma and weighing 8 g was cut and homogenized at 2° in 65 ml of water. Part of this homogenate (0·08 ml) was incubated with 1·92 ml of water and 2·00 ml of sodium barbital–potassium phosphate–potassium chloride buffer pH 8·2.[6] At zero time 0·4 ml of 2·5% (w/v) acetylcholine bromide was added. The pH was noted at 2·5 and 62·5 min to give a Δ pH/hr value. The normal value for six male dogs of similar age in this laboratory was 1·44 (1·10–1·83). No correction in either case was made for the presence of chlorfenvinphos in the brain homogenates.

Continuous titration method

This method was used for the determination of the pI_{50}'s of chlorfenvinphos with rat and dog brain acetylcholinesterase. Rat brain was obtained after decapitation of a female animal; dog brain was obtained from a healthy beagle hound by the method described for the first dog brain experiment. Fresh whole brain was homogenized at 4° with ten times its weight of water in a Virtis 45 homogenizer, and the resultant homogenate used for the assay. Homogenate (0·5 ml) was mixed with 2 ml of 0·5 M-NaCl, 4·4 ml of water and 3 ml of a mixture of various amounts of 10^{-4} M-chlorfenvinphos in water–acetone (98·4:1·6, v/v) made up with the same water–acetone mixture. The mixture was adjusted to pH 7·15 with 0·01 N-NaOH in a Radiometer titrator and allowed to incubate at 30° for 30 min. A solution of 0·4 M acetylcholine bromide (0·1 ml) was added to the Radiometer cell, the contents of which were stirred under CO_2-free nitrogen. The rate of hydrolysis of the substrate was measured from the rate of addition of alkali. The ratio of rate in presence of inhibitor: rate in absence of inhibitor was plotted against inhibitor concentration and thence the molarity of inhibitor required to halve the normal rate was found and expressed as its negative logarithm (pI_{50}).

The action of Plasma on [^{14}C]chlorfenvinphos

Solutions of [^{14}C]chlorfenvinphos (10^{-4} M) in fresh dog or rat plasma (8 ml) were incubated at 37°, 1 ml samples were withdrawn at various times and added at 10 ml of methanol. The metahanol extracts were evaporated and analysed by thin-layer chromatography on Kieselgel G with chlorfenvinphos, 2,4-dichlorophenacyl chloride, and 2-chloro-1-(2′,4′-dichlorophenyl)vinyl ethyl hydrogen phosphate[1] as over-spotted markers. The chromatograms were developed with benzene:methanol (96:4, v/v) and the compounds located by spraying with phenoxyethanol-$AgNO_3$. The spots containing the unhydrolysed insecticide and the two hydrolysis products were removed from the plates into scintillator for counting. Dog plasma hydrolysed 5 per cent of the insecticide in 6 hr and rat plasma, 4 per cent in 16 hr. The product of the slight hydrolysis in both plasmas migrated on the plates as the vinyl hydrogen phosphate metabolite (R_f 0·05).

Distribution of [^{14}C]chlorfenvinphos between plasma and erythrocytes

[^{14}C]Chlorfenvinphos was dissolved in fresh rat or dog heparinized plasma containing 2·8% glucose, or in artificial serum-glucose, to give 10^{-5} M solutions. The artificial serum was composed of glucose (2·8%), sodium chloride (0·4%) and bovine serum albumin (3%) (Armour Pharmaceutical Co. Ltd., Eastbourne). Erythrocytes from 5 ml of blood were prepared by washing twice with 0·9% sodium chloride. The

packed erythrocytes were then carefully mixed with 2·5 ml of plasma containing the labelled insecticide and the mixtures incubated in a water-bath at 37°. At various times 1 ml portions were withdrawn and centrifuged at 1000 rpm for 10 min and 0·1 ml portions of the plasma assayed for radioactivity by blending directly with 10 ml of Bray's scintillator. In each case there was a rapid loss of ^{14}C from the plasma during the first 15 min but after this time the distribution was stable over the 5- or 6-hr period of the experiment. Fresh packed washed erythrocytes (1·5 ml) from each species were mixed with 1·5 ml of each plasma containing 10^{-5} M-[^{14}C]chlorfenvinphos in centrifuge tubes. After 1 hr at 37°, the tubes were centrifuged for 10 min. The plasma was separated and the erythrocytes washed twice with 1·5 ml portions of saline, and the washings combined with the plasma. The erythrocytes were haemolysed by making up to 5 ml with distilled water. Plasma and cell contents were assayed for radioactivity by the extraction of 1 ml portions with Bray's scintillator as described above. Recoveries of radioactivity in systems containing dog plasma and artificial plasma were about 100 per cent. In experiments involving rat plasma, recoveries were 75–80 per cent; radioactivity could not be detected in the precipitated erythrocyte content protein but was found in the precipated rat plasma protein. In three separate experiments in which dog erythrocytes were incubated in rat plasma. extensive haemolysis occurred and the results were discarded. In other mixtures, however, erythrocytes appeared to be as stable as under normal conditions. The distribution of radioactive insecticide between erythrocytes and plasma in whole rat and dog blood at an initial concentration of 10^{-5} M was also studied by the method described.

Paper electrophoresis of chlorfenvinphos in serum

Solutions of 10^{-5} M-[^{14}C]chlorfenvinphos in rat or dog serum (50 μl) were streaked on to Whatman No. 3 MM paper and subjected to electrophoresis at 10 v/cm for 16 hr in a Shandon high voltage paper electrophoresis apparatus with water-cooled plates. Buffers used were veronal sodium pH 8·6, $I = 0{\cdot}1$ and sodium acetate pH 4·5, $I = 0{\cdot}1$. After drying, one half of the strip was stained for protein by immersing in 0·02% bromocresol green containing 3% trichloroacetic acid, and washing with 0·1% HCl. The other half was cut into 1 cm widths which were assayed for radioactivity by scintillation counting.

Dialysis experiments

Dog and rat sera containing 5×10^{-6}M [^{14}C]chlorfenvinphos were dialyzed in 5 ml batches in Visking tubing against stirred horse serum (Oxoid Ltd, London, S.E.1) diluted with an equal volume of water to a total vol. of 200 ml. The system was maintained at about 4°. Samples were taken from the dialysis bags at various times and assayed for ^{14}C. Fall in radioactivity in the test sera, expressed as time (hr)/per cent of initial ^{14}C remaining, were as follows: dog serum 0/100, 1/100, 2/100, 2·5/98, 24/85 rat serum 0/100, 1/99, 2/99, 2·5/99, 24/75. Similar figures were obtained by dialysing at 2°, against undiluted horse serum (20 ml) without stirring for 48 hr.

Gel-filtration of chlorfenvinphos in serum

Sephadex G-25 (coarse grade) (2·5 g) was equilibrated with 0·1 M potassium phosphate buffer, pH 7·4 and transferred to a column of 1 cm diameter. V_0 was determined

with 0·2% blue dextran solution [Pharmacia (G.B.) Ltd.]. [^{14}C]Chlorfenvinphos (10^{-5} M) in 0·2 ml of 0·1 M potassium phosphate pH 7·4 was applied to the column and eluted with the same buffer. The resulting K_D value of 2·33 indicated strong interaction between insecticide and sephadex.[7] The column was washed thoroughly with horse serum and V_0 redetermined with naphthalene black eluted with horse serum. The column was then washed with rat serum and 0·2 ml of a solution of [^{14}C]chlorfenvinphos (2×10^{-5} M) in rat serum applied and eluted with rat serum. The K_D value, 0·07, indicated strong binding of insecticide to a serum component. When the insecticide in rat serum was eluted with the phosphate buffer, the K_D value was 2·3 showing that the protein binding was reversed on dilution. Similar results were obtained with dog serum as solvent and eluent.

Ultracentrifugation

Solutions of [^{14}C]chlorfenvinphos (10^{-5} M) in the two sera (4·1 ml) were pipetted into 5 ml polypropylene centrifuge tubes. Sodium bromide (0·756 g) was dissolved in the mixture. Under these conditions, most of the lipoprotein appears in the top 1-ml fraction of the tube after ultracentrifugation in an M.S.E. Superspeed 40 preparative ultracentrifuge at 135,000 *g* (40,000 rpm) at 4° for 16 hr.[8] The tubes were then sliced into four fractions of 1·2, 0·7, 0·7 and 1·6 ml respectively and 0·2 ml portions assayed for radioactivity. In duplicate experiments, average values for the ratios of concentration of radioactivity in the four fractions were: in dog serum 0·70, 0·78, 0·74, 1·00; and in rat serum 0·46, 0·60, 0·65 and 1·00.

RESULTS

Chlorfenvinphos concentrations in the blood of orally treated rats and dogs

When 25–30 mg of chlorfenvinphos/kg body wt. was administered orally to young adult rats of both sexes, toxic symptoms commenced 25 min after dosing and the pattern of intoxication (for details, see Methods section) culminated in death between 70 and 85 min after dosing. For both sexes, a concentration of $0{\cdot}1 \times 10^{-6}$M or less in peripheral blood was lethal (Table 2). Higher concentrations of chlorfenvinphos in the blood, which were attained at various times in different animals, were not regarded as being particularly significant. Late in intoxication, between 50 and 70 min after dosing, there was a tendency for female rats to achieve higher concentrations of insecticide in the plasma than those for males (Table 2). The biological variation in the results for both sexes is commensurate with oral dosing.

It is noteworthy that at the lethal dose level (25–30 mg of chlorfenvinphos/kg body wt.), two male rats showed only mild symptoms of intoxication and for these animals, the blood concentration of extractable insecticide was 1 and 3×10^{-8} M respectively. There is a strong supposition that rats survive when the concentration of extractable chlorfenvinphos in the blood is less than 1×10^{-8} M, but that higher concentrations ($0{\cdot}1$–$1{\cdot}0 \times 10^{-6}$ M) are associated with severe toxic symptoms and death.

A beagle bitch with a cannula in the hepatic portal vein, which was treated orally with chlorfenvinphos (88 mg/kg body wt.), showed no toxic effects, and higher concentrations of unchanged insecticide were found in portal blood than in peripheral blood, where the concentration remained roughly constant at $0{\cdot}21 \times 10^{-6}$ M between

1 and 6 hr after dosing (Table 3). This experiment showed that chlorfenvinphos was absorbed substantially as unchanged insecticide, and also that some unmetabolised insecticide was present in circulating blood. Massive oral dosing (3180 mg/kg body wt.) produced high concentrations of chlorfenvinphos in peripheral dog blood without harmful effects: 8·5 (2 hr), 3·7 (4 hr) 2·4 (6 hr) and 0·32 $\times$ 10^{-6} M (24 hr).

TABLE 2. CONCENTRATIONS OF EXTRACTABLE CHLORFENVINPHOS IN THE PERIPHERAL BLOOD OF TREATED RATS

Time after dosing (min)	30	50	70	Sex
Concentration of extractable chlorfenvinphos in blood $\times$ 10^{-6} M	0·14, 0·19, 0·06	0·61, 0·14, 0·06 0·22, 0·14, 0·03*	0·14, 0·06, 0·14 0·01*	♂
	0·17, 0·44, 0·08	0·56, 0·33, 0·61	0·78, 1·00, 1·17	♀

Insecticide was measured in 1-ml samples of blood withdrawn by cardiac puncture at various times after oral administration (25–30 mg/kg body wt.)
* These animals showed minimal symptoms.
Each figure represents the mean of duplicate determinations on one animal.

TABLE 3. CONCENTRATION OF EXTRACTABLE CHLORFENVINPHOS IN THE PORTAL AND PERIPHERAL BLOOD OF A TREATED DOG

Time after dosing (hr)	Concentration of chlorfenvinphos in blood ($\times$ 10^{-6} M)	
	Portal	Peripheral
0·25	0·15	0·02
0·5	0·33	0·04
1·0	1·00	0·26
2·0	0·87	0·14
4·0	1·09	0·30
6·0	0·68	0·16

Insecticide was measured in 1-ml samples of blood withdrawn via an hepatic portal cannula and from a front leg vein at various times after oral administration (88 mg/kg body wt.).

Thus ingestion of chlorfenvinphos by dogs (88 mg/kg) and rats (30 mg/kg) afforded similar concentrations of extractable insecticide in the circulating blood, but the absolute concentration of chlorfenvinphos in dog blood was about one-fifth of that in the blood of rats that had received one-third of the (oral) dose.

Brain uptake of chlorfenvinphos and the effect on cerebral acetylcholinesterase

When groups of rats that had been treated orally with a lethal dose of chlorfenvinphos (25–30 mg/kg body wt.) were killed at various times thereafter, the range of

values for the concentration of extractable radioactivity in the brain cells remained roughly constant, with a mean value of $5{\cdot}0 \times 10^{-6}$ M, irrespective of the selected time after dosing (Table 4). This radioactivity, extracted from the bulked brain tissue, was shown by chromatography to be due to unchanged chlorfenvinphos. Throughout the period of chlorfenvinphos intoxication culminating in death, the activity of cerebral AChE progressively diminished (Table 4).

TABLE 4. EFFECT OF BRAIN UPTAKE OF (^{14}C) CHLORFENVINPHOS ON CEREBRAL AChE IN RATS

Time (min)	Concn of ^{14}C in brain (expressed as molarity, mM/g tissue, of chlorfenvinphos $\times 10^{-5}$)	AChE (% of normal)
	0·18	67
	0·05	98
	1·54	77
25	0·17	94
	0·45	37
	0·33	76
	0·73*	10
	0·65	13
50	0·33	11
	0·26	69
	0·52	52
	0·50	46
	0·56	48
	0·43*	58
65–85	1·20*	11
	0·39	11
	0·29	17
	0·37	8

Female rats treated orally with 5 mg of (^{14}C) chlorfenvinphos (6·05 or 5·48 μc) were killed at various times and brain homogenates were assayed for radioactivity and AChE.
* These animals died.

Hence, either brain-uptake of chlorfenvinphos and inactivation of cerebral acetylcholinesterase are unrelated, which seems unlikely, or brain-enzyme inhibition during insecticide intoxication *in vivo* is a time depended reaction.

When a beagle hound ingested [^{14}C]chlorfenvinphos (80 mg/kg body wt.), the concentration of unchanged insecticide in peripheral blood was 0·55 (0·33 hr), 0·70 (0·67 hr) and $0{\cdot}83 \times 10^{-6}$ M (1 hr after dosing), whereas the concentration of ^{14}C in the blood at 1 hr was $30{\cdot}0 \times 10^{-6}$ M. This thirty-six fold disparity indicates the presence of a high proportion of ^{14}C-labelled metabolite in blood, and suggests that the metabolism of [^{14}C]chlorfenvinphos is rapid. On the other hand, the concentration of ^{14}C in a 10·5 g transverse slice of the brain 1 hr after dosing was $2{\cdot}4 \times 10^{-6}$ M, but chromatographic examination suggested that not all of the radioactivity may have been due to insecticide, but this could not be proved, because of the small quantity of ^{14}C material relative to brain cell constituents. Thus a dose level, that produced a higher concentration of unchanged insecticide in the blood of dogs than in that of

rats, gave a lower concentration of insecticide in the brain of the dog than in that of the rat.

Dog-brain AChE activity measured 1 hr after oral administration of chlorfenvinphos was the same as the control values. This prompted investigation of the effect of pre-incubation of dog- and rat-brain homogenate with various concentrations of insecticide on the rates of hydrolysis of acetylcholine bromide *in vitro*. The potency of organophosphates to inhibit ChE may be defined by the pI_{50}, which is the negative logarithm of the molar concentration of organophosphate required to inhibit activity of the enzyme by 50 per cent. The pI_{50} for chlorfenvinphos in respect of rat-brain homogenate (5·9) and dog-brain homogenate (5·1) shows that AChE in rat-brain homogenate is almost ten times more sensitive than that in dog-brain homogenate, under the prevailing experimental conditions (for details, see Methods section).

Efficient anticholinesterase behaviour of chlorfenvinphos *in vitro* suggests that in our *in vivo* experiment, the effects may take several minutes to develop after an effective concentration of insecticide had accumulated in the brain, and this is in agreement with other observations.[9] Further substantiation of AChE behaviour *in vivo* was derived from E.E.G. recordings of a rat treated intravenously with chlorfenvinphos (50 mg/kg body wt). Five minutes after injection, the trace showed signs of activation and desynchromization, and at 20 min, high voltage 10 c/s waves and some 5 c/s spike wave complexes were seen.

Distribution of [^{14}C]chlorfenvinphos between erythrocytes and plasma constituents

Preliminary experiments showed that dog and rat plasma had little action on chlorfenvinphos, when 10^{-5} M solutions were incubated for 2 hr at 37°. For measurement of chlorfenvinphos-uptake by erythrocytes, a plasma concentration of 10^{-5} M was chosen. This concentration, which was amongst the highest found in circulating blood *in vivo*, might nevertheless be regarded as realistic, whereas lower concentrations could not be used without making difficult the measurement of ^{14}C in erythrocytes, because of the low specific activity of [^{14}C]chlorfenvinphos. As in other transport studies, a plentiful supply of glucose was essential for maintenance of membrane integrity.

From a solution of bovine serum albumin, chlorfenvinphos-uptake by both dog and rat erythrocytes was high, and the similar distributions for the two species (42:58 and 45:55) (Table 5) indicate that dog and rat erythrocyte stroma show no significant difference in membrane permeability towards the insecticide. When the solution of bovine serum albumin was replaced by dog or rat plasma, the biological situation became more complex, and there was a threefold difference between the uptake of chlorfenvinphos by rat erythrocytes from rat plasma and that by dog erythrocytes from dog plasma (Table 5); this was confirmed by the distributions between erythrocytes and plasma in the whole blood of the two species. Since there is no difference in membrane permeability, the different availabilities of chlorfenvinphos in rat and dog plasma might be due to the different binding of the insecticide with the plasma lipoproteins and proteins of the two species. In agreement with diminished insecticide availability in dog plasma, rat erythrocytes incorporate less chlorfenvinphos from dog plasma than from rat plasma.

The properties of plasma/serum solutions of chlorfenvinphos

The different availabilities of chlorfenvinphos in dog and rat plasma, prompted investigation of possible bindings to plasma constituents,[10] and 10^{-5} M solutions in

plasma were used for this work. Much of the insecticide was shown to be strongly but reversibly bound to plasma constituents by gel filtration (for details, see Methods section), operated so that the K_D value gave an estimate of the extent of insecticide binding,[7] this method did not distinguish between the degrees of insecticide binding by the two plasmas.

TABLE 5. DISTRIBUTION OF (^{14}C) CHLORFENVINPHOS BETWEEN ARTIFICIAL MIXTURES OF ERYTHROCYTES AND PLASMAS

Erythrocytes suspended in:	Ratios of ^{14}C Erythrocyte: plasma	
	Dog	Rat
Dog plasma + 2·8% glucose	9:91	15:85
Rat plasma + 2·8% glucose	*	23:77
Bovine serum albumin (3%) NaCl (0·4%) + glucose (2·8%)	42:58	45:55
Whole blood + 2·8% glucose	11:89	35:65

Washed rat or dog erythrocytes were suspended in solutions of (^{14}C)chlorfenvinphos (10^{-5} M) in rat or dog plasma containing 2·8% glucose, or in bovine serum albumin-saline-glucose solution for 1 hr at 37°. Separated erythrocytes and plasmas were assayed for radioactivity.

* Interaction between dog erythrocytes and rat plasma prevented the measurement of the distribution ratio.

The partition of [^{14}C]chlorfenvinphos between rat and dog serum proteins after separation at pH 8·6 and 4·5 by electrophoresis (Fig. 1) shows that the patterns of peaks of radioactivity are not greatly altered by this change in pH. At pH 8·6, there are two peaks of radioactivity, which in rat serum move with the mobility of the α-globulins and γ-globulin, and which in dog serum extend over the region occupied by the globulins. Two incompletely resolved peaks of radioactivity are seen in rat serum and one in dog serum after electrophoresis at pH 4·5, where the proteins are also incompletely resolved. Since during paper electrophoresis at pH 4·5 or 8·6 chlorfenvinphos does not migrate *per se*, these experiments exemplify apparent binding to plasma lipoproteins and/or proteins, and at the serum concentrations employed, plasma albumin is not involved in the binding mechanism.

When a solution of [^{14}C]chlorfenvinphos in dog or rat serum was suspended in a Visking sac within a large volume of horse serum, the rate of dialysis was slow, but after 24 hr, 15 per cent of the ^{14}C had been released from dog serum and 25 per cent from rat serum. Unlike the plasma proteins, the lipoproteins have densities from 0·93 to 1·16 (g/ml), and three density steps can be selected which cause flotation in the preparative ultracentrifuge of, respectively, the low-density lipoproteins, the low-density plus high-density lipoprotein-2, and the low density plus total high-density lipoproteins.[11] Absence of radioactivity at the top of the tubes under conditions approximating to the first density step (1·063) suggests that chlorfenvinphos is not associated with the chylomicra and lipomicra of dog and rat blood. Under conditions such that most of the lipoprotein appears in the top 1 ml fraction of the tube after

ultracentrifugation,[8] there was a significantly higher concentration of radioactivity associated with this fraction of dog plasma compared with that of rat plasma, whereas the distributions of ^{14}C amongst the other fractions were alike. The different availabilities of chlorfenvinphos in dog and rat blood may accordingly reflect a difference in binding properties of the plasma lipoproteins.

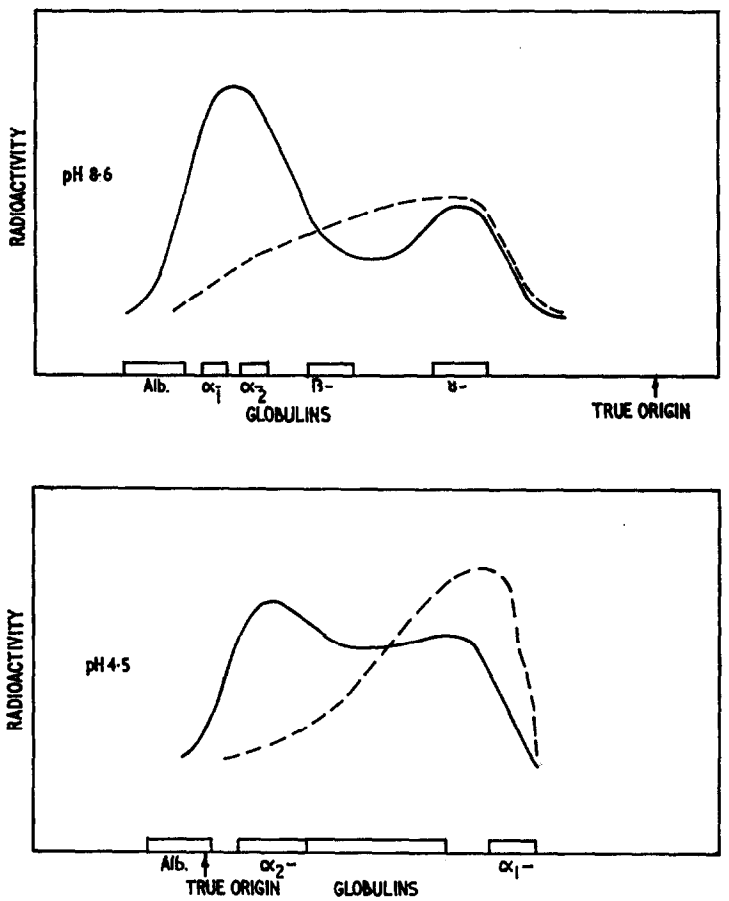


FIG. 1. Partition of (^{14}C) chlorfenvinphos between rat serum proteins (———) and dog serum proteins (.....) after electrophoretic separation at pH 8·6, 4·5. The bars represent the relative positions of the major serum proteins. Insecticide was applied to the paper as 10^{-5} M solutions in the sera.

DISCUSSION

Interaction of several factors would be expected to account for the pronounced difference in acute oral toxicity shown by dogs and rats towards chlorfenvinphos. The physiological and biochemical implications of relatively small differences in each of the parameters that may be involved are therefore of interest.

Absorption/metabolism rates

The concentration of unmetabolised chlorfenvinphos in the peripheral blood of dogs was about one-fifth of that found in rats which had been treated at one-third

of the dose level. This indicates a fifteen-fold difference in the combined absorption-metabolism properties of the two species. This observed difference is made up of the combined differential rates of absorption and metabolism. Unchanged chlorfenvinphos is absorbed from the gastro-intestinal tract of dogs and rats, and ninety, and seventy per cent, respectively, of a single oral dose is excreted as metabolites in the urine. No direct measurements of intestinal absorptions have been made but in dogs a variable proportion of the dose is eliminated in the faeces as unchanged insecticide, dependent on the level of dosing (e.g. 35 per cent was not absorbed at a dose level of 525 mg/kg),[1] thus it is clear that at higher dose levels a limited fraction of the compound is absorbed. The very rapid detoxification and elimination of absorbed chlorfenvinphos as 2-chloro-1-(2′,4′-dichlorophenyl)vinyl ethyl hydrogen phosphate, compared with the more complex pattern of metabolism in rats[1] suggests that a more efficient detoxification mechanism may also be operative in the dog.

Availability of chlorfenvinphos in blood

That the erythrocyte stroma of dogs and rats exhibit no significant difference in permeability towards the insecticide is seen by the fact that dog and rat erythrocytes take up the same proportion of chlorfenvinphos from a solution in bovine albumin. Since "unbound" foreign organic compounds are transported across membranes, the threefold uptake of chlorfenvinphos by rat erythrocytes from rat plasma compared with that by dog erythrocytes from dog plasma is accordingly a measure of the different availabilities of the insecticide in the blood of the two species.

Brain-uptake rates

In chlorfenvinphos intoxication, the brain is involved, (a) since electroencephalographic recordings during intoxication are consistent with (organophosphate) inhibition of neurohumoral transmission, and (b) since severe toxic symptoms are associated with brain uptake of the insecticide. The toxic agent in chlorfenvinphos intoxication therefore appears to be unmetabolized insecticide, and this is also consistent with the non-toxicity of all the chlorfenvinphos metabolites.[1] Hence, the mode of action of this insecticide in animals would seem explicable in terms of brain-uptake of unchanged chlorfenvinphos and of its inhibitory effect on cerebral acetylcholinesterase; this is held to be important. In some animals, complicating symptoms might also be caused by peripheral effects, but these have not been studied.

A dose level of [^{14}C]chlorfenvinphos, which produced a lower concentration of unchanged insecticide in the circulating blood of rats than dogs, afforded a two to three times higher brain-uptake of insecticide in rats than in dogs, 1 hr after dosing. (This measure of the difference in brain-uptake rates may be too low; although all of the radioactivity in the brain of rats was due to unchanged insecticide, this could not be established in the case of the dog.)

Effects on brain AChE

There is a significant difference in the behaviour of the cerebral AChE of the dog and rat towards chlorfenvinphos *in vivo*, and work with homogenates showed that the rat-brain enzyme was almost ten times more sensitive than dog-brain enzyme. There will be a tendency *in vivo* for chlorfenvinphos to associate with the parts of the neurons that are rich in cerebral lipids, and only a small proportion of insecticide

molecules in the brain would be available for complexing with neural acetylcholinesterase. This may account for the apparent insensitivity of dog cerebral acetylcholinesterase towards chlorfenvinphos *in vivo*, and for the fact that in rats, *in vivo* effects take several minutes to develop after an effective insecticide concentration has accumulated in the brain.

Species difference in acute oral toxicity

In rats and dogs, the concerted interaction of the observed proximate differences in the rates of absorption and metabolism, the availability in blood, the rate of brain uptake and the sensitivity of brain AChE towards chlorfenvinphos would account for a 900–1350 fold difference between the acute oral toxicities of the insecticide in the two species. No claim is made for the accuracy of the values for the difference in the various parameters, which seemed to us to be important, but the fact that our analysis accounts for such a large difference in oral toxicity in the two species appears to justify this approach.

The different sensitivities of mammalian brain acetylcholinesterases towards chlorfenvinphos are reminiscent of the different rates of inhibition of the cholinesterases of resistant and susceptible insect strains by organophosphates, which are due to single gene mutants.[12, 13] Insensitivity of a mammalian enzyme towards a suitable substrate, for example dog-brain acetylcholinesterase towards chlorfenvinphos, is almost certainly due to a recessive gene,[14] and genetical considerations might also be applicable to the initial reactions of that insecticide, which determine the course of its metabolism in dogs and rats.[1] Well-authenticated correlations between enzymic activity and genetic make-up in mammals could be important for the future, as they might help in predicting the position of man from toxicological experiments with animals.

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