TOXICITY AND METABOLISM OF DIELDRIN IN RATS

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The main constituent of the insecticide dieldrin has been studied in male and female rats. The LD_{50} s depend on the route of injection and, after intravenous infusion, on the rate of infusion. After oral injection of a 4% solution in arachis oil, deaths take place within six hours or from two to seven days later; but the first convulsion occurs at 40 min. over a wide range of doses. For further studies ^{36}Cl -dieldrin was used. Absorption from the gastrointestinal tract varies with the vehicle and is via the portal vein, not the thoracic lymph duct. Distribution is initially general, but within a few hours of injection it is heavily in favour of fat. Excretion of ^{36}Cl averages about 5% per day. It is markedly increased by a restriction of diet, which reduces the body fat. From the cannulated bile duct, excretion accelerates as the rat loses weight, exceeding 10% per day after a few days. Only 3% of the ^{36}Cl is excreted as dieldrin unless the bile is cannulated, when 10% may be excreted. The remainder is in metabolites, 90% in the faeces and 10% in the urine. Many metabolites have been demonstrated but not identified. The most important, containing about 60% of the total ^{36}Cl , is excreted in the bile, probably as a glucuronide, and reconstituted in the intestine before being excreted in the faeces.

From these findings one can interpret many of the toxic effects of dieldrin, including the effects of starvation on toxicity and the prolonged sensitization to a second dose (Barnes and Heath, 1964) in terms of the low solubility of dieldrin in water, its high solubility in fat, and its mobilization when fat is utilized. Toxic effects are related to the dieldrin mobilized; there is no need to postulate that dieldrin produces a long-lasting lesion in the central nervous system. The findings and conclusions are shown to be relevant to the toxic effects of dieldrin in man.

Dieldrin, one of the group of chlorinated hydrocarbon insecticides based on hexachlorocyclopentadiene, has not proved especially dangerous in use when compared with some other insecticides, notably parathion, at least in the short term. Although, however, there have been very few deaths, epileptiform convulsions, the typical symptoms of poisoning, have often been observed, and in some spraying operations up to 20% of the operators have been affected (Hayes, 1957). Moreover, such epileptiform seizures may occur weeks after the last exposure (Hayes, 1957, 1959).

One possible explanation of the delayed effects is that dieldrin stored in fat is mobilized in a period of weight loss whether this is due to ill health or to a lack of food; but the possibility that dieldrin produces a more long-lasting lesion in the central nervous system must be considered (Hayes, 1959).

Although the symptoms caused by dieldrin are well known (Hayes, 1957; Patel and Rao, 1958),

nothing is known of its mechanism of action, and it is beyond the scope of the present work to put forward views on this. Our aim has been, by the study of the factors affecting the toxicity of dieldrin and of its metabolic fate, to see which, if either, of the two hypotheses about delayed effects is plausible. The rat is a suitable experimental animal for this, as the effects of a single oral dose persist for at least three weeks, during which time female rats were more susceptible than usual to further dieldrin given orally (Barnes and Heath, 1964).

When this work was started, two insecticides of the dieldrin type were available, isotopically labelled aldrin and dieldrin. As aldrin is converted to dieldrin in mammals (Bann, DeCino, Earle, and Sun, 1956; Hunter, Rosen, Williams, Reynolds, and Worden, 1960a) and probably owes much of its toxic action to the dieldrin produced, dieldrin was preferred: in effect a study of dieldrin is a study of both insecticides.

Strictly dieldrin is the accepted name for an insecticidal mixture containing not less than 85% of 1,2,3,4,10,10 - hexachloro - *exo* - 6,7 - epoxy - 1,4,4a,5,6,7,8,8a - octahydro - 1,4 - *endo*, *exo* - 5,8 - dimethanonaphthalene (H.E.O.D.) with the structure I.

For our work only pure specimens of H.E.O.D., unlabelled or labelled with ^{36}Cl , were used, but they are referred to as dieldrin throughout.

The work on toxicity has been reported elsewhere (Vandekar, 1962) and is only summarized here. The work on excretion and some on metabolism has been the subject of a preliminary communication by one of us (Vandekar, 1961).

Materials and Methods

Dieldrin.—Both unlabelled and ^{36}Cl -labelled dieldrin were gifts from Shell Research Ltd., Sittingbourne, Kent. Unlabelled dieldrin was recrystallized twice from ethanol, m.p. 175.5°. ^{36}Cl -dieldrin, uniformly labelled, of specific activity 96 $\mu\text{Ci/g.}$, was at least 98% pure by isotope dilution and chromatography (Heath, 1960).

Other Compounds.—Shell also gave a specimen of *exo*-6-*exo*-7-dihydroxydihydro-aldrin, *i.e.*, the compound with two hydroxy-groups instead of the epoxy group of structure I.

Rats.—Rats were male and female albinos, both originally of Wistar strain, but maintained for about 15 years as separate closed colonies in England and Yugoslavia. Acute toxicities were determined on the English colony, except for those obtained by intravenous infusion. Distribution and excretion studies were carried out on female rats from the English stock, and metabolic studies on male rats from the Yugoslav stock.

Evaluation of Toxicity.—Mortality up to four weeks after dosing was recorded. LD_{50} values with fiducial limits were calculated as described by Thompson (1947) and Weil (1952) or by probit analysis (Finney, 1947).

Intravenous Infusions.—These were carried out into the vena jugularis as described by Vandekar and Heath (1957).

Cannulations.—The bile duct was cannulated as described by Barnes and Magee (1958) and the thoracic lymph duct as described by Gowans (1957). Operations

were carried out under ether anaesthesia lasting 20 to 30 minutes.

Collection of Excreta.—Urine and faeces were collected from single rats in metabolic cages supplied by Microchemical Specialities Co., Berkeley, California, except when ducts were cannulated; then the rats were placed in restraining cages with free access to food and water (Bollman, 1948).

Estimation, Extraction, and Separation of Metabolites.—Estimations were carried out on urine and faeces and, from rats suitably cannulated, on bile or lymph. All ^{36}Cl -determinations were carried out in liquid-counting tubes, and the results were corrected for background and variable absorption of radiation by solvents of different densities. The Geiger-Müller tubes recorded about 4 c.p.m./ $\mu\text{g.}$ of ^{36}Cl -dieldrin.

Quantities are expressed either as counts/min. or as the weight of dieldrin equivalent to the ^{36}Cl found.

Estimation of Total ^{36}Cl Excreted.—The volumes of urine, lymph, and bile were recorded, and the liquids were counted. Bile was centrifuged first. The solid residue contained negligible ^{36}Cl .

Faeces were dried for 24 hr. in a desiccator, ground with anhydrous Na_2SO_4 , and extracted in a Soxhlet apparatus by one of two methods. (a) For distribution and excretion studies faeces were extracted for 24 hr. with *n*-hexane, and then for another 24 hr. with methanol. Both extracts were concentrated to 10 ml. and counted. (b) For metabolic studies faeces were extracted for 3 hr. with methanol. Less than 2% more activity could be extracted in a further hour. The methanol was concentrated to 5-7 ml., diluted to 50 ml. with 0.1N NaOH, and extracted once with 50 ml. of light petroleum, b.p. 30-50°. (A further extraction removed only about 7% as much activity as the first.) The aqueous layer was then acidified with 5 ml. concentrated HCl and extracted once with 50 ml. of ether. This extracted most of the remaining counts. The sodium sulphate in the Soxhlet thimble was dissolved in the minimum quantity of water and counted. This fraction contained up to 10% of the total activity and contained those labelled compounds not extractable from the contents of the thimble by methanol.

Separation of Metabolites.—Metabolites were separated by solvent extraction, and distribution properties were used to characterize them. This was more practicable than chromatography in view of the very high ratio of natural products to metabolites and the low specific activity of the ^{36}Cl -dieldrin.

The process consists of extracting material in one solvent with successive portions of another, immiscible solvent. For a pure solute the concentrations in the two solvents after equilibration by vigorous shaking together are in a constant ratio to each other, *i.e.*, if the concentrations in solvents 1 and 2 are denoted by C_1 and C_2 , $C_1/C_2 = K$, where K is the distribution coefficient. If now solvent 1 is removed and replaced by an equal volume of the same solvent, and the process is repeated, it can be shown that the quantity Q_A in the first extract

is related to the quantity Q_B in the second extract by the relationship:

$$Q_A/Q_B = KV_1/V_2 + 1, \dots \dots \dots (1)$$

where V_1 and V_2 are the volumes of solvents 1 and 2. For Q_A and Q_B the concentrations in the two extracts can be substituted, and these can be determined directly in a liquid-counting tube. If now the first extract with solvent 1 is extracted successively with one or more equal volumes of solvent 2, the concentrations in successive extracts with solvent 2 obey the relationship:

$$Q_A/Q_B = V_1/V_2 K + 1, \dots \dots \dots (2)$$

Finally, K can be determined directly by estimating the concentrations in the two solvents in equilibrium, but this is less reliable as the result may be biased by the presence in one solvent of some inextractable radioactivity. For ease of interpretation solvents should be chosen such that K is in the range 0.5 to 2.0.

Substantially the results fell into one of four groups:

(a) The ratios Q_A/Q_B were constant throughout several extractions, both of solvent 2 with solvent 1, and of the first extract of solvent 1 with solvent 2. Also the concentrations in both solvents gave a value of K consistent with that determined from equations 1 and 2. Then only one compound was likely to be present and was characterized by the value of K found. K is more characteristic of a compound than a melting point (Heath, 1961).

(b) The ratios Q_A/Q_B were constant, but the ratios of the concentrations in the two phases after several extractions gave a different value of K . This indicated that two compounds were present, one with the partition coefficient calculated from equations 1 or 2, and another inextractable from one solvent by the other.

(c) The ratios Q_A/Q_B were not initially constant but became so after a few extractions. The final constant ratio demonstrated the presence of a compound characterized by its value of K . The initial higher ratios might have indicated the presence of a second more extractable compound; but as the extracts contained appreciable quantities of natural products, which were also separated by the distribution procedure and which might therefore have carried with them some ^{36}Cl , inconstant ratios could not be taken as evidence of a second compound without confirmation in other ways.

(d) Haphazard results were obtained. In principle this could be due to decomposition of a labelled solute during partitioning. In practice it was always associated with the presence of interfacial material on which solute was presumably adsorbed.

Detailed procedures are given in the Results section.

Estimation of Dieldrin in Extracts.—Dieldrin was estimated by isotope dilution. Carrier dieldrin was added to the extract, which was evaporated to near dryness, and dissolved in *n*-hexane. In one case the *n*-hexane was extracted with methyl cyanide to separate the dieldrin from fat (Jones and Riddick, 1952), and the methyl cyanide was removed by evaporation and the residue dissolved in *n*-hexane. The dieldrin was then recrystallized from *n*-hexane and/or ethanol, and the specific

activity was determined after each recrystallization. The ^{36}Cl -dieldrin in the original sample was calculated from the steady specific activity finally achieved.

Estimation of Chloride Ion.—Chloride ion was estimated in some sodium sulphate residues in the Soxhlet thimbles and in a bulked specimen of urine. The sodium sulphate residue was dissolved in water. Thereafter both sodium sulphate residues and urine were treated in the same way. Each specimen was acidified with H_2SO_4 , extracted twice with two equal volumes of *n*-butanol, boiled to remove the residual *n*-butanol, and centrifuged. The clear supernatant was made 1N with respect to HNO_3 , 1 ml. of N HCl was added as carrier, the solution was heated to 60–80°, and 0.5M AgNO_3 was run in until the precipitate of AgCl coagulated. The precipitate was filtered, dissolved in concentrated NH_3 solution, and counted.

Results

Acute Toxicity.—In Table 1 it is shown that dieldrin was about equally toxic to rats whether given orally or intraperitoneally. As found by other workers (Treon and Cleveland, 1955), dieldrin given orally was more toxic to female rats. The difference

TABLE 1
ACUTE TOXICITY OF DIELDRIN IN RATS

Route of Administration*	Sex	Age (mth)	LD_{50} (mg./kg.)	95% Fiducial Limits (mg./kg.)
Oral	F	3-4	50.8	41.3 - 62.5
	M	3	63.5	54.3 - 74.2
Intraperitoneal	F	3	55.9	47.9 - 65.3
Intravenous	F	1	8.4	7.9 - 8.9
	F	3	8.9	7.6 - 10.3

*Dieldrin was given orally in arachis oil (40 mg./ml.) and intraperitoneally and intravenously in glycerol formal.

was not significant in either set of experiments, but the combined results suggest that it is real. Deaths took place either in the first six hours after injection, or from two to seven days after, and only one later. The observations after oral injections are summarized in Table 2. The results confirm earlier work by J. M. Barnes (personal communication), in which, however, the division between early and late deaths was not quite so complete. After intraperitoneal injection 17 out of the 20 deaths took place in the second period. Death was always shortly preceded by an epileptiform seizure.

The first symptoms were always observed at the same time, 40 ± 10 min. ($\pm$ S.D.) after oral injection regardless of the dose (20 to 120 mg./kg., 24 rats per dose). When the rats were starved for 24 hr. before dosing the onset of symptoms was delayed about 20 min., a significant time at every dose. As

TABLE 2
CUMULATIVE DEATH RATES OF FEMALE RATS AFTER
SINGLE ORAL INJECTIONS OF DIELDRIN

Dose (mg./kg.)	Time after Injection (days)					
	0.25	1	2	7	14	28
20	1	1	1	2	3	3
40	9	9	9	13	13	13
80	10	11	11	15	15	15
160	12	12	13	21	21	21
Total	32	33	34	51	52	52

Groups of 24 rats were used at each dose.

symptoms follow intravenous injection within two to five minutes, these observations suggest that the absorption of dieldrin is controlled by the amounts of some naturally occurring materials in the gut which enable dieldrin to be absorbed, and not by the quantity of dieldrin present. Symptoms were still more delayed in rats given dieldrin intraperitoneally, presumably because absorption was slower (Vandekar, 1962).

Dieldrin was much more toxic by intravenous injection (Table 1); symptoms appeared within a few minutes, and death took place after lethal doses within 40 min., always immediately after the fourth, fifth or sixth epileptiform seizure. The rats that survived soon recovered and showed no delayed effects. Mice responded similarly (Heath, 1960).

FIG. 1.—Tolerance of dieldrin at different rates of infusion into the vena jugularis of male rats (240-260 g.). The boxed circles represented rats which survived after the infusion was stopped. The remaining rats died.

When, however, dieldrin was infused slowly into the vena jugularis, rats tolerated much larger quantities, the amount depending on the rate of infusion (Fig. 1). Rats could tolerate nearly as much by this route as orally; but they then resembled rats treated orally by showing delayed symptoms and sometimes by dying a few days after the infusion had been completed (Vandekar, 1962, and observations made on male rats in the present work).

Absorption.—A very full study has been made by Hunter *et al.* (1960b) to whom the reader is referred for many details. These workers suggested that absorption was spread over several days. We found that very little remained for as long as 24 hr. in the gut of our rats (Table 3), but that quite high proportions were excreted rapidly in the faeces. It is shown later that much of this material must have been unabsorbed dieldrin.

TABLE 3
PERCENTAGE OF ¹⁴Cl INJECTED ORALLY AS DIELDRIN
(25 mg./kg.) FOUND IN THE GASTROINTESTINAL TRACT
AND FAECES AT VARIOUS TIMES AFTER INJECTION

Material	¹⁴ Cl as Dieldrin (% of initial dose) found after				
	Hours		Days		
	1	2	1	2	4
Stomach	27	19	2	7	0.6
Small intestine	10	40	1	2	
Large intestine	37	15	3	3	
Faeces	6	0	30	10	56

To discover the route of absorption from the gut the thoracic lymph duct was cannulated and the lymph was collected for 24 hr. after an oral injection. The results are shown in Table 4. Of the dieldrin

TABLE 4
ABSORPTION OF DIELDRIN FROM THE GUT AFTER
ORAL INJECTION*

Material	Dieldrin Recovered (%)
Faeces	20
Urine	0
Stomach and intestine	3
Remaining tissues	50
Lymph	8

*A male rat (275 g.) was cannulated the previous evening and given 25 mg./kg. of dieldrin in arachis oil (25 mg./ml.). Lymph, faeces, and urine were collected for 24 hr. The rat was then killed and the total ¹⁴Cl determined.

absorbed, only about one-seventh was recovered from the lymph. Thus most of the dieldrin was absorbed via the portal vein. (The total recovery was only 81%. The figure most likely to be in error is that for the dieldrin in the 'remaining tissues', *i.e.*, the fraction in the lymph should be even lower.)

The rate of absorption through the lymph was

FIG. 2.—³⁶Cl estimated as dieldrin in lymph after oral injection of (A) 25 mg./kg. in arachis oil, and (B) 50 mg./kg. in glycerol formal.

slowed considerably when dieldrin was given in glycerol formal even at a double dose (Fig. 2), *i.e.*, the solvent used influenced the rate of absorption, a common observation.

Distribution of ³⁶Cl after Injection of Dieldrin.—

The results obtained after oral injection and intravenous infusion are shown in Table 5. After oral injection maximum concentrations were reached in one to two days, the highest concentrations appearing in the fat. Some redistribution took place in favour of fat within four days. After infusion the distribution was similar to that shortly after oral injection.

TABLE 5

DISTRIBUTION OF ³⁶Cl AS DIELDRIN (μg./g.) AFTER ORAL INJECTION AND INTRAVENOUS INFUSION

Tissue	Oral Injection (25 mg./kg.)					I.V. Infusion	
	Hours		Days			A*	B*
	1	2	1	2	4		
Brain	5	6	3	4	1	8	9
Liver	16	11	13	17	5	23	36
Kidney	13	23	20	17	6	7	8
Heart, lungs, and spleen	2	8	5	6	5	—	—
Muscle	2	4	8	13	2	4	5
Skin and subcutaneous fat	3	6	26	29	7	—	—
Genital organs and fat	4	4	27	33	18	—	—
Mesenteric fat	18	29	70	116	22	—	—
Retroperitoneal fat	17	18	78	97	48	15	34

*A, 8 mg./kg./hr. for 1 hr.; B, 4 mg./kg./hr. for 4 hr. Both rats were killed immediately after infusion.

A Recoveries were over 90% in the first two hours and over 77% up to four days.

Excretion of ³⁶Cl as Dieldrin or its Metabolites.—

The rate of excretion of ³⁶Cl was studied in female rats given ³⁶Cl-dieldrin by slow intravenous infusion. This mode of administration was preferred to oral injection because the results could not be complicated by the excretion of unabsorbed dieldrin, which can account for a high proportion of the total dose (see Table 3). There is also reason to believe, from the variable response to oral dosing and from the results in Table 3, that very variable amounts are absorbed by this route. After infusion an exactly known quantity was absorbed. It was preferred to intravenous injection because more dieldrin could be injected (Fig. 1) and there was less risk that dieldrin would be precipitated in the blood stream, as may occur after rapid intravenous injection (Heath, 1960).

Female rats (205 to 215 g.) were therefore infused at 680 μg./hr. for 150 to 300 min., thus receiving total doses of 8 to 16 mg./kg., and faeces and urine were analysed daily for ³⁶Cl. By the end of the experiments, 28 and 42 days respectively, 70% of the injected dose had been recovered from the faeces (Figs. 3 and 4). Faecal excretion is therefore much the most important route of elimination. Only about 10% of the dose was excreted in the urine.

At the end of the experiments the rats were killed and the ³⁶Cl remaining in them was estimated. One rat (Fig. 3) contained 3.7% of the initial dose, the other (Fig. 4) 17.5% of the initial dose, giving total recoveries of 85% and 92%, respectively. As shown in the next section, the method of estimating ³⁶Cl in the faeces was likely to give recoveries about 10% low. This error probably accounts for much of the deficit.

The effect of food restriction on excretion is shown in Figure 3. The rat was given 8 g./day, about one-third of its normal consumption, for the third week of the experiment. During this period the rat lost 25% of its weight, and after the fourth day the rate of excretion increased considerably. When, however, during the fourth week the rat was given unrestricted access to food it soon regained its original weight and the excretion of ³⁶Cl almost ceased. Excretion in the urine was influenced in a similar way.

The effects of short periods of starvation are shown in Figure 4. As was to be expected, excretion fell during two days' starvation since excretion of faeces nearly ceased; but when faeces were produced afterwards the excretion rate was immediately greatly increased. The overall effect of short periods of starvation was to increase the average rate of excretion. The effect was confirmed in male rats.

In order to find the route by which dieldrin and/or

FIG. 3

FIG. 4

FIG. 3.—Excretion of ^{36}Cl from a female rat weighing 210 g. and given 16.2 mg./kg. of ^{36}Cl -dieldrin. —●— in faeces; —▲— in urine; — body weight.

FIG. 4.—Excretion of ^{36}Cl from a female rat weighing 210 g. and given 10.3 mg./kg. of ^{36}Cl -dieldrin. —●— in faeces; —▲— in urine.

its metabolites are excreted into the gut some experiments were carried out on rats with cannulated bile ducts (Fig. 5). All rats showed the effects of starvation due to the disturbed absorption of food in the absence of bile, and lost 30% of their weight in five to 10 days. Accordingly the rate of excretion was accelerated. As shown in Fig. 5A, a high proportion of the dose could be collected in the bile in seven days, but whether the duct was cannulated before or seven days after injection some ^{36}Cl was always excreted in the faeces via the intestinal mucosa (Figs.

5B and 5C). In males the intestinal mucosa contributed 10% or less of the total excreted, about one-third of the contribution in females.

Metabolites.—In this part of the work we tried to characterize the metabolites rather than to estimate their relative proportions, which are therefore given only roughly. Work was carried out on bulked samples from male rats weighing 300 g. to obtain enough ^{36}Cl -activity.

FIG. 5.—Recovery of ^{36}Cl in faeces and bile from female rats given ^{36}Cl -dieldrin. A, rat (205 g.) given 8 mg./kg. (faeces were not collected); B, rat (210 g.) given 11.0 mg./kg.; C, rat (210 g.) given 11.4 mg./kg. Amounts excreted in bile —○—; in faeces —●—.

Faecal Extracts from Uncannulated Rats.—When the bile duct was not cannulated the faeces yielded four extracts containing the following percentages of ^{36}Cl : light petroleum (57%); ether (31%); aqueous residue (2%); and material retained in the Soxhlet thimble (sodium sulphate residue, 10%). This last fraction was probably not extracted by the method used to get the results in the last Section, which are therefore probably about 10% low.

(a) The petroleum extract was shown by isotope dilution to contain less than 2% of dieldrin. On extraction with 0.1N NaOH no ^{36}Cl was extracted, i.e., the extract contained no acidic compounds.

The petroleum was evaporated from a specimen, which was then dissolved in *n*-hexane, and extracted with successive equal volumes of 10% aqueous

methanol (10 vol. water + a little HCl made up to 100 vol. with methanol. The acid aided separations by reducing the emulsifying properties of the extract but did not affect the experiments in any other way). The results indicated that 98% of the ^{36}Cl was present as one compound, with a distribution coefficient (K), aqueous methanol/*n*-hexane, of 4.0 ± 0.1 . For dieldrin $K = 0.3$. The experiment was repeated with 30% aqueous methanol with the results shown in Table 6. The values of the partition coefficient calculated are shown. The first four are

TABLE 6
EXTRACTION OF PETROL EXTRACT OF FAECES IN
n-HEXANE BY 30% AQUEOUS METHANOL*

Extracts	Counts/min.	K ($\pm$ S.E.)
First aqueous-MeOH	245.6	0.69 ± 0.07
Second aqueous-MeOH	145.2	
Third aqueous-MeOH	97.8	
Fourth aqueous-MeOH	59.5	
Fifth aqueous-MeOH	38.7	0.52 ± 0.03
<i>n</i> -Hexane in equilibrium with fifth aqueous-MeOH	74	
First aqueous-MeOH after <i>n</i> -hexane extraction	96	0.76 ± 0.04
<i>n</i> -Hexane in equilibrium	126	

*The specimen of faeces extract in *n*-hexane was extracted five times with 30% aqueous methanol, and count rates were recorded in counts/min. The counts/min. in the *n*-hexane in equilibrium with the fifth aqueous methanol extract are also given, as well as the counts/min. in the two phases when the first aqueous extract was partitioned with *n*-hexane. The value of the distribution coefficient, K, aqueous-MeOH/*n*-hexane, are given for each set of results.

the most reliable (see under 'Materials and Methods') and give an average value of 0.59 ± 0.04 . The presence of about 1% dieldrin, $K = 0.037$, probably explains the low value of 0.52. The last value, 0.77, suggested that about 3% of the ^{36}Cl was in compounds which could not be extracted by *n*-hexane. Thus 96% of the ^{36}Cl behaved as a single compound. The value of K for this compound was found to vary between 0.59 and 0.77 in different determinations, probably due to differences of temperature. The temperature was not controlled in these exploratory experiments.

A portion of the specimen was heated in 2N NaOH at 100°C. for two hours. After this treatment 92% of the ^{36}Cl was still extractable by *n*-hexane from 2N NaOH.

The metabolite with these properties will be referred to as metabolite I.

(b) From the ether extract 40% of the ^{36}Cl could be extracted by aqueous sodium hydroxide, *i.e.*, was in acidic metabolites. These were not appreciably extractable by 2N HCl from ether and did not therefore contain the conjugate found in bile (see later). The very large quantities of natural compounds in

this extract, however, made a detailed study impracticable.

The alkaline extraction also removed from the ether most of the naturally occurring emulsifying agents in it. Probably because of this, when the ether was replaced by *n*-hexane, the whole of its ^{36}Cl content (60%) was inextractable from *n*-hexane by either acidic or alkaline solutions. Thus compounds which could not be extracted from an aqueous extract of faeces by hydrocarbon solvents could be extracted when some of the natural products were removed.

When this portion, the neutral ethereal material dissolved in hexane, was extracted five times with 30% aqueous methanol, 5 to 7% was inextractable and was shown by isotope dilution to be dieldrin; 30% was not extractable by hexane from aqueous methanol; and the remainder distributed as metabolite I and was equally stable in 2N NaOH at 100°C. The inextractable portion was difficult to handle because of the presence of interfacial material. However, since about half of it was decomposed by 2N NaOH at 100°C. in two hours, this much at least was not metabolite I, entrained by natural products, but another compound.

(c) Of the aqueous residue the whole was extractable from acidified solution with *n*-butanol. This fraction was not further investigated as the count rates were too low.

(d) The ^{36}Cl in the sodium sulphate, which remained in the thimble after Soxhlet extraction, could not be extracted by light petroleum or acetone and was not present as chloride ions. The metabolites present were obviously highly polar organic chlorine compounds. They were not further investigated.

To summarize, in the faeces about 65% of the ^{36}Cl was present as a single compound, metabolite I, which was neutral and somewhat more polar than dieldrin, although like dieldrin it was extracted quantitatively from water by hydrocarbon solvents. In addition, 6% of the ^{36}Cl was probably present as another neutral, but considerably more polar compound, 12% was present as an acidic compound, about 3% was present as dieldrin, and the remaining 14% consisted of metabolites too polar to extract from aqueous acid with ether. No chloride ions derived from dieldrin were present.

Faecal Extracts from Cannulated Rats.—When the bile duct was cannulated, 80% to 90% of the ^{36}Cl in the faeces was extracted by light petroleum from the alkaline faecal extract. The residue was too inactive to study. At least 80% of the ^{36}Cl in this extract was as dieldrin, by isotope dilution. The remainder was adsorbed on interfacial material and could not be studied further.

Bile.—When the bile was made 2N with respect to HCl and extracted successively with portions of ether, the quantities extracted by the second and subsequent extractions suggested the presence of one compound with a distribution coefficient, K , ether/(acidified bile), of 0.44, and that all but 4% of the ^{36}Cl content of the bile was in this and more ether extractable compounds. A total of 79% appeared to be this one compound, with $K = 0.44$.

To separate a specimen of the compound, acidified bile was extracted twice with 0.5 vol. of ether. These extracts were rejected. The bile was then extracted three times with 2 vol. of ether, and the combined extracts were concentrated to 10 ml. At this stage it was found that about 10% of the ^{36}Cl could not be extracted from this concentrate by dilute hydrochloric acid. Later experiments confirmed this finding: if the compound was concentrated in ether which had been treated with acid, about 10% was converted to a less polar compound. The compound was therefore further separated as follows. The ether concentrate was extracted twice with 2 vol. of 2N HCl, and the acid extract was extracted twice with 2 vol. of ether. The ether was then neutralized with NaOH solution and concentrated. To recover the material in ether the product was then acidified. If acidification was omitted the compound remained on the walls of the vessel even though no aqueous phase could be observed.

The product, in ether, was shown to be one compound by extracting its ethereal solution with successive equal volumes of 2N HCl, and the first HCl extract with ether. The product distributed as a single compound, with K , ether/2N HCl, of 0.73 ± 0.03 (the removal from bile had raised the value of K from 0.44).

The whole was extractable by 0.1N NaOH, *i.e.*, the compound was acidic.

This fact, and the large quantity found in bile, suggested that it might be a conjugate of metabolite I. A portion was therefore heated with 2N HCl for one hour at 100°C. After this treatment about 15% of the ^{36}Cl partitioned on successive extraction from *n*-hexane with 30% aqueous methanol with $K = 0.9 \pm 0.2$. A little metabolite I, extracted from faeces, was added to the extraction system and gave $K = 0.77$. The errors were large because of the low ^{36}Cl content of the specimen. Another specimen was obtained by taking down the bile product in acidified ether. This gave $K = 0.6 \pm 0.1$. On treatment for two hours at 100°C. with 2N NaOH $80 \pm 10\%$ remained extractable with *n*-hexane. Thus this bile metabolite was a conjugate of metabolite I and was converted to metabolite I by acid.

As metabolite I was without acidic properties, and dieldrin possesses no potentially basic groups, it was

probably a hydroxy-compound. The most likely conjugates were therefore glucuronides or sulphates. Glucuronides have pK_a s of about 3.8, and sulphates have pK_a s of less than 1.0. The pK_a of the bile product was therefore determined.

A solution in ether was equilibrated with neutral buffer, when most of the ^{36}Cl was in the aqueous layer. Both layers were then counted and returned to the separating funnel, and sufficient of the acid constituent of the buffer was added to change the pH by a known amount. The layers were shaken together, separated, and counted. The process was then repeated several times. Finally the mixture was made strongly acid, and the concentrations in both layers were again determined. The anionic form of the conjugate was not extractable by ether. The acid form was partly extractable, by an amount determined in strong acid. Half as much would be extracted from buffer of pH equal to the pK_a as from strong acid. The results obtained using citric acid-sodium hydrogen phosphate buffers (McKilvaine's) are shown in Figure 6. The estimated pK_a was 3.88. This is only a rough result and may be up to 0.2 low because, to conserve material, the ionic strength of the buffer was not maintained constant throughout the determination; the buffer capacity of the natural products present was not quite negligible, so that concentrated buffers, ionic strength of about 0.2, had to be used; and the buffers increased the partition coefficient in favour of ether. (The last was a salting out effect, also produced by sodium chloride.) Such

FIG. 6.— pK_a of the major bile metabolite. Experimental points —●—; theoretical line - - - -. The pK_a is the pH at which the experimental line cuts the horizontal line at $\log [\text{salt}]/[\text{acid}] = 0$, *i.e.*, at which the salt and acid concentrations are equal.

effects are expected to reduce the slope of the curve in Fig. 6 from that calculated on the appropriate simple theory, as observed. The value obtained, however, shows that the compound present was probably a glucuronide.

An attempt was made to study the more ether-soluble materials in bile (see the beginning of this Section). Some of the material was extractable by aqueous NaOH, *i.e.*, was acidic, but much of the ^{36}Cl was adsorbed onto solid material and could not be investigated. About one-third was neutral and extractable from water in iso-octane. Of this, less than 10% was dieldrin (isotope dilution).

Thus in bile very little (< 1.5%) was dieldrin, and 78% was a single compound, probably the glucuronide of metabolite I.

A possible structure for metabolite I appeared to be *exo-6-exo-7-dihydroxydihydroaldrin* (see 'Materials and Methods, Other Compounds'), but this compound was shown to distribute between 30% aqueous methanol and *n*-hexane with $K \approx 4$, and not 0.7. The compounds were also shown to be different by isotope dilution.

Urine.—Only about 10% of the total ^{36}Cl was excreted in the urine. Successive extractions yielded four fractions: in light petroleum from alkaline solution (24%); in ether from acidified solution (16%); in *n*-butanol from acid solution (30%); and an inextractable residue (30%).

The ^{36}Cl in the petroleum fraction was dissolved in *n*-hexane and extracted successively with 30% aqueous methanol. About 40% was inextractable and was shown by isotope dilution to be mainly dieldrin. The rest distributed as one compound, with a distribution coefficient (K , 30% aq.-MeOH/*n*-hexane) of 0.86 ± 0.07 . In the same solvent system metabolite I gave $K = 0.72 \pm 0.04$. These values are probably different ($P = 0.05$), but not with enough certainty to establish that the metabolites are in fact different. This was established by treatment of the urine metabolite with 2N NaOH at 100°C. for two hours, when over 90% of the ^{36}Cl appeared in compounds which were not extractable by hexane.

In the ethereal extract, most of the labelled material was acidic, *i.e.*, was extractable by 2N NaOH. Interference by natural products discouraged further investigation of this fraction and also of the *n*-butanolic fraction.

About half of the ^{36}Cl in the aqueous residue was as chloride ion.

Discussion

The toxic manifestations of dieldrin are controlled by its very low solubility in water and its high solubility in fat.

This is shown most clearly by certain effects noticed when the oral toxicity is investigated. First, the response of the rat to oral injection is very variable: some rats die after only 20 mg./kg. whereas others survive 160 mg./kg. (Table 2). This is plainly not due to variations in the sensitivities to dieldrin of different rats; their response is substantially uniform both after intravenous injection (Table 1) and after slow intravenous infusion (Fig. 1). Secondly, the time of onset of symptoms after oral injection is uniform for a given solvent and concentration in it regardless of the dose. These results imply that for a given solvent and concentration the rate of absorption is constant, but that the quantity finally absorbed depends upon the contents of the rat's gastrointestinal tract and its rate of peristalsis. Thirdly, Barnes and Heath (1964) have shown that the LD_{50} depends upon the concentration in the solvent; and in this paper we have shown that the time to onset of symptoms was significantly longer when glycerol formal was substituted for arachis oil as the solvent. All these effects are to be expected of a compound which is not soluble in water, is not freely absorbed, and which depends upon the contents of the gastrointestinal tract for its absorption. It may be concluded also that absorption is substantially confined to the upper part of the gastrointestinal tract, as the time to onset of symptoms is very short compared with the time required for the excretion of a high proportion of the unabsorbed dieldrin (Table 3 and Hunter *et al.*, 1960b).

Further evidence appears from the distribution studies. Shortly after injection by any route a relatively high proportion of the dieldrin appears in the brain and other organs rather than in the fat, though the proportion in the brain is higher the faster the rate of absorption. It is, for example, particularly high after rapid intravenous infusion. After absorption has ceased a further redistribution in favour of fat takes place (Table 5), but this is quite slow, although dieldrin is appreciably 'soluble' in blood (as Moss and Hathway (1964) have shown, it is transported in the blood adsorbed on lipoproteins and on certain proteins). This explains why to load a rat with dieldrin intravenously one has to infuse the dieldrin slowly, since in this way the fat is loaded rather than the central nervous system. This does not imply that there is always a direct correlation between toxic effects and the concentrations of dieldrin found in rats' whole brains. It appears likely from Table 5 that transport within the brain to the affected sites is fairly slow, so that in short experiments during which the blood concentration is changing rapidly, there is little correlation.

The rates of excretion also fit in well with the general hypothesis. The rate of excretion is greatly

influenced by the nutritional state of the rat (Figs. 3 and 4). If the diet is restricted or the rat starved, so that it loses weight, the excretion rate increases. The obvious inference is that the concentration of dieldrin in the blood stream is increased when the fat is mobilized from its depots. In a rat with a cannulated bile duct this effect is particularly obvious. As fat absorption is stopped completely by the cannulation, the rate of excretion of ^{36}Cl in bile accelerates (Fig. 5), and the quantity of dieldrin excreted unchanged in faeces is greater than in a normal rat. As the bile was collected separately and the rats loaded with dieldrin intravenously the dieldrin in the faeces must have been released from the intestinal mucosa.

When metabolism is considered the detailed picture is complex, but certain features stand out. First, very little dieldrin is excreted as such: probably never more than 10% (when the bile duct is cannulated) and normally only about 3%. Secondly, the bile duct excretes polar metabolites very rapidly. As in addition the production of metabolites increases rapidly when weight is lost, there must be either an excess capacity to metabolize dieldrin or the metabolizing enzyme must be readily stimulated. It appears more likely that the disposal of dieldrin is not controlled by the capacity of the rat to metabolize dieldrin but by the rate of flow of dieldrin to the places where it is metabolized, which, from the concentrations of metabolites found in the bile, appear to be in the liver.

The metabolic processes cannot be described in detail. The main metabolite, metabolite I, is excreted in the faeces. It is somewhat more polar than dieldrin but is still a compound of low polarity, extractable quantitatively from water by hexane. The main compound excreted in bile is possibly the glucuronide of this compound. An attempt to hydrolyze it with glucuronidase is under way. Thus probably dieldrin is oxidized in the liver of the rat to a hydroxy-compound, metabolite I, which is conjugated there with glucuronic acid, secreted in bile into the gastrointestinal tract, and re-formed by the action of glucuronidase before excretion. In the urine, a compound with a very similar distribution coefficient to metabolite I but less stable to alkalis is the main compound excreted. The similarity in distribution coefficient suggests that it may be an isomeric hydroxy-compound (Heath, Park, and Lane, 1955). Other compounds are also excreted in the urine and faeces. Some have acidic properties but they do not seem polar enough to be conjugates and may well be compounds in which one of the rings of the dieldrin molecule has been opened with the formation of carboxylic acids. Others are highly polar and are likely to be conjugates. The hexa-

chlorocyclopentadiene moiety in dieldrin is fairly stable since less than 2% of the ^{36}Cl is excreted as chloride ion, but the rest of the molecule is obviously open to attack in several ways once it reaches an organ capable of metabolizing it. Dieldrin persists in the body only because it is not readily transported to these organs.

The excretion and metabolism of dieldrin has also been studied extensively by Ludwig, Weiss, and Korte (1964, and personal communication). They gave their rats very small daily doses of ^{14}C -dieldrin or ^{14}C -aldrin orally over long periods. Their results are in excellent agreement with ours in all main particulars: after the cessation of dosing nearly all the label is excreted within a month, about 90% of it in the faeces; the main faecal metabolite is a little more polar than dieldrin and, as they suggested, differs from the main urine metabolite by its greater stability to alkalis. Very little dieldrin is excreted unchanged. They also found acidic and highly polar metabolites and that a polar metabolite was excreted in the bile one to ten hours after injection. Thus the course of metabolism is not dependent upon the method of dosing nor upon the quantity of dieldrin in the animal. This last in itself is strong evidence that excretion and metabolism are controlled by physical properties, such as relative solubilities and rates of absorption and desorption and not by biochemical factors, which are usually found to be concentration-dependent when a wide range of concentrations is studied.

We can now come to some conclusion about the genesis of delayed effects in rats described by Barnes and Heath (1964) and in the present paper. On the present view, from a healthy rat the rate of excretion of dieldrin metabolites should fall exponentially, as Ludwig *et al.* (1964) found. From rats given a toxic dose the rate of excretion may remain steady (Fig. 3) or even increase during the first four to seven days, *i.e.*, the concentration of dieldrin in the blood-stream does not fall while the rat remains underweight from loss of fat. After this period the rat regains weight, the rate of excretion falls, and deaths rarely take place. During the middle period, two to seven days, dieldrin is redistributed in the body in favour of fat via the blood-stream (Table 5). The less fat available, the less will be taken up by it, and the greater the exposure of the central nervous system. Accordingly, the rats that lose most weight in the first two days are most likely to die subsequently. The situation is analogous to that in rats under-fed before dosing, and which are considerably more susceptible. The high toxicity of a second dose given within three weeks of the first dose cannot be explained so simply, as deaths after a second dose tend to take place in the first few hours after it is given, before much fat

can be utilized. With some reasonable assumptions about the rates of the various processes some explanation can, however, be attempted. The rate of loss of weight after an injection of dieldrin is very rapid, up to 20 g. from a 200-g. rat in one day, perhaps due to the convulsive activity and anorexia. In a rat previously dosed with dieldrin this fat releases dieldrin, both towards blood and towards the fat not yet mobilized. In effect a concentration barrier is interposed between the blood and the fat, so that the dieldrin coming in from the fresh dose is diverted away from fatty tissues, and much more reaches the central nervous system. It is also possible that a high fatty acid concentration in the blood may increase the solubility of dieldrin in blood. The effect does not depend upon the total proportion of fat in the body but upon its rate of mobilization. As the rate falls after the first day, the situation described earlier is reached, and redistribution in favour of fat can take place in survivors, a normal proportion, 10 to 20%, of which die later.

In man the most important route of uptake is in practice percutaneous (Hayes, 1957, 1959). Percutaneous absorption is well simulated by very slow intravenous infusion, except that probably more is held in subcutaneous fat in the former case. The toxic effects are rather similar in that the most severe symptoms are likely to be observed a few days after exposure has ceased and often develop after a few successive exposures, *i.e.*, after a situation not unlike the double-dosing experiments described by Barnes and Heath (1964). Dieldrin has been found in many specimens of human fat (Hunter, Robinson, and Richardson, 1963; Dale and Quinby, 1963), but there is little evidence as to its persistence. Such serial analyses of dieldrin in blood as there are (Kazantzis, McLaughlin, and Prior, 1964) indicate, however, that dieldrin persists about eight times as long as in the rat. On this basis, symptoms should not appear later than two to four months after the last exposure unless perhaps fat depots are greatly decreased by illness or starvation, and there should be no special sensitivity to a second dose after six months. This accords with observation. There appears to be no case of symptoms appearing longer than about four months after exposure (Hayes, personal communication), and although many cases

of poisoning have been noted without any follow-up being carried out, enough have been followed up to make it unlikely that symptoms ever occur later. The evidence, therefore, is that in the rat and in man dieldrin does not produce a long-lasting lesion in the central nervous system. The toxic effects can be accounted for by the dieldrin mobilized in the body at the time they take place, and they disappear with the dieldrin.

The only positive evidence against this view is provided by a single rat, which convulsed at intervals throughout its (normal) life span after a regimen involving successive dosing with dieldrin (Hayes, 1957; and personal communication). This effect has never been reproduced, and it is at least doubtful if it should be attributed solely to the action of dieldrin (Hayes, personal communication).

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