Interestingly, the percentage of motoneurons afflicted (not more than 1 in 61) was very small. The authors propose a neuronal migration from the injected muscle into the representative parts of the spinal cord. Their observations are concordant with ours.

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Biodegradation of Alpha-Hexachlorocyclohexane *

III. Decrease in Liver Non-Protein Thiol after Intragastric Application of the Drug

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Summary. 1. Alpha-hexachlorocyclohexane (alpha-HCH) reduces total non-protein thiol in the liver of the rat through a reduction in glutathione (GSH) content. Brain and kidney non-protein thiol is not affected.

2. Maximum reduction was by about 50% 18 h after 400 mg/kg, the highest dose tested. At this time, the decrease in liver non-protein thiol was linearly related to dose and to alpha-HCH-concentration in liver as a whole and in the liver cytosol fraction.

3. The effect is thought to be causally related to GSH-dependent degradation of alpha-HCH in the liver, previously shown to take place in vitro.

Key words: Alpha-Hexachlorocyclohexane — Biodegradation — Liver Glutathione.

Studies in vitro have implicated glutathione (GSH) as a cofactor in enzymic reactions by which alpha-hexachlorocyclohexane (alpha-HCH) is converted to hydrophilic substance in the rat (Kraus *et al.*, 1973). The liver cytosol fraction, in particular, was shown to contain enzymic activity that catalyses a reaction between GSH and alpha-HCH resulting, presumably, in the formation of a GSH-conjugate (Portig *et al.*, 1973). This paper reports on experiments the result of which is thought to add some weight to these observations in that injection of alpha-HCH into the rat's stomach is shown to be followed by a transitory decrease in the GSH-content of the liver.

Methods

Alpha-1,2,3,4,5,6-hexachlorocyclohexane (alpha-HCH) was from the batch used by Portig *et al.* (1973). L-glutathione (GSH) was purchased from E. Merck AG, Darmstadt, yeast glyoxalase I from Boehringer Biochemica, Tutzing. Ellman's reagent was obtained through Serva Chemicals, Heidelberg, methyl glyoxal through Roth Chemicals, Karlsruhe.

Animal Experiments

Female Wistar rats of 120–180 g body weight (7 to 8 weeks old) supplied by Zentralinstitut für Versuchstierzucht, Hannover, were used. In the laboratory

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to "Altromin I 10" pellets and tap water.

Alpha-HCH was dissolved in olive oil (Oleum Olivarum DAB 7) and administered by stomach tube. The vehicle was found to produce a decrease in liver non-protein thiol content when given alone (Table 1). Because of this, the dose of oil was kept constant at 10 ml/kg and HCH-concentration in the oil was varied.

The animals were killed between 9.00 and 10.00 in the morning, the time of pretreatment being set accordingly. In order for the assays of tissue thiol to be completed in 2 h, the maximum number of rats killed on any one day was eight. Killing was by decapitation; after cessation of reflex movements, the organs to be examined were dissected out and weighed. 2 g of liver was assembled from small pieces cut off each lobe and was put into ice-cold perchloric acid for estimation of non-protein thiol; the remainder of the organ was used for determination of HCH-concentration as appropriate. Brain and kidneys were taken in toto for estimation of thiol content. 1.5 min elapsed between picking an animal up and immersion of tissue in acid.

Analytical Procedures

Tissue non-protein thiol was estimated on extracts prepared as follows: 1 part of tissue was homogenized in 4.5 parts of 1 N $HClO_4$ with an Ultra-Turrax blender and centrifuged at 12000 × g for 10 min. 2.5 ml of the supernatant was added to 0.9 ml of 1.75 M K_2PO_4, left in an ice bath for 15 min, and filtered (Bernt and Bergmeyer, 1970). Aliquots of filtrate were then taken for estimation of total thiol content or of total thiol and of GSH content. Every assay was run in triplicate. Total thiol and GSH content of the extracts was found to remain constant for 3 h after which time it started to decline.

Total thiol was estimated using the method of Ellman (1959). Aliquot volume of extract added to the reaction mixture was 50, 100, and 200 µl for liver, kidneys and brain, respectively. For liver extracts from untreated rats readings taken at 412 nm were 10 to 15 times a water blank. 10 and/or 20 µg of GSH freshly dissolved were routinely tested for colour formation and consistently gave a mean ε_{412} of 13800 (Ellman: 13600). Absorption at 412 nm increased as expected when known amounts of GSH were added to extracts of liver before or after removal of perchlorate, but loss of endogenous thiol was not checked on.

GSH-content of liver was estimated on 100 µl aliquots of extract using the method of Wieland *et al.* (1955) as modified by Bernt and Bergmeyer (1970), 0.1 M phosphate buffer being used instead of water. ε_{340} was 3400–3600.

Alpha-HCH—content of liver was estimated using GLC. Liver was homogenized in 5 vol. of water with a Potter-Elvehjem type glass homogenizer. The homogenate was weighed and divided in portions by weight. One portion was extracted with 2 vol of cyclohexane, the other was spun at 100000 × g (av)—140000 × g (max) for 90 min, and weighed aliquots of the supernatant, which was called the cytosol fraction, were also extracted with 2 vol of cyclohexane. Conditions of assay were those described by Portig *et al.* (1973).

Values for P refer to application of Student's t-test.

Results

Table 1 shows how the non-protein thiol content of rat liver was found to change with time during the 24 h following oral application of 50, 200, and 400 mg/kg of alpha-HCH. Olive oil, the vehicle used for therefore, kept constant.

Table 1. Non-protein thiol content (µEq/g) of rat liver after three different doses of alpha-hexachlorocyclohexane
Figures are mean ± S.D.; number of estimations in brackets

Time after application (h)	Olive oil only (10 ml/kg)	Plus alpha-HCH		
		0.5% (= 50 mg/kg)	2% (= 200 mg/kg)	4% (= 400 mg/kg)
0[a]	7.50 ± 0.70 (63)			
6	6.26 ± 0.56* (12)	5.40 ± 0.46 (4)	5.22 ± 0.42 (4)	5.98 ± 0.58 (4)
12	6.78 ± 0.92* (24)	5.77 ± 1.04** (8)	4.50 ± 0.76** (16)	4.80 ± 1.20** (8)
18	7.15 ± 0.61 (25)	6.53 ± 0.81** (12)	5.55 ± 0.83** (12)	4.07 ± 0.76** (20)
24	7.48 ± 0.50 (16)	7.51 ± 0.32 (4)	6.19 ± 0.53** (8)	6.02 ± 1.01** (9)

[a] Untreated rats.
* $P < 0.025$ for difference to untreated rats.
** $P < 0.025$ for difference to rats given the vehicle only.

Total non-protein thiol in the livers of *untreated* rats varied to some extent (range 5.89–8.86 µEq/g in 63 animals), but this variation as well as the mean (see Table 1) are of the order recently reported from other laboratories (Berthod and Chasseaud, 1970; Jocelyn, 1972) for this species. GSH, estimated with the glyoxalase method, accounted for 87.4 ± 5.7% (N = 19) of total non-protein thiol in the livers of untreated, oil-treated, and alpha-HCH-treated rats, whose total thiol content ranged from 8.32 down to 3.21 µEq/g. This, too, confirms what others have found (see Jocelyn, 1972) and, in the context of this study, means that the fall in liver non-protein thiol brought about by alpha-HCH is due, predominantly, to a fall in liver GSH.

Attention was paid to the influence that reduced intake of food may have on liver GSH content (Lindan and Work, 1953). To monitor the individual rat's feeding behaviour between dosing and killing, body weight and relative liver weight were recorded and were compared to body weight increase or decrease and to relative liver weight in untreated rats that had or had not access to food for the same periods of time.

Injection of olive oil alone into the stomach reduced food intake somewhat for the next 6 h, but the reduction was by no means so large as to explain the fall in liver GSH. When HCH was added to the oil, feeding was impaired more. However, this only became apparent after the large doses, and it could not, by itself, account for the decrease in liver GSH. E.g., the mean decrease 18 h after 400 mg/kg of alpha-HCH (see Table 1), the largest dose tested, exceeded by 1.8 µEq/g the mean decrease seen after 18 h starvation, when the animals had lost on the average 12 g of their initial body weight, and their mean relative liver weight was 3.46 g/100 g as compared to 4.3 g/100 g in non-starved rats of the same age. By contrast, 18 h after 400 mg/kg alpha-HCH the mean reduction in body weight was by only 3.5 g, and mean relative liver weight was 3.95 g/100 g.

Table 2. Drug content (nmoles/g) of rat liver after three different doses of alpha-hexachlorocyclohexane

Figures are mean ± S.D.; number of estimations in brackets. Line a) gives drug content of whole liver, line b) drug content of cytosol fraction (equivalent of 1 g of fresh liver)

Time after application (h)		50 mg/kg	200 mg/kg	400 mg/kg
6	a	133 ± 16 (7)	232 ± 79 (7)	568 ± 88 (6)
	b	8.9 ± 3.0 (3)	21.3 ± 5.3 (3)	49.7 ± 11.0 (3)
12	a	115 ± 14 (7)	394 ± 98 (7)	850 ± 72 (4)
	b	5.1 ± 0.5 (3)	19.5 ± 1.3 (3)	48.7 ± 6.5 (3)
18	a	84 ± 18 (8)	341 ± 65 (8)	654 ± 69 (8)
	b	6.0 ± 0.5 (4)	21.2 ± 4.6 (4)	40.5 ± 5.5 (7)
24	a	79 ± 5 (4)	296 ± 22 (4)	592 ± 23 (3)

The decrease in liver non-protein thiol produced by alpha-HCH was significant at $P<0.025$ or less (for difference to oil-treated animals) with all three doses tested 12 and 18 h after application, and with the two large doses also at 24 h. Although impaired feeding behaviour contributed to some extent, it was clear that the decrease was largely due to a different cause. A likely cause was thought to be consumption of GSH in an enzymic reaction with alpha-HCH, shown previously to take place in vitro (Portig *et al.*, 1973), and it was of interest, therefore, to determine the amount of alpha-HCH held by the liver while the drug passes through it. This was done (Table 2) for liver as a whole and for its soluble fraction (cytosol) which, when tested in vitro, appeared to contribute most to GSH-dependent HCH-degradation in the liver cell (Kraus *et al.*, 1973).

Not surprisingly in view of the hydrophobic character of alpha-HCH, more than 90% of the total amount of drug contained in the organ was always found to be associated with the membranous particles. Also, while drug concentration in the whole organ changed with time, particularly after the two large doses, drug concentration in the membrane-free fraction remained comparatively constant for the first 18 h.

Drug concentration in the whole organ is maximal about 12 h after application (Table 2), and it is after approximately this interval of time that, with increasing dose, the decrease in GSH content becomes most marked. 18 h after application there was a straight-line relationship between drug effect and dose, and between drug effect and drug concentration in the whole of the liver as well as in the liver cytosol fraction (Fig. 1, lower curve). It may be worth noting that this curve intercepts

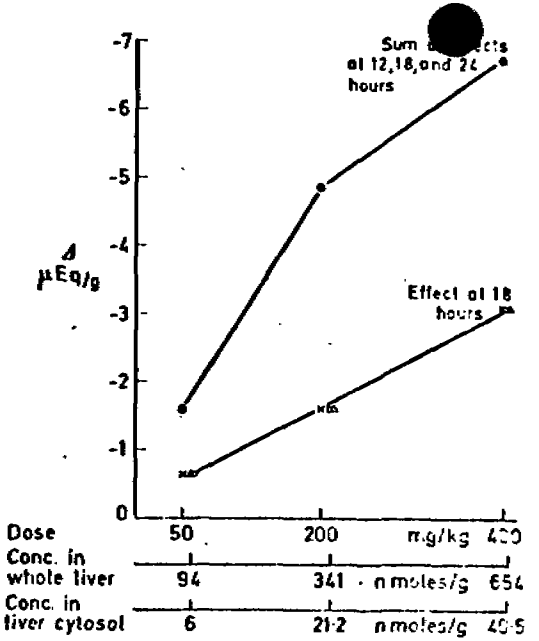

Fig. 1. Alpha-HCH-effect on non-protein thiol content of rat liver in relation to dose and to drug concentration in the organ. Ordinate: Difference in Eq g in liver non-protein thiol content between rats given the drug and those given the vehicle only. Abscissae: Dose of alpha-HCH injected into the stomach (mg/kg); alpha-HCH-concentration in whole liver (nmoles/g), and in liver cytosol fraction (nmoles/g fresh liver equivalent). Upper curve: Sum of effects recorded 12, 18 and 24 h after application in relation to dose. Lower curve: Effect seen 18 h after application in relation to dose (×), to drug concentration in whole liver (•), and to drug concentration in liver cytosol (○)

the ordinate at a point > 0 which might represent an average value up to which factors such as reduced intake of food during the hours following application of the drug contributed to the total drug effect on liver GSH seen in an individual animal. When the drug effects seen 12, 18, and 24 h after application are added up, their relation to dose is as shown by the upper curve in Fig. 1. This relation may be expected to become more nearly linear when a time interval greater than the 24 h tested here is studied, since (see Table 1) the return to normal of liver non-protein thiol after the two larger doses of HCH is not yet complete after 24 h and it is slowest with the largest dose.

Apart from liver, non-protein thiol content was determined in brain and kidneys 12 h after oral application of 200 mg/kg and 18 h after 400 mg/kg of alpha-HCH. This was done because experiments with organ

homogenates had indicated that GSH-mediated degradation of α HCH may occur in these—and in other—organs (Kraus *et al.*, 1973). However, brain content of non-protein thiol was found not to change at all after dosing with the drug, while in the kidneys total non-protein thiol appeared to even increase somewhat but this was not entirely convincing ($P \geq 0.05$ for either dose with $N = 2 \times 8$). It may be added that drug concentration in the brain after oral application of 200 mg/kg was determined in separate experiments and found to change with time exactly as it does in liver; it seems reasonable to suppose that the time-course is not very different in the kidneys.

Discussion

Barnes *et al.* (1959), in the course of their work on mercapturic acid formation in the mammal, were the first to show that administration to rats of halogenated organic compounds, which are mercapturic acid precursors, leads to a fall in liver GSH content, and that the fall in liver GSH is roughly equivalent to the amount of mercapturic acid produced. It was on the basis of this work, which has subsequently been extended (e.g. Boyland and Chasseaud, 1969, 1970) to include other types of mercapturic acid precursor, and in view of evidence (Portig *et al.*, 1973) that rat liver cytosol converts alpha-HCH to a hydrophilic metabolite in a GSH-dependent enzymic reaction, that the experiments described in this paper were undertaken. The finding that liver GSH decreases following injection of alpha-HCH into the rat's stomach, and the good correlation that was found to exist between the extent of the decrease and the amount of drug administered as well as the amount of drug present in the liver, are consequently considered with preference for a causal relationship with HCH-biodegradation. More specifically, the result of this investigation is taken to indicate that a reaction consuming GSH is of some quantitative importance in the biodegradation of alpha-HCH in the rat though, presumably, less so than this appears to be the case in flies (Bradbury and Standen, 1959; Ishida and Dahm, 1965). Information on what proportion of the compound is, in fact, excreted by a rat as product(s) of reaction with reduced sulfur is currently sought to be obtained.

In contrast to liver, brain and kidney content of non-protein thiol appeared to be unaffected when high amounts of alpha-HCH were present in these organs. This may be so either because GSH-mediated conversion of the drug in these tissues, which was found to occur under conditions *in vitro* (Kraus *et al.*, 1973), is of only minor importance, quantitatively, in the living animal or, alternatively, the role of GSH in HCH-degradation in these tissues might not be that of cosubstrate. This issue should be clarified through further investigation.

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