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THE EFFECT OF GAMMA-HEXACHLORCYCLOHEXANE ON RAT LIVER LYSOSOMES

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Abstract—(1). An intraperitoneal injection of gamma-HCH (40 mg/kg, LD_{50}) was shown to induce a significant release of three lysosomal enzymes—acid phosphatase, β-glucosidase and cathepsin D—in rat livers already 1 hr after administration of the agent. From 1 hr up to 24 hr of the observation period the release of selected lysosomal hydrolases consistently increased. In contrast to the biochemical findings, the morphological changes including, hepatic necrosis and fatty infiltration, were established only 24 hr after gamma-HCH treatment. (2). The *in vitro* studies on the latency of two lysosomal enzymes—acid phosphatase and β-glucosidase—in the rat liver lysosome-rich suspension treated with gamma-HCH, showed that the concentrations of 10^{-5} M, 10^{-4} M and 10^{-3} M significantly decreased latency of both enzymes. However, acid phosphatase and β-glucosidase, exhibited differential responses to the *in vitro* effect of gamma-HCH, which might be due to the differential affinities of these enzymes for the lysosomal matrix. The above mentioned concentrations of gamma-HCH did not significantly change the activities of acid phosphatase, β-glucosidase and cathepsin D *in vitro*. (3). No significant alterations were observed in the total activities of enzymes studied after *in vivo* and *in vitro* administered gamma-HCH. (4). The present study suggests that gamma-HCH exerts its effect on rat liver lysosomes by modifying the structural properties of the lysosomal membrane.

Information on the biochemical behaviour and reactions of pesticides in living systems is essential for the assessment of hazards arising from the use of these compounds. Gamma-hexachlorcyclohexane (gamma-HCH), an organochlorine insecticide, is among the most ubiquitous and persistent environmental pollutants [1–6]. Earlier studies on the toxicologic properties of gamma-HCH pointed to hepatic cell necrosis accompanied by increased activity of serum acid phosphatase in rats poisoned with this agent [7, 8]. In fact, the increased amount of serum acid phosphatase appeared to be lysosomal in origin, since enzyme assay utilized β-glycerophosphate as substrate [9–11].

It is well documented that injuries to lysosomal membrane by certain toxic agent lead to the release of acid hydrolases from lysosomes into cytoplasm, and to consequent cell death and necrosis [12–16]. Such pathological processes have been found to be associated with subsequent leakage of lysosomal enzymes into blood serum [17–19].

The present study was therefore undertaken to investigate whether hepatic cell necrosis and increased activity of serum acid phosphatase observed in gamma-HCH-intoxicated rats are causally related to the alterations of liver lysosomal membrane. To test this possibility the latency of lysosomal enzymes in rat liver was studied over a period before the onset of hepatic necrosis which normally develops after gamma-HCH administration. Moreover, parallel experiments were conducted to obtain informations on the *in vitro* effect of gamma-HCH upon rat liver lysosomes.

MATERIALS AND METHODS

Chemicals. Bovine serum albumin and Triton X-100 were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Great Britain. Gamma-HCH (gamma-hexachlorcyclohexane) was purchased from British Drug House Chemicals, Ltd., Poole, Dorset, Great Britain. All other chemicals were of analytical grade from Koch-Light Laboratories Ltd., Colnbrook, Bucks, Great Britain. Haemoglobin was prepared according to Anson [20].

Animals. Male and female albino rats of Wistar strain, weighing 250–300 g were used. Animals were fasted overnight before the treatment but allowed free access to water. Fifty rats randomly divided into two treatment groups were injected as follows: (a) 40 mg/kg (LD_{50}) gamma-HCH dissolved in arachis oil as 0.8% solution according to Grover and Seems [21] and (b) equal-volume doses of the solvent. Time-course of the developing liver injury at the dose used has been well established in the preliminary experiments. Five animals of each group were sacrificed at 1, 4, 8, 12 and 24 hr after treatment. Control group consisted of five untreated rats.

Histology. Animals were sacrificed by decapitation. Livers were immediatelly removed, blotted on filter paper and weighed. Tissue samples were taken from each liver, fixed in phosphate-buffered 10%-formaldehyde solution and stained with haemotoxylin-eosin under light microscopy. Formaldehyde solution-fixed frozen sections were stained with red oil to show fat infiltration, as described by Kimbrough *et al.* [22].

Preparation of liver homogenates. The remaining parts of livers were placed in an ice-cold homogeniz-

ing solution (250 mM sucrose and 1 mM EDTA, pH 7.4). All subsequent preparative operations were carried out at 2–4°. Tissues were then disrupted with 9 vol. of homogenizing solution in an ice-jacketed Potter-Elvehjem homogenizer by three strokes at a speed of 1000 rev/min. Homogenization was checked by phase-contrast microscopy. Tissue homogenates were filtered through a double layer of cheese cloth and diluted to 10% (w/v) suspension.

Preparation of liver lysosome-rich suspension. The lysosome-rich suspension was prepared by the method of de Duve *et al.* [23]. Three particulate fractions (*N*, *M*, *L*) and a final supernatant (*S*) were separated by successive centrifugations at increasing speeds and times: *N*-nuclear (6000 *g* × min, average field g_{max}600), *M*-mitochondrial (33,000 *g* × min, average field g_{max}3300) and *L*-lysosome-rich (250,000 *g* × min, average field g_{max}25,000). Centrifugations were carried out in an MSE High-Speed 25 refrigerated centrifuge (rotor no. 59584) except for the separation of the nuclear fraction. This fraction was separated in an I.E.C. centrifuge (Damon Corp. Needham Heights, MA, model PR-J, head no. 253). The lysosome-rich pellet was washed twice and resuspended in the same medium to give a final suspension containing 250 mg of liver equivalent per ml (L/4). A few biochemical parameters useful for the characterization of the *L* fraction were similar to those reported previously [24].

Test for the in vitro *effect of gamma-HCH on the activities of lysosomal hydrolases.* Before deciding whether a given agent acts by inhibiting or by increasing the release of enzyme from lysosomes, one has to study its effect on the activity of the corresponding enzyme in the presence of Triton X-100 (0.1%, v/v). This concentration of Triton X-100 effectively renders the maximum activity of acid hydrolases available to the substrate. Thus the effects of gamma-HCH upon the activities of three lysosomal marker enzymes, acid phosphatase, β-glucosidase and cathepsin D, were studied in the supernatant of lysosome-rich suspension exposed to Triton X-100 (0.1%, v/v) and centrifuged at 250,000 *g* × min, average field g_{max}25,000. Portions of the supernatant were incubated for 10 min at 37° with the increasing concentrations (10^{-5} M, 10^{-4} M and 10^{-3} M) of gamma-HCH dissolved in isopropanol according to Gertig *et al.* [25]. Appropriate controls with equal volume-doses of isopropanol or without this solvent were also included in the experiments.

Test for the effect on rat liver lysosomes of gamma-HCH administered in vitro. This test was carried out by measuring the release of acid phosphatase and β-glucosidase from rat liver lysosomes into the surrounding medium of lysosome-rich suspension after treatment with gamma-HCH. Portions of lysosome-rich suspension were exposed to increasing concentrations of gamma-HCH (10^{-5} M, 10^{-4} M and 10^{-3} M) as in the preceding experiments. Untreated samples of lysosome-rich suspension and those treated with equal volume-doses of isopropanol served as controls. Before (0) and 5, 10 and 15 min after incubation at 37° all samples were centrifuged at 250,000 *g* × min. average field g_{max} 25,000, and the supernatants were assayed for released enzymes. Total releasable activities of selected enzymes were determined in the aliquots of lysosome-rich suspension treated with Triton X-100 (0.1%, v/v). Enzyme release, is expressed as per cent of total activity [26].

Test for the effect on rat liver lysosomes of gamma-HCH administered in vivo. This experiment was performed by measuring total and unsedimentable activities of acid phosphatase, β-glucosidase and cathepsin D, in 5% whole liver homogenates obtained from untreated rats and those treated with gamma-HCH or equal volume-doses of arachis oil as described in the text of subsection: "animals". The total activity was determined under conditions designed to ensure complete release of enzymes from lysosomal structures by using 0.1% Triton X-100. The reliability of this procedure was verified in specimens treated with repeated freezing and thawing instead of Triton X-100. The unsedimentable activity was determined in the supernatant after the whole liver homogenates (5%, w/v) had been subjected to high centrifugation force 75,000 *g* in an MSE High-Speed 25 centrifuge (rotor No. 59584) for 1 hr at 4° to sediment particulate activity. The ratio of unsedimentable to total activities, expressed as per cent, served as measure for the *in vivo* release of acid hydrolases from lysosomes into cytoplasm [27].

Enzyme assays. Acid phosphatase (EC 3.1.3.2) was measured following the method of de Duve *et al.* [28]. β-glucosidase (EC 3.2.1.2) was assayed according to Beck and Tappel [29] and cathepsin D (EC 3.4.4.23) was determined by the method of Barret [30]. Enzyme activities are expressed in standard units (μmoles of substrates hydrolized per min under assay conditions). Specific activities are defined as μmoles of products formed per mg of protein, or of g of fresh liver per min. The activities of the selected enzymes displayed linearity with respect to the time of incubation and enzyme concentrations.

Protein determination. Protein content was measured by the method of Lowry *et al.* [31] using bovine serum albumin as a standard.

Statistical analysis. The significance of the results was checked by standard statistical techniques [32].

RESULTS

In vitro *effect of gamma-HCH on the activities of acid phosphatase, β-glucosidase and cathepsin D from rat liver lysosomes.* Table 1 shows that there are no significant ($P > 0.05$) changes in the activities of acid phosphatase and β-glucosidase and cathepsin D in samples incubated for 10 min at 37° with increasing concentrations of gamma-HCH (10^{-5} M, 10^{-4} M and 10^{-3} M) compared with controls incubated with equal volume-doses of isopropanol or without this solvent.

The effect on rat liver lysosomes of gamma-HCH administered in vitro. The release of acid phosphatase and β-glucosidase from rat liver lysosome-rich suspension were significantly ($P < 0.05$) increased immediately after addition of all concentration (10^{-5} M, 10^{-4} M and 10^{-3} M) of gamma-HCH used up to 15 min ($P < 0.01$) of incubation at 37° compared to those measured in untreated or isopropanol-treated samples (Fig. 1). These effects of gamma-HCH are dose- and time-dependent, so that increasing concentrations of the agent induce the progressive release of increasing amounts of both enzymes during 15 min

Table 1. Specific activities of rat liver lysosomal enzymes after incubation with increasing concentrations of gamma-HCH or isopropanol for 10 min at 37°

Treatment	Acid phosphatase	β-glucosidase	Cathepsin D
Concentrations of gamma-HCH (M)			
10^{-5}	42.6 ± 4.20	16.4 ± 3.41	99.6 ± 3.45
10^{-4}	38.1 ± 2.73	17.7 ± 2.65	104.2 ± 2.98
10^{-3}	39.9 ± 3.12	18.2 ± 3.32	106.4 ± 3.25
Isopropanol			
1%, v/v	38.9 ± 4.01	15.4 ± 2.59	101.5 ± 4.11
0	39.5 ± 2.54	16.1 ± 1.69	102.3 ± 5.31

Enzyme preparation used was the supernatant obtained from lysosome-rich suspension after treatment with Triton X-100 (0.1%, v/v) as described in Methods. Specific activities are expressed as μmoles of inorganic phosphate, *p*-nitrophenol and tyrosin/min/mg of protein for acid phosphatase, β-glucosidase and cathepsin D respectively. Each value represents the mean ± S.E.M. of three separate experiments.

of incubation period. It is also noteworthy that there is a marked, though not statistically significant, difference in the responses of acid phosphatase and β-glucosidase to the action of gamma-HCH at any time intervals. On the other hand, gamma-HCH did not produce significant change in the total amount of both enzymes under conditions of these experiments.

The effect on rat liver lysosomes of gamma-HCH administered in vivo. The results of time-course study on the *in vivo* effect of gamma-HCH administration on rat liver lysosomes are summarized in Fig. 2. The curves represent the values for unsedimentable activities of acid phosphatase, β-glucosidase and cathepsin D in the liver homogenates of rats that were sacrificed at the time indicated after an intraperitoneal injection of gamma-HCH or arachis oil as described in the section of Materials and Methods. Unsedimentable activities of acid phosphatase, β-glucosidase and cathepsin D in the whole liver homogenates of gamma-HCH-injected rats were significantly (at 1 hr—$P < 0.05$, and from 4–24 hr—$P < 0.01$) greater than those in the animals treated with arachis oil at any of the time intervals. On the other hand, total activities of these enzymes in rat livers following administration of gamma-HCH or arachis oil were not appreciably affected in comparison with data of untreated animals. The results obtained in these experiments indicate that administration of gamma-HCH

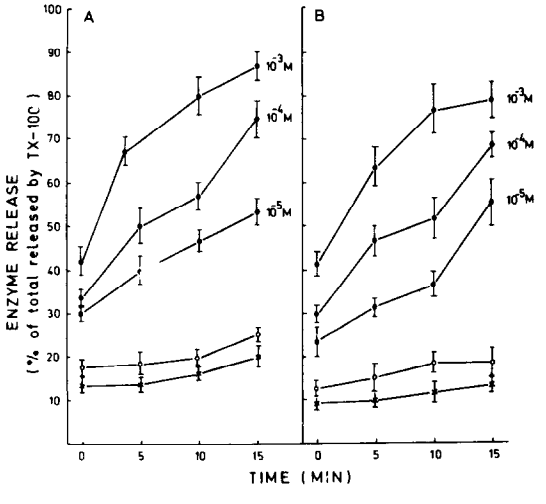

Fig. 1. Time- and dose-dependent release of acid phosphatase (A) and β-glucosidase (B) from rat liver lysosome-rich suspension *in vitro* in the absence (×) and in the presence of gamma-HCH (●) or isopropanol (○). The experimental design is presented in the text. Enzyme release is expressed as a percentage of the total activity released by Triton X-100 (1%, v/v). Each point corresponds to the mean ± S.E.M. of three separate observations.

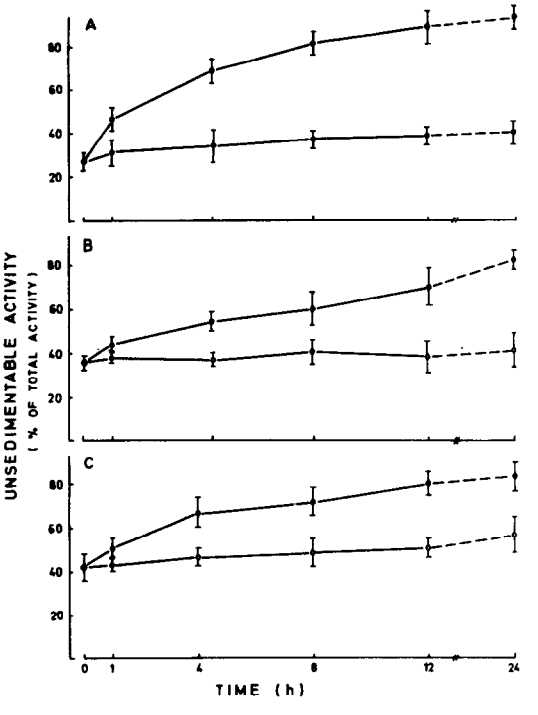

Fig. 2. Unsedimentable activities of acid phosphatase (A), β-glucosidase (B) and cathepsin D (C) expressed as per cent of total activities of these enzymes in the whole liver homogenates before (◑) and at 1 hr intervals up to 24 hr following an intraperitoneal injection of 40 mg/kg gamma-HCH (●) or equal volume-doses of arachis oil (○) into rats. Experimental details are given in the Materials and Methods. Each point represents the mean ± S.E.M. of the values obtained in five animals.

into rats brings about release of acid hydrolases from liver lysosomes into the cytoplasm. This effect of gamma-HCH is in correlation with increasing time periods. In contrast to the lysosomal changes observed already 1 hr after gamma-HCH administration, the examination by light microscopy of rat liver showed fatty infiltration and centrilobular necrosis only 24 hr after treatment with this agent.

DISCUSSION

The experiments described in the present paper clearly show that striking release of acid phosphatase, β-glucosidase and cathepsin D from rat liver lysosomes precedes by many hours the onset of a typical gamma-HCH-induced hepatic necrosis. These observations suggest that damage to the lysosomal membrane may be an event responsible for the pathogenesis of the liver injury by gamma-HCH. The question arises whether gamma-HCH exerts its effect selectively on the lysosomal membrane within lysosomes, or whether the free radicals, resulting from gamma-HCH conversion through homolytic route by interaction with the microsomal electron transport chain, peroxidize nonselectively the lipid constituents of the intracellular membrane system including the lysosomal one. It is currently accepted that hepatotoxicity induced by some toxic substances is causally related to their conversion to a free radicals [33–37]. However, the evidence that addition of gamma-HCH to the lysosome-rich suspension induces time and dose-dependent release of two lysosomal enzymes—acid phosphatase and β-glucosidase—favours the belief that gamma-HCH itself causes the alterations in the properties of the lysosomal membrane. This conclusion is supported by the recent observations on the *in vitro* labilizing effects of DDT, DDE and PCB upon rat liver lysosomal membrane [38]. Moreover, the enzymes studied exhibited differential responses to the *in vitro* effect of gamma-HCH. Such phenomena have been observed previously with these or other acid hydrolases in lysosomes from rat liver [39] or rat brain [40–42]. These later investigations suggested differential structural affinities of the acid hydrolases for the lysosomal matrix. Thus it seems possible that gamma-HCH may effect the solubilization of acid phosphatase to a higher extent than those of β-glucosidase which is firmly attached to the lysosomal membrane.

On the other hand, the way by which gamma-HCH enters lysosomes is highly speculative. One suggestion is, that gamma-HCH reaches lysosomal system by permeation which involves passive membrane diffusion through both plasma and lysosomal membrane. The other possibility is that gamma-HCH binds plasma protein in the form of complex which is then taken up within lysosomes by endocytosis, as was observed for many pharmacologically active, toxic and cancerogenic substances [43–45]. After intralysosomal proteolysis gamma-HCH is set free to exert its effect upon lysosomal membrane. Further studies are necessary to resolve this problem. Also, the mechanism by which gamma-HCH modifies the structural properties of the lysosomal membrane remains to be clarified.

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