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THE EFFECT OF D-PENICILLAMINE ON THE RELEASE OF ACID HYDROLASES FROM RAT LIVER LYSOSOMES INDUCED BY GAMMA-HEXACHLORCYCLOHEXANE

OLGA CAREVIĆ

Institute for Medicine and Psychology of Traffic, Šarengradska 3, 41000 Zagreb, Yugoslavia * and Research Institut "PLIVA", Pharmaceutical and Chemical Works, Ive Lole Ribara 89, Zagreb 41000, Yugoslavia

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Abstract—D-Penicillamine administered p.o. at a dose of 800 mg/kg, exerts *in vivo* a stabilizing effect on lysosomal membrane in rat liver. This was demonstrated in normal rats and in the animals whose lysosomes had been rendered fragile by an intraperitoneal injection of 40 mg/kg gamma-HCH. Under these experimental conditions, D-penicillamine protected rat liver against cell necrosis induced by gamma-HCH and prevented, therefore, the efflux of β-glycerophosphatase from the organ to the blood plasma. D-Penicillamine also significantly relieved the *in vitro* labilizing effect of gamma-HCH upon lysosomal membrane in the lysosome-enriched liver suspension. On the other hand, D-penicillamine did not significantly affect the activities of the three acid hydrolases, β-glycerophosphatase β-glucosidase and cathepsin D, chosen as lysosomal marker enzymes, nor their total activities in rat liver. The possible mechanisms by which D-penicillamine exerts protective action on gamma-HCH-induced alterations of rat liver lysosomes are discussed.

Recent studies demonstrated that a striking release of acid hydrolases from rat liver lysosomes precedes by many hours the onset of a typical cell necrosis which develops after intoxication with gamma-hexachlorcyclohexane (gamma-HCH), an organochlorine insecticide [1]. Moreover, evidence was obtained that gamma-HCH *in vitro* induces a time- and dose-dependent discharge of lysosomal enzymes in the lysosome-enriched liver suspension. This favours the belief that labilization of rat liver lysosomal membrane may be an event responsible for the pathogenesis of the liver injury by gamma-HCH.

Since the results of several laboratories [2, 3] as well as our own preliminary observations (unpublished data) indicated that D-penicillamine, β-β-dimethylcystein, stabillizes the membrane of isolated rat liver lysosomes, it was of interest to evaluate its effectiveness in relieving the gamma-HCH-induced labilization of lysosomal membrane and thereby protecting the liver against morphological alterations.

MATERIALS AND METHODS

Chemicals. Gamma-HCH was purchased from British Drug House Chemical, Ltd., Poole Dorset, Great Britain. D-Penicillamine ("Metalcaptase") was supplied by "PLIVA" Pharmaceutical and Chemical Works, Zagreb, Yugosalvia. All other chemicals employed were reagent-grade from Koch Light Laboratories Ltd., Colnbrook, Bucks, Great Britain. Haemoglobin was received as a gift through courtesy of Mr. P. Tausik from Koch Light Laboratories Ltd.

Animals and treatments. Male and female albino rats of Wistar strain, weighing 250–300 g were used in the experiments. Animals were fasted overnight before treatment but allowed free access to water. Forty rats, randomly divided into 4 groups of ten animals each, were treated as follows: (a) 40 mg/kg gamma-HCH dissolved in arachis oil as a 0.8% solution according to Seems and Grover [4], (b) equivalent volume-doses of arachis oil were injected intraperitoneally (i.p.), (c) 800 mg/kg D-penicillamine dissolved in distilled water as a 10% solution was given p.o. and (d) D-penicillamine dissolved in distilled water as in the preceding experiment was given 1 hr after an i.p. injection of 40 mg/kg gamma-HCH. The time-course of the developing liver injury by gamma-HCH used was well established in the previous work [1]. The results of these experiments also demonstrated that an intraperitoneal injection of arachis oil neither affects the integrity of liver lysosomes nor the activities of selected lysosomal enzymes. Therefore, the arachis oil-treated rats served as a control group in the present study. The doses of D-penicillamine were chosen on the basis of the preliminary observations. The animals were sacrificed 24 hr after treatment with gamma-HCH or arachis oil and 23 hr after administration of D-penicillamine.

Preparation of blood plasma. β-Glycerophosphatase assay was performed with plasma prepared in detail as proposed by Zuretti and Baccino [5]. The preparation was carried out with care to prevent clotting or haemolysis and to remove platelets during blood clotting as well as from damaged cells. The blood, withdrawn with siliconized needles and syringes from abdominal aorta under nembutal anaestesia, was mixed with 1/9 vol. of 135 mM sodium citrate in the siliconized centrifuged tubes as suggested by the above mentioned authors. The plasma was obtained by spinning at 2000 g_{av} for 30 min, and the supernatant resulting from a first run at 3500 g_{av} for 10 min. The lack of structure-linked latency of β-glycerophospatase activity in plasma, taken as an index for the absence of appreciable

* Address for reprint requests.

amounts of platelets, was checked by performing assays both in the absence and in the presence of 0.1% (v/v) Triton X-100. Plasma level of β-glycerophosphatase was determined 24 hr after injection of gamma-HCH or arachis oil and 23 hr after administration of D-penicillamine into rats, as described in text of "Animals and treatments".

Histological techniques. Animals were sacrificed by decapitation. Livers were immediately removed, blotted on filter paper and weighed. Tissue samples were excised from the same region of the median lobe, fixed in phosphate-buffered 10%. Formaldehyde solution-fixed frozen sections were stained with Sudan Black for visualization of lipids [6].

Preparation of liver homogenates. The remaining parts of the livers were placed in an ice-cold homogenizing solution (250 mM sucrose and 1 mM EDTA, pH 7.4). The techniques used thereafter followed the method originally developed by de Duve *et al.* [7]. The livers were homogenized with 5 vol. of the same sucrose/EDTA solution in an ice-jacketed Potter–Elvehjem homogenizer by three strokes at a speed of 3000 rev./min. Homogenization was checked by phase-contrast microscopy. Tissue homogenates were filtered through a double layer of cheese cloth and diluted to a 10% (w/v) suspension.

Preparation of liver lysosome-enriched suspension. The 10% homogenates were centrifuged at 6000 $g \times$ min, (field g_{max} 600) in an I.E.C. centrifuge (Damon Corp. Needham Heights, MA model PR-J, head no. 253), to remove nuclei and cell debris. The supernatant was then centrifuged at 33,000 $g \times$ min, (average field g_{max} 3000) to separate mitochondria, and further at 250,000 $g \times$ min (average field g_{max} 25,000) to obtain a lysosome-enriched pellet. These centrifugations were carried out in an MSE High-Speed refrigerated centrifuge (rotor no. 59584). The lysosome-enriched pellet (L) 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 [8, 9].

Test for the in vitro *effect of* D-*penicillamine on the activities of β-glycerophosphatase, β-glucosidase and cathepsin D.* In the examination of the stability of the lysosomal membrane to various chemicals, it is possible that the variation of the enzyme activity in the supernatant fraction might not reflect the permeability of the membrane but results from a direct action of these compounds on the released enzyme. Therefore the effects of D-penicillamine upon the activities of β-glycerophosphatase, β-glucosidase and cathepsin D, chosen as lysosomal marker enzymes, were studied. The lysosome-enriched liver suspension exposed to Triton X-100 (0.1% v/v) was centrifuged at 250,000 $g \times$ min (average field g_{max} 25,000). This concentration of Triton X-100 effectively renders maximum activity of β-glycerophosphatase and cathepsin D available to the substrates. Since Triton X-100 was found to inhibit liver lysosomal β-glucosidase [10], the total activity of this enzyme was measured in the specimens with repeated freezing and thawing instead of the detergent. The portions of the resulting supernatant were incubated for 10 min at 37° in the absence (controls) and in the presence of D-penicillamine dissolved in distilled water in the concentrations ranging from 10^{-6} to 10^{-3} M.

Test for the in vivo *effects of gamma-HCH and* D-*penicillamine on the release of acid hydrolases from rat liver lysosomes.* These experiments were performed by measuring the total and unsedimentable activities of β-glycerophosphatase, β-glucosidase and cathepsin D in the 5% whole liver homogentates obtained from rats treated according to the procedure described in the section "Animals and treatments". Total activity was determined under conditions designed to ensure complete release of enzymes from lysosomal structures by using 0.1% (v/v) Triton X-100. In the experiments with β-glucosidase the specimens were submitted to a repeated freezing and thawing as in the preceding test. The unsedimentable activity was determined in the supernatant after the whole liver homogenates (5%, w/v) had been subjected to a high centrifugation force 75,000 g in an MSE High-Speed 25 centrifuge (rotor no. 59584) for 1 hr at 4°, in order to sediment particulate activity. The ratio of unsedimentable to total activities expressed as per cent, served as a measure for the *in vivo* released of acid hydrolases from lysosomes into the cytoplasm as described previously [11, 12].

Test for the in vitro *effects of gamma-HCH and* D-*penicillamine on the release of β-glycerophosphatase from rat liver lysosomes in the lysosome-enriched liver suspension.* The *in vitro* release of β-glycerophosphatase from lysosomes was measured in the surrounding medium of the lysosome-enriched liver suspension after incubation for 10 min at 37° with: (a) gamma-HCH dissolved in 1% (v/v) isopropanol solution, according to Gertig *et al.* [13], (b) D-penicillamine dissolved in distilled water or (c) gamma-HCH plus D-penicillamine at varying molar concentrations. Appropriate controls with equal volume-doses of isopropanol or without this solvent were also included in the experiments. At a definite time interval, the incubation mixtures were centrifuged at 250,000 $g \times$ min (average field g_{max} 25,000) and the resulting supernatants were assayed for the released enzyme activity. Total activity of β-glycerophosphatase was determined in the aliquots of lysosome-enriched liver suspension treated with Triton X-100 (0.1%, v/v). Enzyme release is expressed as percentage of the total activity [14, 15].

Enzyme assay. Enzyme activities were assayed as follows: β-glycerophosphatase (EC 3.1.3.2) as indicated by de Duve *et al.* [16], β-glucosidase (EC 3.2.1.2) according to Beck and Tappel [17] and cathepsin D (EC 3.4.23.5) as described recently by Smith and Turk [18]. Suitable enzyme and substrate blank assays were included. One unit or enzyme activity corresponds to the transformation of 1 μmole of substrate/min under the present assay conditions. Specific enzyme activity is defined as μmoles of products formed per mg of protein, or g of fresh liver per min. The activities of the selected enzyme displayed linearity with respect to the time of incubation and enzyme concentrations.

Determination of protein. Protein concentrations were determined by the method of Lowry *et al.* [19] with bovine serum albumin as standard.

Statistics. Statistical analysis of data was performed by the use of Student's t test [20]. Significance levels were chosen as $P < 0.05$. The number of experiments is indicated in the individual tables and figure.

Table 1. Effect of D-penicillamine on the activities of β-glycerophosphatase, β-glucosidase and cathepsin D

D-Penicillamine (M)	Specific activities β-Glycerophosphatase	β-Glucosidase	Cathepsin D
0	41.8 ± 2.85	17.3 ± 2.20	91.5 ± 3.16
10^{-6}	42.9 ± 2.09	16.8 ± 2.87	89.8 ± 4.31
10^{-5}	39.7 ± 3.37	17.0 ± 3.04	90.9 ± 3.70
10^{-4}	40.5 ± 2.91	15.9 ± 2.73	88.6 ± 5.60
10^{-3}	38.8 ± 3.98	16.0 ± 2.66	88.3 ± 4.12

The enzyme preparation used was the supernatant obtained from lysosome-enriched liver suspension after treatment with Triton X-100 and centrifugation at 250,000 g × min as described in Materials and Methods.

Specific activities are expressed as μmoles of inorganic phosphate, p-nitrophenol and tyrosin/min/mg of protein for β-glycerophosphatase, β-glucosidase and cathepsin D respectively. Each value represents the mean ± S.E.M. of five separate experiments.

RESULTS

In vitro *effects of* D-*penicillamine on the activities of β-glycerophosphatase, β-glucosidase and cathepsin D*

Table 1 shows that D-penicillamine in the concentrations of 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M did not significantly ($P > 0.05$) affect the activities of β-glycerophosphatase, β-glucosidase and cathepsin D over a 10 min incubation period at 37° with respect to the corresponding controls.

In vivo *effects of* D-*penicillamine administration on gamma-HCH-induced release of β-glycerophosphatase, β-glucosidase and cathepsin D from rat liver lysosomes*

Previous work demonstrated that unsedimentable activities of the three lysosomal hydrolases, β-glycerophosphatase, β-glucosidase and cathepsin D, in the liver homogenates of gamma-HCH-injected rats were significantly (at 1 hr $P < 0.05$ and from 4 to 24 hr $P < 0.01$) higher than those in the untreated or arachis oil-treated animals at any of the time intervals. On the other hand, equivalent volume-doses of the solvent did not cause significant ($P < 0.05$) changes in the ratio between unsedimentable and total activities of these enzymes in rat livers when compared with control values. Examination by light microscopy of liver showed fatty infiltration and cell necrosis only 24 hr after treatment with gamma-HCH. In the present studies the rats from A and B groups (see Fig. 1) were treated with arachis oil (A) and gamma-HCH (B) under identical experimental conditions as in the above mentioned work.

It can be seen from data in Fig. 1 that gamma-HCH, again markedly enhanced ($P < 0.01$) the unsedimentable activities of the three lysosomal enzymes in rat liver. However, D-pencillamine administered 1 hr after gamma-HCH significantly ($P < 0.01$) decreased the unsedimentable activities of these enzymes, but still did not reach the control values excepts the one for β-glucosidase. In the animals treated with D-penicillamine alone, this parameter was somewhat lower but statistically significant ($P < 0.05$) when compared with observations obtained in arachis oil-injected rats. Total activities of β-glycerophosphatase, β-glucosidase and cathepsin D in rat liver after administration of gamma-

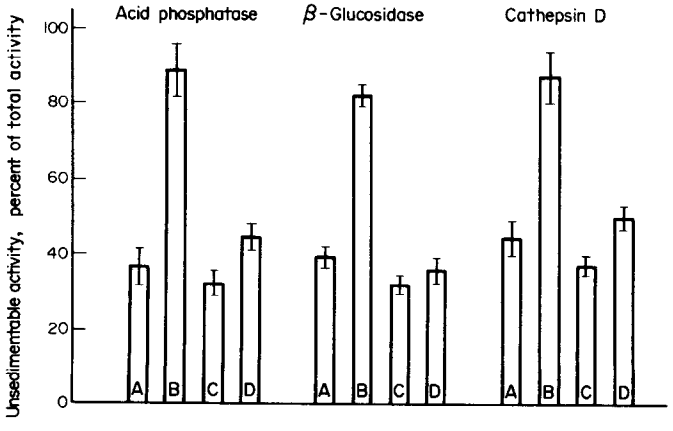

Figure. 1. Unsedimentable activities expressed as per cent of total activities of three lysosomal enzymes in rat liver after administration of: A—arachis oil (equivalent volume-doses, i.p.), B—gamma-HCH (40 mg/kg, i.p.), C—D-penicillamine (800 mg/kg, p.o.), D—D-penicillamine (800 mg/kg, p.o.) 1 hr after gamma-HCH (40 mg/kg, i.p.). The figures represent the means and bars the S.E.M. of the observations obtained in ten animals 23 hr after treatment with D-penicillamine. Other details are given in Materials and Methods.

Table 2. Effect of D-penicillamine on the plasma level of β-glycerophosphatase in rats previously treated with gamma-HCH

Treatment*	β-Glycerophosphatase†
Untreated	20.98 ± 1.98
Arachis oil	21.05 ± 2.36
Gamma-HCH	39.15 ± 7.31
D-Penicillamine	19.87 ± 4.21
Gamma-HCH + D-Penicillamine	21.42 ± 3.62

* The animals were treated according to the description presented in Figure 1. Experimental details are given in Materials and Methods.

† β-Glycerophosphatase activity is expressed as nmoles of β-glycerophosphate hydrolyzed/min/ml. The values are the means ± S.E.M. of ten separate experiments.

HCH plus D-penicillamine were not appreciably ($P < 0.05$) affected, in comparison with the data for arachis oil-treated animals.

In accordance with the inhibitory effect of D-penicillamine on the relase of acid hydrolases from rat liver lysosomes induced by gamma-HCH, the histological inspection of the liver showed that D-penicillamine protected it against cell necrosis which develops between 12 and 24 hr after poisoning with gamma-HCH. However, D-penicillamine alone did not produce any changes in the normal architectural pattern of the rat liver during 24 hr after its administration.

Effect of D-penicillamine administration on plasma level β-glycerophosphatase in rats previously treated with gamma-HCH

Table 2 summarizes the relationship among the results obtained in the plasma level of β-glycerophosphatase 24 hr after administration of gamma-HCH or arachis oil and 23 hr after treatment with D-penicillamine as described in Materials and Methods. Untreated animals were included as control group.

A significant ($P < 0.01$) increase in the level of β-glycerophosphatase activity occurs in the blood plasma of gamma-HCH-injected rats as compared with values of untreated or arachis oil-treated animals. However, when administered 1 hr after gamma-HCH, D-penicillamine significantly ($P < 0.01$) decreased the elevation in β-glycerophosphatase activity produced by gamma-HCH alone. On the other hand, no significant ($P < 0.05$) differences in the activity of β-glycerophosphatase in blood plasma were established between untreated rats and those treated with D-penicillamine or arachis oil.

In vitro *effects of D-penicillamine on gamma-HCH-induced release of β-glycerophosphatase from rat lysosome-enriched liver suspension*

Table 3 shows that increasing concentration (10^{-6}–10^{-3} M) of gamma-HCH dissolved in isopropanol induced a significant ($P < 0.01$) dose-dependent release of β-glycerophosphatase from lysosomes into the surrounding medium of lysosome-enriched liver suspension after incubation at 37° for 10 min. Tests carried out with isopropanol alone under the same experimental conditions demonstrated that the solvent does not affect the release of this enzyme when compared to the data of untreated samples. These results are in agreement with our previous report [1]. On the other hand, D-penicillamine in the equimolar concentrations (10^{-6}–10^{-3} M) slightly, but significantly ($P < 0.05$) retarded the discharge of β-glycerophosphatase from lysosomes which occurs spontaneously during incubation of the lysosome-enriched suspension at 37° for 10 min in the presence of 0.01 M acetate buffer, pH 5.0. When the lysosome-enriched suspension was incubated with both compounds either in the concentrations of 10^{-6} M gamma-HCH and 10^{-5} M D-penicillamine, or 10^{-5} M gamma-HCH and 10^{-4} M D-penicillamine, the enzyme activity release was significantly ($P < 0.01$) reduced in

Table 3. Release of β-glycerophosphatase in the lysosome-enriched liver suspension incubated at 37° for 10 min with isopropanol, gamma-HCH, D-penicillamine and gamma-HCH plus D-penicillamine, or without the solvent and drugs

Treatment	Concentrations	β-Glycerophosphatase (% of enzyme release)
Untreated	0	16.7 ± 0.92
Isopropanol	1%, v/v	17.2 ± 1.03
Gamma-HCH	10^{-6} M	26.1 ± 2.01
	10^{-5} M	35.4 ± 3.93
	10^{-4} M	52.9 ± 6.12
	10^{-3} M	74.1 ± 6.98
D-Penicillamine	10^{-6} M	13.0 ± 1.01
	10^{-5} M	12.9 ± 1.95
	10^{-4} M	12.4 ± 1.35
	10^{-3} M	12.0 ± 1.08
Gamma-HCH + D-penicillamine	10^{-6} M + 10^{-5} M	17.7 ± 2.30
Gamma-HCH + D-penicillamine	10^{-5} M + 10^{-4} M	21.2 + 2.87

The experimental design is presented in Materials and Methods. Enzyme release is expressed as a percentage of that released by 0.1% (v/v) Triton X-100. Values are means ± S.E.M. from five individual observations.

comparison with the values obtained in the gamma-HCH-treated samples. Treatments with isopropanol, gamma-HCH, D-penicillamine or gamma-HCH plus D-penicillamine did not significantly ($P < 0.05$) change the total activity of β-glycerophosphatase.

DISCUSSION

The increased discharge of three lysosomal enzymes, β-glycerophosphatase, β-glucosidase and cathepsin D, which occurs in gamma-HCH-injected rats was found to be significantly decreased after oral administration of D-penicillamine. Moreover, the addition of D-penicillamine to the lysosome-enriched liver suspension inhits the release of β-glycerophosphatase from lysosomes induced by gamma-HCH. This effect of D-penicillamine was also effective in reducing the extent of hepatic cell necrosis that develops in gamma-HCH-treated rats and preventing, therefore, the efflux of β-glycerophosphatase from organ to plasma.

These results indicate that D-penicillamine counteracts the effects of gamma-HCH upon rat liver lysosomal membrane and protects the liver against gamma-HCH-produced cell necrosis. Because of the very complex nature of the factors influencing the developing of cell necorsis, data obtained in the present study are not sufficient to determine whether stabilization of rat liver lysosomal membrane may play a role in the protective effect of D-pencillamine against hepatic necrosis in gamma-HCH-treated rats. It is also difficult to explain the mechanisms by which gamma-HCH and D-penicillamine exert their antagonistic effects upon lysosomal membrane.

However, in the previous study [1] an assumption was considered that free radicals, resulting from gamma-HCH conversion through homolytic route mediated by microsomal electron transport chain, peroxidize double bonds of unsaturated fatty acids in phospholipids that form part of the intracellular membranes including the lysosomal one.

Peroxidation of unsaturated fatty acids in the lysosomal membrane leads to serious consequences such as: disintegration of carbon chain, rupture of the lipoprotein, release of acid hydrolases from lysosomes into the cytoplasm subsequent autolysis, cell death and necrosis [21–23]. Moreover, it was suggested that even an initially small quantity of peroxide in the biomembranes may initiate a chain process of autooxidation catalyzed by metal forming redox system for example Cu^{+}/ Cu^{2+} [24–26]. On the other hand, thiol compounds have been found to keep metalic ions in the reduced state. In the case of D-penicillamine, Walsche [27] pointed out that this chelating agent coordinates to copper ions *in vivo*, as can be judged by its cupriuric activity. Recently Younes *et al.* [28, 29] demonstrated that red-violet copper–penicillamine complex proved to be a potent inhibitor of some reactions mediated by superoxide.

In considering these data and results obtained in the present study one gains the impression that lipid peroxide formation in the lysosomal membrane may be related to, if not the prime cause of, gamma-HCH damage to rat liver lysosomes. In accord with this hypothesis is the possibility that D-penicillamine counteracts the labilizing action of gamma-HCH on lysosomal membrane by its ability to act as a superoxide dismutating agent.

However, other possible mechanisms for the antagonistic effects between gamma-HCH and D-penicillamine upon rat liver lysosomal membrane cannot be excluded. Further experiments are under way in order to clarify the present observations.

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