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The Release of β-Glucuronidase from Rat Liver by the Administration of Diazinon, the Organophosphorus Insecticide

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β-Glucuronidase activity in rat serum was found to be considerably elevated by a single injection of organophosphorus insecticides. In order to elucidate this mechanism, the intracellular distribution of β-glucuronidase in the liver was investigated. The extent of increase of β-glucuronidase in serum of diazinon treated rat was shown to correlate with the degree of decrease in microsomal fraction of liver. After the administration of diazinon, β-glucuronidase in the microsomal fraction was held constant at low level for 10 days and a period over 30 days was required to recover the normal level.

Alteration in serum β-glucuronidase activity have been reported in animals after administration with several drugs.[2] Williams reported the increase of the activity in rat serum caused by the administration of pesticides and hepatotoxic agents.[3] We have obtained the results that the injection of some organophosphorus esters including both insecticidal and noninsecticidal compounds produced a remarkable elevation of serum β-glucuronidase activity.[4] The maximum activity occurred within 3 hours and reached 70 or more times the normal level.

It has been demonstrated that β-glucuronidase is found mainly in the liver and localized both in lysosomes and microsomes at the subcellular level.[5] Isolated lysosomes are known to release their enzymes in the presence of organophosphorus insecticides.[6] However, the increase in serum β-glucuronidase activity mentioned above did not accompany the increase of other marker enzymes of lysosomes, so it could not be attributed to the release from lysosomes. The assumption that the increased serum β-glucuronidase would originate from endoplasmic reticulum of liver is more likely.

In the present paper we investigated the origin of β-glucuronidase increased in serum and the mode of change of the activity in the original site.

Experimental

Materials——Pure diazinon [O,O-diethyl O-(2-isopropyl-4-methyl-6-pyrimidinyl)phosphorothioate] was kindly furnished by Dr. H. Miyazaki, Nihonkayaku Co., Ltd. All other materials were of reagent grade and were purchased commercially.

Animals and Treatment of Drugs——Female Wistar rats weighing 150 to 200 g were used. Rats were injected intraperitoneally with diazinon dissolved in corn oil in a dose of 75 mg/kg. After intervals, the rats were killed by decapitation.

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2) *a*) T.F. Slater and A.L.Greenbaum, *Biochem. J.*, **96**, 484 (1965); *b*) K. Saito and E. Suter, *J. Exp. Med.*, **121**, 739 (1965); *c*) G. Weissman, J.W. Uhr and L. Thomas, *Proc. Soc. Exptl. Biol. Med.*, **112**, 284 (1963).
3) C.H. Williams, *Toxicol. Appl. Pharmacol.*, **14**, 283 (1969).
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Differential Centrifugation——Liver was homogenized with 9 volumes of 0.25M sucrose containing 1 mM ethylenediaminetetraacetic acid (EDTA). The homogenate was successively centrifuged at 1000 ***g*** for 10 min, at 5000 ***g*** for 10 min, at 17000 ***g*** for 15 min and finally at 105000 ***g*** for 60 min to obtain nuclear, mitochondrial, lysosomal and microsomal fractions, respectively. The resulting supernatant was called as the soluble fraction. The microsomal fraction was washed once by resuspending it in 0.25M sucrose and cen trifuging it again at 105000 ***g*** for 60 min. Each fraction was resuspended in an adequate volume of 0.25M sucrose.

Density Gradient Centrifugation——According to the method of Ganshow and Paigen,[7] 0.5 ml of the cytoplasmic particles which were prepared by centrifuging a 10% liver homogenate at 1000 ***g*** for 10 min was layered over a linear 4.5 ml gradient of sucrose ranging in concentration from 0.4M to 2.1M and buffered at pH 7.5 with 0.02M glycylglycine–NaOH. This was centrifuged at 32650 ***g*** for 30 min using 40PS swinging rotor (Hitachi Co., Ltd., Tokyo, Japan) and 0.5 ml fractions were collected from the bottom.

Osmotic Shock to Microsomal Fraction——The microsomal fraction was resuspended in 10 volumes of distilled water in order to solubilize lysosomal enzymes. The suspension was then centrifuged at 105000 ***g*** for 60 min to separate microsomal enzymes from lysosomal ones. The sediment was resuspended in the original volume of sucrose solution.

Determination of Enzyme Activity——β-Glucuronidase (E.C. 3.2.1.31) activity was assayed using *p*-nitrophenyl β-glucuronide as substrate.[8] The incubation medium consisted of 0.08M acetate-acetic acid buffer, pH 4.2, 1 mM substrate and enzyme preparation containing 0.01% (w/v) Triton X-100 in a final volume of 1 ml. The reaction proceeded at 37° was ceased by the addition of 1 ml of 0.5N NaOH. After dilution with 3 ml of distilled water, the absorbance of *p*-nitrophenol was measured at 420 nm. Acid phosphatase (E.C.3.1.3.2) activity was assayed using *p*-nitrophenyl phosphate disodium as substrate.[9] The incubation medium contained 0.08M acetate–acetic acid buffer, pH 5.0, 0.01M substrate and enzyme preparation containing 0.1% Triton X-100 in a final volume of 1 ml. Following the same method as β-glucuronidase, *p*-nitrophenol was determined colorimetrically. Glucose-6-phosphatase (E.C.3.1.3.9) activity was assayed using glucose-6-phosphate disodium as substrate.[10] The incubation medium contained 0.1M maleic acid–NaOH buffer, pH 6.5, 0.02M substrate and enzyme preparation. After incubation at 37° for 15 min, the reaction was stopped by the addition of trichloroacetic acid and inorganic phosphorus liberated was determined by the procedure of Fiske-Subbarow.[11]

Protein Determination——Protein was determined by the procedure of Lowry, *et al.* using bovine serum albumin as standard.[12]

Result

Effect of Diazinon Injection on the Intracellular Distribution of β-Glucuronidase in Rat Liver

Two hours after the administration of diazinon in a dose of 75 mg/kg, total β-glucuronidase activity in the liver was decreased to 70—80% of control activity. Table I shows that this decrease of total activity arose from the decrease of total activity in nuclear and microsomal fractions. Considering specific activity, β-glucuronidase activity in lysosomes and microsomes from treated animal decreased to 83 and 25% of control, respectively. To exclude the activity of lysosomal β-glucuronidase contaminated to microsomes, microsomal fraction obtained above was exposed to hypotonic condition. From the observation of Ganshow, *et al.*[13] and Mameli, *et al.*[14] lysosomal β-glucuronidase is solubilized almost quantitatively.

Table II shows that microsomal β-glucuronidase was separated into soluble and particulate portions by hypotonic treatment. Since most of the activity of control microsomes remained in the sediment, the β-glucuronidase is a part of the microsomal and not the lysosomal fraction. The effect of diazinon treatment on microsomal β-glucuronidase level is again obvious.

These facts reveal that the elevation of serum β-glucuronidase caused by diazinon treat-

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Table I. Cellular Distribution of β-Glucuronidase Activity in the Liver of Control and Diazinon Treated Rats

Treatment	Fractions					
	Homogenate	Nuclei	Mitochondria	Lysosomes	Microsomes	Supernatant
Control						
total activity	173.0 ± 5.7	60.3 ± 8.6	35.2 ± 2.2	27.9 ± 5.0	13.6 ± 0.7	3.6 ± 0.7
specific activity	0.60 ± 0.03	—	0.58 ± 0.02	1.54 ± 0.13	0.61 ± 0.02	0.05 ± 0.01
Diazinon						
total activity	141.3 ± 6.5	47.5 ± 7.9	41.6 ± 4.3	27.3 ± 1.4	3.6 ± 0.7	5.0 ± 0.7
specific activity	0.42 ± 0.02	—	0.69 ± 0.02	1.28 ± 0.11	0.15 ± 0.01	0.05 ± 0.01

Subcellular fractions were obtained from the liver of rat killed 2 hr after the administration of Diazinon (75 mg/kg, *i.p.*) as described in the text. Total and specific activities are expressed as μmole substrate hydrolyzed/hr/g of liver and μmole substrate hydrolyzed/hr/mg protein, respectively. Values are the mean ± S.D. of 4 rats.

Table II. Distribution of β-Glucuronidase Activity in Microsomes after Hypotonic Treatment

Treatment	Microsomal fractions			Recovery
	Suspension	Supernatant	Sediment	
Control				
protein content	2.73	0.39	2.43	2.82(112%)
β-glucuronidase activity	1.81	0.27	1.69	1.96(108%)
Diazinon				
protein content	2.63	0.42	2.37	2.79(106%)
β-glucuronidase activity	0.36	0.19	0.21	0.40(111%)

Microsomal fraction obtained as Table I was treated hypotonically as described in the text. Protein content and β-glucuronidase activity are expressed as mg/ml and μmole substrate hydrolyzed/hr/ml, respectively.

ment takes place consequently with the loss of microsomal β-glucuronidase. Such explanation was supported by subcellular fractionation using density gradient technique.

Fig. 1 shows the distribution profile of acid phosphatase, glucose-6-phosphatase and β-glucuronidase in each fraction. After the administration of diazinon in a dose of 75 mg/kg, β-glucuronidase activity in microsomal zone (Fr. No. 8—10) decreased significantly. However, β-glucuronidase activity in lysosomal zone (Fr. No. 5—7) did not change by diazinon treatment. No significant change was observed in both acid phosphatase and glucose-6-phosphatase activities between control and diazinon treated rats.

Correlation between Serum and Microsomal β-Glucuronidase Activities after the Administration of Diazinon

The effect of various doses of diazinon on serum and microsomal β-glucuronidase activities are shown in Fig. 2. The increase of serum β-glucuronidase showed a good correlation with the decrease of microsomal β-glucuronidase. These results support the conclusion that the increased serum β-glucuronidase originates from the endoplasmic reticulum of the liver.

β-Glucuronidase Activity in Liver for a Long Term after the Administration of Diazinon

Subcellular distribution of β-glucuronidase activity in liver was examined for a long period after the administration of diazinon. As shown in Fig. 3, β-glucuronidase in the microsomal fraction remained at a low constant level between 2 hours and 10 days after the administration and then gradually returned to 90% of control level by 30 days. On the other hand, β-glu-

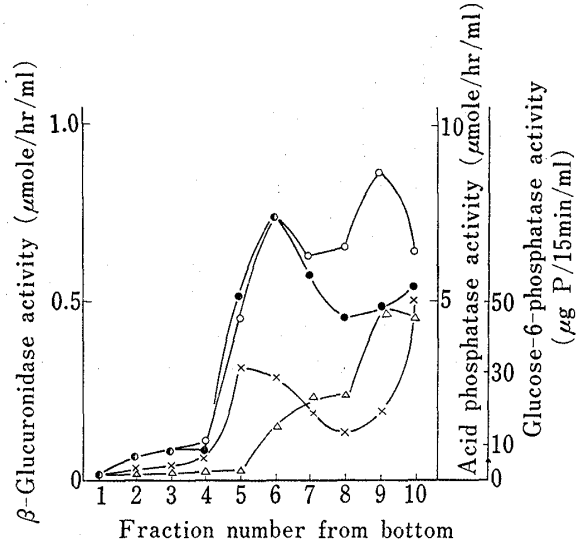

Fig. 1. Distribution of Enzyme Activities after Density Gradient Centrifugation of Rat Liver Cytoplasmic Particles

Cytoplasmic particles were prepared from the liver of rat killed 2 hr after the administration of diazinon (75 mg/kg, *i.p.*).
β-glucuronidase, ○—○ control, ●—● diazinon treatment; acid phosphatase, ×—× control and diazinon; glucose-6-phosphatase, △—△ control and diazinon

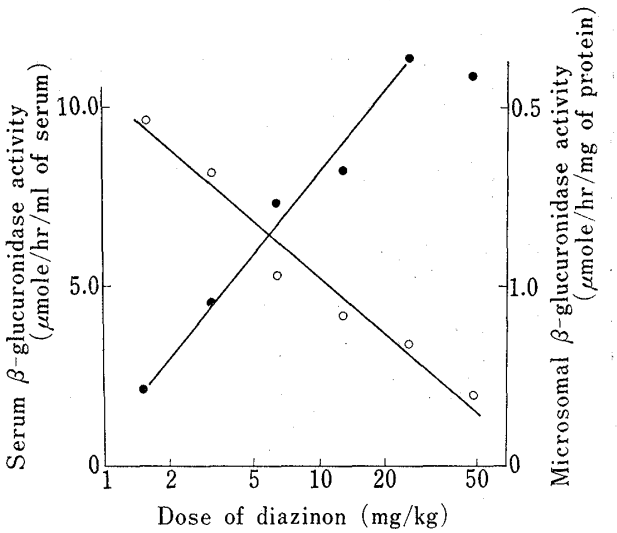

Fig. 2. Relationship between Serum and Microsomal β-Glucuronidase Activities

Serum and microsomal fractions were prepared from the rats killed 3 hr after administration of various amounts of diazinon. Each point represents the mean of 4 rats.
serum, ●—●; microsome ○—○

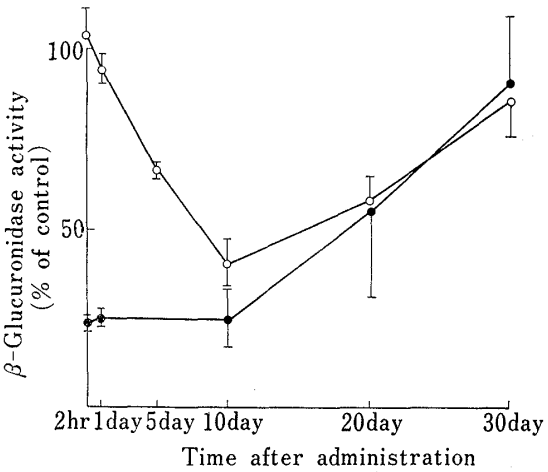

Fig. 3. Time Course of the Alteration of β-Glucuronidase Activity in Liver Subcellular Fractions after Administration of Diazinon (75 mg/kg, *i.p.*)

Mitochondrial-lysosomal and microsomal fractions were prepared from the rats killed 3 hr after the administration of diazinon. The activity is expressed as percentage in specific activity of that of untreated rats. Each point represents the mean±S.D. of 4 rats.
mitochondrial-lysosomal fraction, ○—○; microsomal fraction ●—●

curonidase activity in lysosomal fraction was not altered 2 hours after the administration but decreased to 40% of control level by 10 days and then gradually increased to 85% of control by 30 days.

Discussion

Williams reported that serum β-glucuronidase activity was markedly increased 2, 4 and 6 hours after the administration of sublethal dose of paraoxon and liver β-glucuronidase was slightly increased.[3] These results were not in agreement with our results in liver enzyme level. These disagreement may be mainly due to the difference of dose level of administration. Paraoxon is highly toxic for animals and an allowable dose can not exceed 2 mg/kg, while 75 mg/kg of diazinon is not lethal. The same author also described that repeated treatment of parathion daily for 4—15 days resulted in the decrease of β-glucuronidase content in liver by using biochemical and histochemical techniques.[15] As we will report elsewhere, potency of drugs to increase serum β-glucuronidase was independent of their inhibitory potency of cholinesterase or toxicological signs.

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Transport of this enzyme from endoplasmic reticulum to serum is still not completely elucidated. Lancker and Lentz[16] and Kato, *et al.*[17] reported the observation that microsomal β-glucuronidase was the precursor of lysosomal β-glucuronidase. From this hypothesis, the delayed decrease in lysosomal β-glucuronidase after the administration of diazinon is though to be due to the lack of microsomal β-glucuronidase which would be transported to lysosomes. However, it is also probable that by the administration of organophoshate β-glucuronidase in endoplasmic reticulum was readily transformed to serum type and rapidly released into blood. Recently, Swank and Paigen[18] described that the localization of β-glucuronidase in endoplasmic reticulum was controlled by a genetic locus separated from a structural gene locus and β-glucuronidase in endoplasmic reticulum could not serve as a precursor of lysosomal one. In a elucidation of the toxicity of organophosphates, the effect on the transport of β-glucuronidase in liver remained to be defined.

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