Organophosphate Degrading Enzymes in the Crude Supernatant Fraction from the Rat Liver

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Attempts were made to classify the enzymes which degrade insecticidal organophosphates in the crude supernatant fraction (*i.e.* 20,000 × *g* supernatant) of the rat liver. On the basis of cofactor and ion activation characteristics of the enzymes, four distinctly different types of enzymes have been observed. The enzymes which degraded malathion and parathion were detected by the electrophoresis method. Electrophoresis treatments did not, however, facilitate the detection of Ca^{2+} or GSH-stimulated enzymes. Most of the esterases detected in the rat liver 20,000 × *g* supernatant by using 1-naphthyl acetate do not appear to play any significant role in degrading organophosphates.

There are two major groups of enzyme systems known to degrade toxic organophosphorus esters in the rat liver: the microsomal oxidative system and nonoxidative enzymes such as esterases and dealkylating enzymes. While the former has been the subject of recent intensive studies, the latter group of enzymes has only been examined by a relatively few workers. Examples of such works are described in the papers by Mounter (1955) on DFPase, by Hodgson and Casida (1962) and by Main and Braid (1962) on malathion hydrolyzing aliesterase, by Fukami and Shishido (1963) and Shishido and Fukami (1963) on dealkylating enzymes for dimethyl organophosphates, by Suwanai and Shishido (1965) and Yang *et al.* (1971) on diazinon degrading enzymes, and by Kojima and O'Brien (1968) on paraoxon degrading enzymes. In brief, from the above works there appears to be at least several types of such enzymes: an esterase degrading diethyl phosphates and phosphorothioates, which is stimulated by Ca^{2+}; a DFPase which is stimulated by Mn^{2+} and Co^{2+}; a malathion carboxylesterase; and a dealkylation enzyme which requires reduced glutathione.

Electrophoresis studies on rat liver esterases are scarce, except for the work by Schwark and Ecobichon (1968, 1969) who detected 13 bands of esterases in rat liver homogenates by using a starch gel electrophoresis.

This paper describes an attempt to classify and characterize these enzymes by using electrophoresis and cofactor and ion activation techniques.

MATERIALS AND METHODS

Albino male rats were obtained from Rolfsmeyer Co., Madison, Wis. The livers were removed and washed immediately with cold standard buffer solution, 0.02 *M* sodium phosphate buffer at pH 7.4. After weighing, the sample was homogenized with the same volume of standard buffer as the weight of the liver by using a Teflon-glass Potter–Elvehjem homogenizer of which the pestle was motor-driven at approximately 1000 rpm. To facilitate a clear separation, the homogenate was first briefly centrifuged at 10,000 × *g* for 10 min, and the resulting supernatant was centrifuged at 20,000 × *g* for 1 hr to obtain a clear supernatant. This supernatant was used directly as the enzyme source throughout.

Inhibitors and labeled insecticides used in this study were identical to the ones described by Sakai and Matsumura (1968) except for the ring-labeled Famphur [*o,p*-(dimethylsulfamoyl)-phenyl *O,O*-dimethyl phosphorothioate] which was obtained from American Cyanamid Co.

The agar gel electrophoresis method employed was essentially that of Ogita (1964). The supporting agar was prepared by adding 0.7 g each of PVP (polyvinyl pyrrolidone, 360,000 mol wt) and agar (Noble agar, Difco) to 100 ml of phosphate buffer (ionic strength 0.015 μ). All enzyme sources were introduced to the agar bed by using pieces of filter paper (Whatman No. 40, 1.5 × 0.2 cm each). To study the effect of various inhibitors on esterases, the enzyme source in solution was first incubated with the inhibitor at 37° C for 30 min. This treated enzyme solution was used for electrophoresis studies as described above.

For degradation studies of the insecticidal substrate on the electrophoretically developed enzyme bands, the method developed by Matsumura and Sakai (1968) was modified as follows. The agar piece separated from the plate was transferred to a test tube containing 1 ml of the standard buffer, with or without cofactor or divalent cations. Radio-labeled insecticides (10^{-3} *M*) were added with 10 μl of absolute ethanol, and the system was maintained at 37° C for 2 hr with shaking.

To study the direct degradation activity of the liver 20,000 × *g* supernatant, a 10 μl aliquot of the insecticidal substrate in absolute ethanol was added to 0.5 ml of the dilute 20,000 × *g* supernatant (prepared from the original homogenate containing 4% weight by volume of liver tissue, except for malathion which required 0.5%), 0.3 ml of the standard buffer, and 0.2 ml of the buffer with the cofactor, the inhibitor, or ions. The system was incubated for 2 hr at 37° C with shaking.

All incubation products were analyzed as before (Matsumura and Sakai, 1968), except that the chloroform extraction process was repeated three times for all tests. The media incubated with Famphur, however, were extracted once with 3 ml of toluene. All electrophoresis experiments have been repeated at least four times.

RESULTS

A typical zymogram of rat liver 20,000 × *g* supernatant on the agar gel electrophoresis is shown in Figure 1, along with the numerical designation of the bands used throughout this study. It should be mentioned here that the true origin of the

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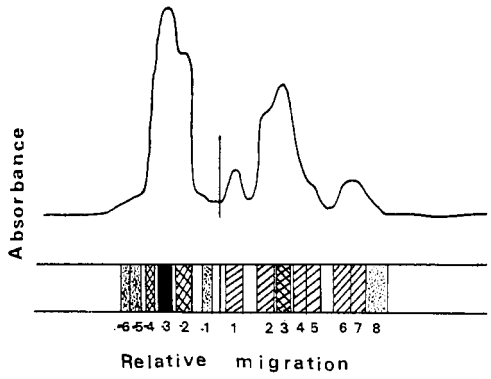

Figure 1. Thin-layer agar gel electrophoresis of the rat liver esterases hydrolyzing 1-naphthyl acetate. Zymogram developed diazo blue B was measured by a densitometer (upper graph)

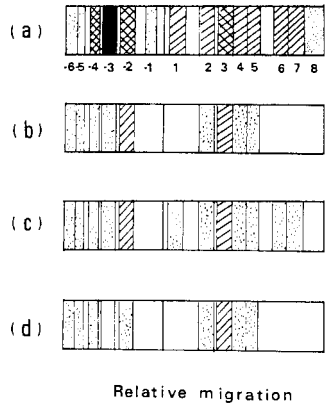

Figure 2. The effect of some inhibitors on the rat liver esterases separated by agar gel electrophoresis. (a) Control; (b) $10^{-4}M$ DFP; (c) $10^{-2}M$ TOCP; (d) $10^{-4}M$ dichlorvos

zymogram exists around band E_{-3} and E_{-4} because of the displacement of the origin during electrophoresis. Altogether 14 bands, including six anodically moving bands, were recognized by this method, in contrast to the results of similar studies by Schwark and Ecobichon (1969), who recorded 13 bands by using a starch gel electrophoresis technique.

To characterize these esterases various inhibitors were used. The results shown in Figure 2 indicate that band $E_{-3,-1,1,6,7}$ and E_8 are B-type esterases being susceptible to both DFP and dichlorvos. Band $E_{-6,5}$ and E_3 are of resistant type. $E_{1,6}$ and E_7 are susceptible to DFP and dichlorvos, but are resistant to TOCP.

To study the properties of degradation enzymes present in this 20,000 $\times$ g supernatant preparation, the effects of various

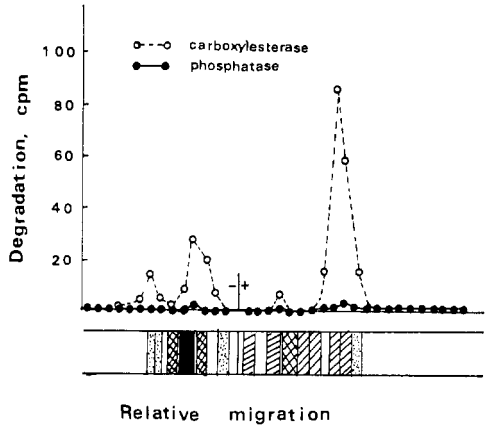

Figure 3. Degradation zymogram of ^{14}C-malathion by the rat liver esterases

ions, sesamex, and reduced glutathione on the rate of degradation were investigated (Table I). The absence of any inhibition of sesamex clearly indicates that the enzyme activities in consideration are not of general oxidase origin. GSH (reduced glutathione) strongly activated the degradation activities of Famphur, while the effect on diazinon was modest. Also evident was the activity of the carboxylesterase on malathion. Under these experimental conditions, however, neither Mn^{2+} nor Co^{2+} stimulated DFPase (Mounter *et al.*, 1955). Ca^{2+} did not appear to stimulate the esterase activity for dichlorvos (Hodgson and Casida, 1962). It is possible that the latter enzyme's localization is strictly limited to the mitochondria and that the centrifugal process eliminated the enzyme from this preparation. On the other hand, the degradation of diazinon was slightly enhanced by Ca^{2+}, but strongly inhibited by Hg^{2+}. This contrasts with the desmethylation process of malathion which was activated by Hg^{2+} without being affected by Ca^{2+}.

To correlate the above enzyme activities to the esterase positions on the electrophoresis plates, the agar pieces corresponding to esterase bands were separated and were incubated with various insecticidal substrates. The zymogram shown in Figure 3 indicates that the peaks of the malathion degrading activities aligned well with some of the 1-naphthyl acetate hydrolyzing activities. The highest carboxylesterase activity coincides with the band position of $E_{6,7}$ and E_8, all of which have been already shown to exhibit characteristics of the B-type esterase. The position of the second peak appears to align with band E_{-3} which also is a B-type esterase.

The zymogram obtained for parathion, however, does not show such clear alignment against the esterase bands which

Table I. Degradation Activities of Insecticidal Substrates by Rat Liver 20,000 $\times$ g Supernatant[a]

		Cofactor[b]–ion[c]–inhibitor[c]						
Substrate	Control	GSH	Ca^{2+}	Mn^{2+}	Co^{2+}	Cu^{2+}	Hg^{2+}	Sesamex
DFP	1.5	1.2		1.1	1.5			1.0
Famphur	1.1	7.8	1.6					0.7
Dichlorvos	11	12	9	9	9			10
Diazinon	3.5	5.7	4.2		3.8		0.13	3.3
Malathion[d] P	4.7	5.2	4.2		5.3	5.7	17.5	5.4
C	155	147	156		158	152	28.6	150
Parathion	2.5		2.0					2.7

[a] Data expressed in nmole hydrolyzed per mg protein per hr, at 37° C. Blank spaces indicate that the experiment was not performed. The experiment has been repeated at least twice for each insecticidal substrate. [b] 4×10^{-3} M final concentration. [c] 10^{-3} M final concentration. [d] "P" for desmethyl phosphate products. "C" for carboxylesterase products.

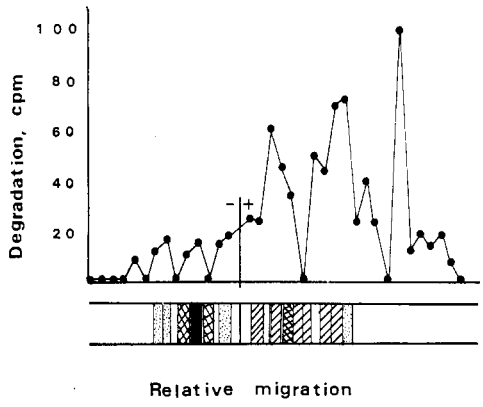

Figure 4. Degradation zymogram of ^{14}C-parathion by the rat liver esterases

are detected by using 1-naphthyl acetate. Enzyme activities could be observed even where no hydrolysis activities for 1-naphthyl acetate could be found. Only the area which coincided with the organophosphate resistant band area was that of E_2 and E_3. Essentially the pattern of zymogram was obtained for parathion in the presence of Ca^{2+}, confirming the previous finding *in vitro* that the Ca^{2+}-stimulated enzyme system is missing in this enzyme preparation (Table I).

No degradation activities were observed when dichlorvos, Famphur, and diazinon were used as substrates for the same electrophoresis studies either in the presence or absence of GSH. Nor were DFP degradation activities apparent with or without Mn^{2+}.

DISCUSSION

It is apparent that the original 20,000 × *g* supernatant preparation in the current study did not contain all enzymes described by other workers. Missing are, for instance, the specific Mn^{2+}- and Co^{2+}-dependent DFPase, and Ca^{2+}-stimulated enzyme for dichlorvos. It is, therefore, not surprising that these enzymes are not detected in the electrophoretically prepared agar plates.

There was, however, a strong GSH-stimulated enzyme for Famphur. The absence of a GSH-stimulated enzyme activity on the electrophoretically prepared plates could indicate that this electrophoresis method, at least, is not suited to detect some of the labile types of enzymes. Since the radioactive Famphur used herein was labeled at the ring position, extraction of the degradation products with toluene is expected to extract most of the known metabolic products such as *N*-demethyl Famphur (O'Brien *et al.*, 1965) except *O*-demethyl Famphur, which stays in the aqueous phase. It is likely, therefore, that the GSH stimulated process represents a dealkylation reaction such as the one described by Hollingworth (1969) for methyl parathion in the mouse liver systems.

The similar desmethylation reaction for malathion, however, appears to be carried out by a desmethyl phosphatase, since this process is not activated by GSH, and since desmethyl malathion is the only known phosphatase product which should carry radioactivity (malathion is labeled at 1,2-succinyl carbons). This esterase activity is not affected by any other factors except Hg^{2+}. It is, however, difficult to conclude whether this stimulatory effect is a genuine enzyme activation since, in the case of malathion, the inhibition of carboxylesterase by Hg^{2+} automatically increases the availability of this substrate to other enzyme systems.

In summarizing, the use of cofactor and ions helped identify at least four basic types of degradation enzymes: a GSH-stimulated dealkylation enzyme; a Ca^{2+}-stimulated diazinon degrading enzyme (Suwanai and Shishido, 1965); a desmethyl phosphatase of malathion which is not stimulated either by GSH nor Ca^{2+}; and at least three malathion carboxylesterases.

The use of this particular electrophoresis technique was helpful in studying malathion carboxylesterase, but the approach does not appear to be useful in detecting the GSH-stimulated dealkylation activities. Only four of the esterase groups could possibly be related to degradation activities of these phosphorus esters. The use of acrylamide gel electrophoresis did not facilitate the detection of organophosphorus degrading enzymes. Most of the strong esterase activities detected in the rat liver 20,000 × *g* supernatant by the use of 1-naphthyl acetate do not appear to be correlated to the processes of insecticidal degradation. These are mostly B-type esterases (*e.g.*, $E_{-3,1,6,7}$), but the presence of organophosphate-resistant types (*e.g.*, $E_{-6,-5,3}$) was also noticed. It was surprising that the latter enzymes, at least, did not show any abilities to degrade organophosphates except for the weak degradation activities against parathion. This marks a great contrast to the results obtained for mouse brain A-type esterase (Sakai and Matsumura, 1968), where most of A-type esterases separated by similar electrophoretic means appeared to be correlated to organophosphate degradation activities. It is likely that the majority of the 1-naphthyl acetate degrading esterases in the liver 20,000 × *g* supernatant are esterases which do not participate in degrading organophosphate esters.

LITERATURE CITED

Fukami, J., Shishido, T., *Botyu-Kagaku* **28,** 77 (1963).
Hodgson, E., Casida, J. E., J. AGR. FOOD CHEM. **10,** 208 (1962).
Hollingworth, R. M., J. AGR. FOOD CHEM. **17,** 987 (1969).
Kojima, K., O'Brien, R. D., J. AGR. FOOD CHEM. **16,** 574 (1968).
Main, R. A., Braid, P. E., *Biochem. J.* **84,** 255 (1962).
Matsumura, F., Sakai, K., *J. Econ. Entomol.* **61,** 598 (1968).
Mounter, L. A., *J. Biol. Chem.* **215,** 705 (1955).
Mounter, L. A., Dien, L. T. H., Chanutin, A., *J. Biol. Chem.* **215,** 691 (1955).
O'Brien, R. D., Kimmel, E. C., Sferra, P. R., J. AGR. FOOD CHEM. **13,** 366 (1965).
Ogita, Z., *Med. J. Osaka Univ.* **15,** 141 (1964).
Sakai, K., Matsumura, F., J. AGR. FOOD CHEM. **16,** 803 (1968).
Schwark, W. S., Ecobichon, D. J., *Can. J. Physiol. Pharmacol.* **46,** 207 (1968).
Schwark, W. S., Ecobichon, D. J., *Biochem. Pharmacol.* **18,** 915 (1969).
Shishido, T., Fukami, J., *Botyu-Kagaku* **28,** 69 (1963).
Suwanai, M., Shishido, T., "Experimental Results on the Resistance to Insecticides of Insects," (in Japanese) "Nihon Shokubutsu Boeki Kyokai," Japan 1965, p 116.
Yang, R. S. H., Hodgson, E., Dauterman, W. C , J. AGR. FOOD CHEM. **19,** 10 (1971).

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