Esterases of Mouse Brain Active in Hydrolyzing Organophosphate and Carbamate Insecticides

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A number of hydrolytic enzymes of mouse brain are detected by a thin-layer agar gel electrophoresis technique. Each of the 13 bands of esterase activity was tested against various substrates, including insecticidal, radiolabeled organophosphates and carbamates, to characterize the abilities of these esterases to degrade these insecticides and their analogs. Three cathodally moving and eight anodally moving bands show appreciable amounts of insecticide-degrading activity. The latter bands can be subdivided into three major groups, one of which is particularly active in hydrolyzing malathion carboxylesters.

Organophosphate and carbamate insecticides owe their toxicity against animals to effects upon the nervous system through their inhibitory properties against cholinesterases. Little is known, however, about the ability of the nervous systems to detoxify these toxicants, except in isolated instances (Mounter et al., 1955).

Enzymes involved in detoxification of organophosphate and carbamate insecticides have been extensively investigated with mammalian plasmata and livers. Aldridge (1953) discovered that serum esterases can be divided into two major groups by using an organophosphate inhibitor—i.e., the A, or aryl esterases, which are not only unaffected by $10^{-3}M$ paraoxon but degrade the phosphate, and the B, or aliesterase, which is inhibited by $10^{-7}M$ paraoxon. Studies by Bergmann et al. (1953) reveal the presence of a third group of esterases (the C-esterases) that were neither inhibited by organophosphate inhibitors nor degrade them. Though the above classification is important, discovery of various isozymes and related enzymes following the development of powerful electrophoresis techniques (Smithies, 1955) has necessitated further studies on these esterases.

Eränkö et al. (1962) electrophoretically separated esterases of the rat brain into five distinct bands. Barron et al. (1963) distinguished 13 esterase bands in the human brain by using vertical starch gel electrophoresis. The mouse brain esterases also were found to be separable into seven visible bands by starch gel electrophoresis (Lemkey, 1962).

Attempts were therefore made to study the degradation pattern of insecticidal esters by various brain esterases that have been separated by electrophoresis. A thin-layer agar gel electrophoresis technique was adopted because of its superior resolution capability and the ease of recovering the enzyme from the bed through extraction with buffer.

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EXPERIMENTAL

The brains, consisting of frontal, parietal, occipital, midbrain, and hypothalmic regions, from adult female mice were used throughout the study. The brain tissues were dissected out, washed in distilled water, and blotted on a filter paper to remove blood, and the homogenates were prepared with a Teflon-glass homogenizer at $0°$ C. using a pestle which was motor-driven at a speed of approximately 1000 r.p.m. Homogenates in distilled water consisted of 1 to 2 dilution (grams of wet tissue per milliliter) and were freeze-thawed five times in rapid succession prior to centrifugation at 25,000 × G at $0°$ C. for 60 minutes. The clear supernatant thus obtained was concentrated to 20 to 25% of the original volume through freeze-drying, and used as the final enzyme source.

The thin-layer electrophoresis technique employed for esterase separation was essentially that of Velthuis and Van Asperen (1963) and Ogita (1964) with a medium containing 0.7 gram of agar (Noble agar, Difco) and 2 grams of PVP [poly(vinyl pyrrolidone), molecular weight 360,-000, Nutritional Biochemicals Co., Cleveland, Ohio] in 100 ml. of veronal buffer (pH 8.4) of ionic strength 0.025. The medium was gelatinized on a microscope slide (1 × 3 inches) in such a way as to produce a 0.8- to 0.9-mm. thick layer with a very smooth surface. A constant current of 2.0 ma. per cm. width of the gel layer was applied for 40 minutes.

For starch electrophoresis studies the method of Barron et al. (1963) was directly followed without modification. For the detection of esterases, 1-naphthyl acetate (1 × $10^{-3}M$) was used throughout with diazo blue B (Nutritional Biochemicals Co.) as the coloring reagent for the end product of hydrolysis, 1-naphthol (Gomori, 1953). The same technique was applied for all the substrates that yield 1- or 2-naphthol as the end product. To determine the activity of cholinesterases, the method of Ellman et al. (1961) with acetylthiocholine was used. Breakdown of

insecticides was studied by first sectioning the corresponding zymogram into 35 to 38 pieces (usually 2-mm. width), and by homogenizing each piece with 2 ml. of 0.1 mM KH$_2$PO$_4$–NaOH buffer at pH 7.0 in a 15-ml. test tube. Each tube was then incubated with the desired isotope-labeled insecticide at 30° C. for 60 minutes. The reaction products were first extracted with an equal volume of chloroform to separate "solvent-soluble" compounds from "water-soluble" metabolites. In the case of carbamate insecticides, the chloroform fraction was further analyzed by thin-layer chromatography by using silica gel H (Merck AG, Darmstadt, Germany) with a mixture of n-hexane and ether (1 to 4) as the mobile phase. This treatment clearly separates carbaryl (R_f 0.64 ± 0.01) and its hydrolysis product, 1-naphthol (R_f 0.88 ± 0.01), or Baygon, o-isopropoxyphenyl N-methylcarbamate (R_f 0.22 ± 0.01), and its hydrolysis product, o-isopropoxyphenol (R_f 0.53 ± 0.01). The labeled insecticides used were parathion (diethyl H^3-labeled), DDVP (dichlorvos, dimethyl C^{14}-labeled), malathion (1,2-succinyl C^{14}-labeled), DFP (diisopropyl H^3-labeled), carbaryl (naphthyl ring H^3-labeled), and Baygon (isopropoxy-1,3-C^{14}-labeled).

The radioactivity of each sample was assessed by a liquid scintillation counter with a 10-ml. aliquot of counting solution: a mixture of toluene (0.5 liter), ethylene glycol monomethyl ether (0.5 liter), PPO (5.5 grams), and dimethyl POPOP (300 mg.). All counting vials with the liquid scintillation phosphor were precounted at least five times before the addition of the radioactive samples. The electrophoresis tests were repeated at least four times for each insecticidal substrate to ensure the reproducibility of the degradation zymograms.

RESULTS

By the method employed, 1-naphthyl acetate is hydrolyzed by 10 anodally moving bands, numbered consecutively E_1 to E_{10}, E_{10} migrating farthest from the origin. Three cathodally moving enzymes, designated E_{-1} to E_{-3} and separable with varying degrees of sharpness, are also encountered (Figure 1). Generally, the activity of

the zymogram for the anodally moving esterases is concentrated in three major zones: a quadruplet close to the origin (bands E_1 to E_4), a closely approximated midanodal triplet (bands E_5 to E_7), and a far-moving anodic triplet (bands E_8 to E_{10}). The degree of separation of the E_8–E_{10} triplet is variable. The intensity of staining a band E_{10} is inconsistent, and this may affect the clarity of its separation from the E_8–E_9 zone. Zymograms obtained with 2-naphthyl benzoate are almost identical to those obtained with 1-naphthyl acetate. Apparently, the occasional loss of activity in the region of E_5–E_7 was caused by denaturation of the enzymes due to aging. In a few cases, the cathodally migrating bands are less sharply separated than depicted. Band E_1 is variable because it is arbitrarily defined as the band immediately adjacent to the origin.

Results on the mouse brain esterases hydrolyzing 1-naphthyl acetate and related esters are shown in Figure 2. With acetylthiocholine iodide as the substrate, four bands of activity are encountered on the anodal side (Figure 2d). Two bands (E_1 and E_3 in the diagrammatic representation) are completely inhibited, while E_2 is partially inhibited by DFP ($10^{-4}M$).

DDVP at the concentration used does not result in complete inhibition of any band active against naphthol esters. However, the data indicate the degree of resistance of the soluble esterolytic activities hydrolyzing 1-naphthyl acetate. The resistant enzymes—e.g., bands E_2, E_5, and E_7—are classified as A-type esterases after the terminology of Aldridge (1953). Band E_{10}, which is often weakly reactive with 1-naphthyl acetate, is slightly activated by p-hydroxymercuribenzoate (p-HMB) (Sigma Chemical Co., St. Louis, Mo.) at $10^{-3}M$, indicating the presence of a C-type esterase in the zone corresponding to E_{10}. Bands E_5, E_6, and E_7 were very susceptible to p-HMB, and with other evidence (Table I) these esterases may be classified as A-type esterases.

To correlate our zymographic results with those of Lemkey (1962), the studies of starch electrophoresis were also made with 1-naphthyl acetate as the test substrate (Figure 3a). For the characterization of the relative enzyme patterns, it is necessary to conduct an inhibition study with DFP (Figure 3c) and acetylthiocholine (Figure 3b) in the manner used by Lemkey (1962). From the zymograms for both agar and starch gel electrophoresis, in
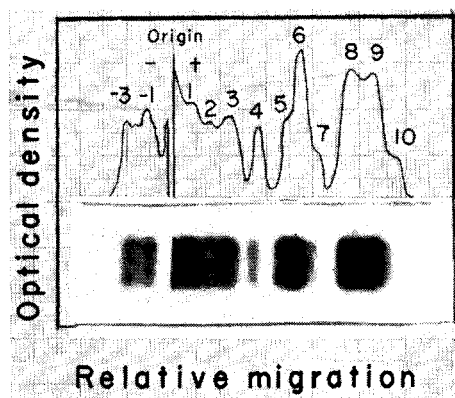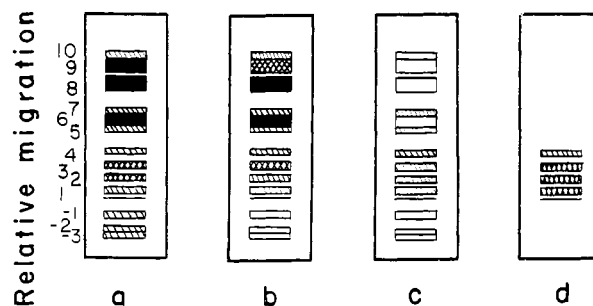

Figure 1. Agar gel electrophoresis of mouse brain esterases with 1-naphthyl acetate as a substrate

Zymogram developed with diazo blue B and relative absorbance of each band measured by a densitometer

Figure 2. Schematic representation of agar gel electrophoresis of mouse brain esterases which hydrolyze

a. 1-Naphthyl acetate
b. 2-Naphthyl butyrate
c. Carbaryl
d. Acetylthiocholine

Inhibitors[a]	Esterase Bands[b]										
	−1 to −3	1	2	3	4	5	6	7	8	9	10
1-Naphthyl Acetate as Substrate											
Control	+	+	+	+	+	+	+	+	+	+	+
DDVP, $10^{-5}M$	−	−	+	−	±	+	+	+	−	−	±
DFP, $10^{-5}M$	−	−	+	−	±	+	±	+	−	−	±
p-HMB, $10^{-3}M$	−	+	+	+	+	−	−	−	±	±	+
Acetylthiocholine as Substrate											
Control	−	+	+	+	+	−	−	−	−	−	−
Eserine, $10^{-3}M$	−	−	±	−	+	−	−	−	−	−	−

[a] DDVP (or dichlorvos, O,O-dimethyl O-dichlorovinyl phosphate), DFP (diisopropyl phosphorofluoridate), and p-HMB (p-hydroxymercuri-benzoate).
[b] + indicates presence, − absence of a staining reaction with diazo blue B (for 1-naphthyl acetate hydrolysis) or 5,5-dithio-bis-7-nitrobenzoic acid (for acetylthiocholine hydrolysis).

general, the separation pattern of 1-naphthyl acetate–hydrolyzing esterases apparently is similar. Nevertheless, a close examination indicates that some minor variations could result from the differences in the separation method: In the starch gel zymograms there is a strong band in front of band E_1.

An attempt was made to estimate the degradation activity of these esterases against parathion and diazinon, two typical diethyl phosphorothioates. The results shown in Figure 4 indicate that the patterns of degradation activities by these esterases against both substrates were similar. There are, however, two distinctly different types—e.g., E_5 and E_7—of degradation esterases for each substrate. Band E_7 has high activity against parathion and a low activity toward diazinon, while band E_5 shows an exactly opposite tendency.

Malathion is a representative of a unique group of organophosphorus compounds that are selective because of the additional hydrolyzable carboxylic ester bands (Krueger and O'Brien, 1959). The results of malathion hydrolysis (Figure 5) indicate that bands E_2, E_6, E_8, and
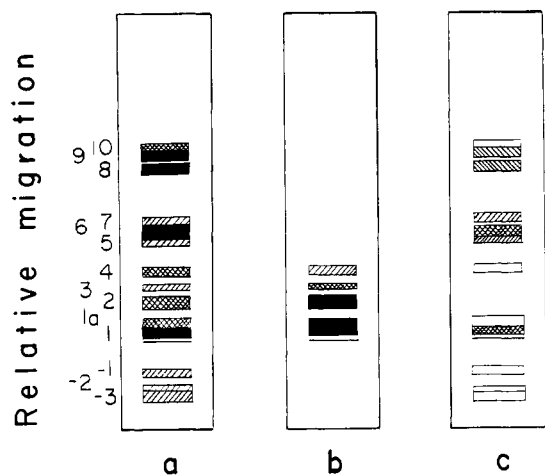
Figure 3. Schematic representation of starch gel electrophoresis of mouse brain esterases which hydrolyze

a. 1-Naphthyl acetate
b. Acetylthiocholine
c. 1-Naphthyl acetate in the presence of $10^{-3}M$ DFP

Relative migration
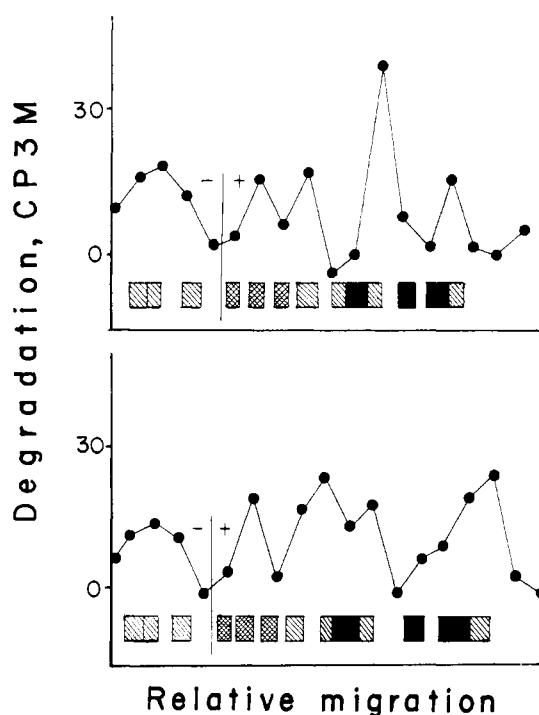
Figure 4. Degradation zymogram of H^3 parathion (upper) and C^{14} diazinon (lower) by mouse brain esterases

Gel was sliced into 19 pieces and each piece incubated with $10^{-5}M$ of radiolabeled insecticide to assess its degradation capacity. Corresponding zymogram is for 1-naphthyl acetate, simultaneously developed to indicate the relative position of each band. Results are expressed by the amount (in terms of H^3 or C^{14} counts per 3 minutes—i.e., CP $3M$) of degradation products formed

E_9 have strong hydrolysis activity at the carboxyester sites, and bands E_2, E_4, E_5, E_7, and E_{10} possess the ability to degrade the malathion molecule at the methoxyphosphate sites.

Studies on the distribution pattern of esterases against DDVP and DFP (Figures 6 and 7) reveal discrete differences between the zymograms of DDVP- or DFP-resistant esterases and those of DDVP- or DFP-degrading enzymes. The pattern for DDVP degradation is almost
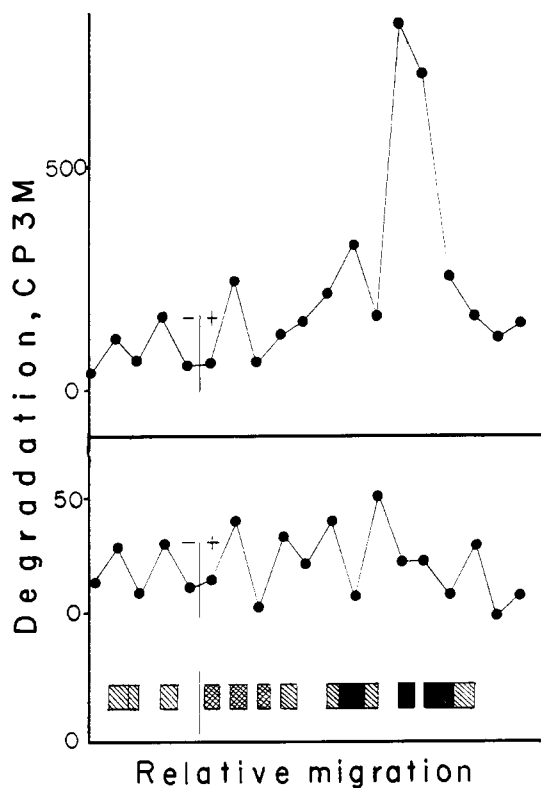
Figure 5. Degradation zymograms of C^{14} malathion at the carboxyester (upper) and methoxyphosphate ester sites (lower figure)
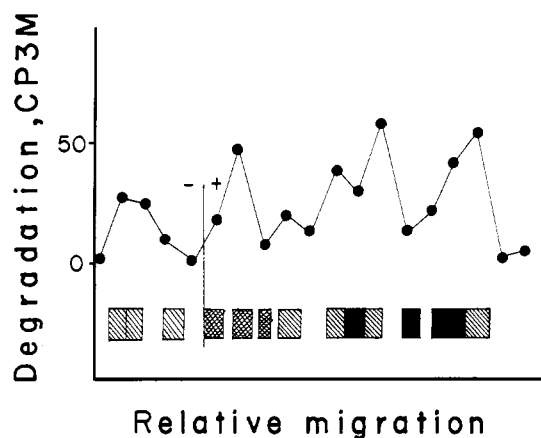
Experimental conditions as in Figure 4

Figure 6. Degradation zymogram of C^{14} DDVP (dichlorvos) by mouse brain esterases

Experimental conditions as in Figure 4

Figure 7. Degradation zymogram of H^3 DFP by mouse brain esterases

Experimental conditions as in Figure 4

Figure 8. Degradation zymograms of H^3 carbaryl (upper) and C^{14} o-isopropoxyphenyl N-methylcarbamate (Baygon, lower figure) by mouse brain esterases

Experimental conditions as in Figure 4

identical to that for diazinon. The band between bands E_7 and E_8 appears to be specifically active in degrading the DFP molecule. The three bands migrating toward the cathode in agar gels are very similar in mobility, substrate specificity, and inhibitor sensitivity. It could be that these three cathodic bands are multiple forms of the same enzyme—i.e., isozymes.

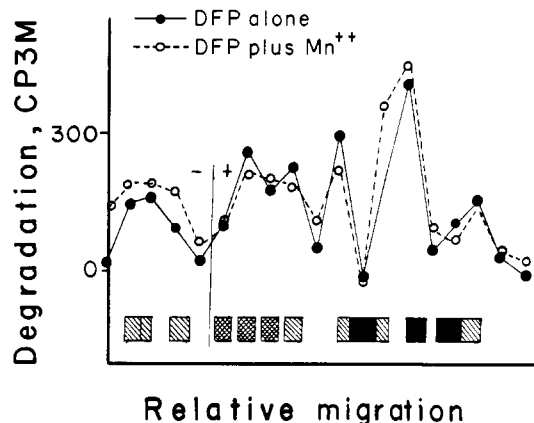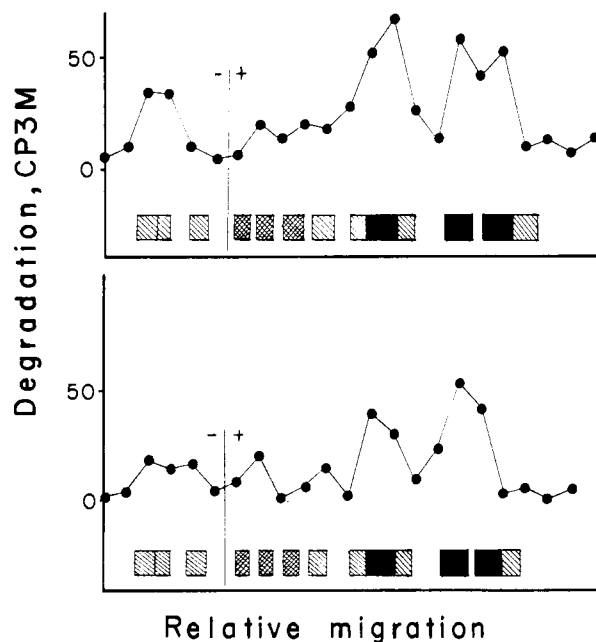Similar tests using H^3-carbaryl (Figure 8) indicate that

enzymes in bands E_6, E_8, and E_9 are active in hydrolyzing carbaryl. The degradation pattern of Baygon is somewhat different; here band E_8 is particularly active in hydrolyzing the molecule to yield o-isopropoxyphenol.

DISCUSSION

Despite the excellent reproducibility of the degradation patterns by the method used to determine insecticide hydrolysis, the major analytical method, which is based upon the simple incubation and extraction procedures of insecticidal substrates and the enzymes in agar pieces, can involve two types of artifacts relating to physical binding of insecti-

cidal esters: to the agar pieces themselves, and to the proteins in and/or outside the agar pieces. The first appears not to be a problem, because no radioactivity is recovered from the agar pieces themselves after they have been incubated with labeled insecticides. To test the second possibility of insecticide binding with soluble proteins, the incubation medium was analyzed with a Sephadex G-50 column (Matsumura and O'Brien, 1966). None of the media contained insecticide-bound soluble proteins. Paper chromatographic analyses of the aqueous phase after extracting twice with ether failed to detect any measurable amount of the parent compound remaining in the medium. Other control experiments to study the validity of the test method employed were: homogenate-free controls for both naphthyl esters and insecticide-degradation tests, substrate-free controls for diazo blue B coloration artifacts, zero-minute incubation controls for insecticidal binding tests, and precounting and quenching correction controls for radioassay.

Apart from the methodological problems, there are a number of questions to be answered before classifying the brain esterases that degrade insecticidal esters. The first is the heterogeneity of each band, which appears to be single by the zymogram obtained with 1-naphthyl acetate. For instance. band E_2 exhibits characteristics of both A-type and B-type esterases, which could mean that it is a mixture of at least two esterases: an acetylthiocholine-hydrolyzing and organophosphate-susceptible B-type esterase and a DDVP and DFP-hydrolyzing, organophosphate-resistant esterase. A similar conclusion can be made about the E_4 esterases, though the B-type esterase of the latter is different from that of E_2 esterase in that the E_2 appears to be able to hydrolyze malathion at the carboxylic ester sites while the latter does not. On the other hand, bands E_5, E_6, and E_7 exhibit a typical A-type esterase pattern, being sensitive to p-HMB and resistant to DDVP and DFP. Zone E_6, however, could contain a B-type component, as judged by its susceptibility toward organophosphates. Band E_8 appears to consist of a typical B-type esterase (aliesterase) which can selectively degrade malathion at the carboxylic ester bond. Judging by the reaction toward acetylthiocholine, and by the previous observation by Lemkey (1962), bands E_1 and E_8 involve cholinesterases that hydrolyze acetylthiocholine. Although DFP was degraded by various mouse brain enzymes, the lack of activation with Mn^{2+} appears to indicate the absence of a specific DFPase in the mouse brain preparation.

CONCLUSIONS

A number of brain enzymes degrade insecticidal esters. They can be divided into three major zone groups—i.e., band groups E_2 to E_4, E_5 to E_6 to E_7, and E_9 to E_{10}. Band E_8 had a particularly high carboxylesterase activity against malathion. Three cathodally moving bands degrade all substrates tested. This may mean that these bands contain a number of esterases with various substrate specificities, or consist of related nonspecific enzymes with very broad substrate spectra.

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