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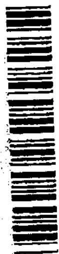

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INITIAL CHARACTERIZATION OF THE ORGANOPHOSPHATE ACID ANHYDRASE ACTIVITY OF THE CHICKEN, *GALLUS DOMESTICUS*

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Abstract—1. Supernatant solutions from kidney and liver homogenates of the chicken, *Gallus domesticus*, were found to hydrolyze the organophosphate (OP) compound diisopropylfluorophosphate (DFP). The activity on DFP as substrate was heat-inactivated and was characterized for temperature and pH optima, enzyme kinetics, and requirements for manganous ion.

2. Gel column chromatography indicated that the DFPase in both tissues is in the range of 82,100 to 93,300 D. This activity is strongly inhibited by N,N′-diisopropylphosphorodia–midofluoridate (mipafox).

3. The chicken has organophosphate acid (OPA) anhydrase activity comparable to other eucaryotic sources in its ability to hydrolyze DFP. Although birds may not have paraoxonase activity comparable to mammalian species, they do not differ significantly in the ability to hydrolyze DFP and probably related compounds.

INTRODUCTION

Recent interest has focused on a group of enzymes that may convey resistance to organophosphate poisoning. These enzymes, the OPA anhydrases, are defined as enzymes capable of hydrolyzing the acid leaving group of an OP. Once the acid leaving group is hydrolyzed, the OP is incapable of inhibiting acetylcholinesterase (AChE). Therefore, the OPA anhydrases act as detoxifying enzymes (Chemnitius *et al.*, 1983).

Avian species are considered generally more susceptible than mammals to organophosphate and carbamate poisoning (Schafer 1972; Brealey *et al.*, 1980). The biotransformation of phosphorothionates (thions) to their oxygen analogs (oxons) is a necessary step before AChE inhibition, and some researchers have concluded that this metabolic activity determines greater susceptibility of some species (Neal, 1967). Investigations by Machin *et al.* (1976) and Brealey *et al.* (1980) also suggest that organophosphate-hydrolyzing esterases play a role in the protection of an organism from organophosphate intoxication. Machin *et al.* (1975) conducted a comparative study of the hepatic microsomal metabolism of diazinon (*O,O*-diethyl *O*-(2-isopropyl-6-methyl)-4-pyrimidinyl phosphorothioate) in five mammals and the turkey, chicken and duck. Machin *et al.* (1975) found that the transformation of diazinon to diazoxon (*O,O*-diethyl*O*-2-(1-methylethyl)-4-pyrimidinyl phosphate) was not generally correlated with the greater susceptibility of birds to diazoxon poisoning. A further investigation (Machin *et al.*, 1976) revealed that hydrolysis of the oxon by esterases in avian sera was slow or undetectable, while mammalian sera exhibited a high activity. Brealey *et al.* (1980) found that mammalian sera had from 13 to 170 times the hydrolyzing activity of 14 species of birds against pirimiphos methyloxon (*O*-2-diethylamino-6-methylpyrimidin-4-yl *O,O*-dimethyl phosphate) and paraoxon. In accordance with these findings, both Machin *et al.* (1976) and Brealey *et al.* (1980) postulated that serum esterases play a primary role in conveying resistance to OPs, thereby being the distinguishing factor between bird and mammal susceptibility to many types of organophosphate poisoning.

Paraoxonase activity, or hydrolysis of paraoxon, has become synonymous with "A-esterase" activity. Despite this, Aldridge (1989) points out that by his original definition (Aldridge, 1953) depicting an enzyme as an A-esterase may not be appropriate if both carboxylic and organophosphorus substrates have not been proven to be hydrolyzed by the same esterase. It appears that at the present time, paraoxonase ("A-esterase") activity is within the defined OPA anhydrase activity, and investigations into A-esterases provide insight into OPA anhydrases. Mackness *et al.* (1987) utilized paraoxon and pirimiphosimethyloxon as substrates for A-esterase activities and phenyl acetate for arylesterase activity to determine that the two groups of activities are expressed by different enzymes. Ten species of birds had no detectable levels of A-esterase activity, yet were capable of arylesterase activity. In fact, the arylesterase activity was inhibited by paraoxon, making it a B-esterase. In ten species of mammals, both activities were apparent, and in the case of human serum, they were completely separated from each other by gel filtration. Mackness *et al.* (1987) propose that the term A-esterase has value in signifying toxicological activity, but that its meaning be clarified. Throughout the remainder of this paper, the term A-esterase will be used if an investigator chose

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to use it to indicate paraoxonase or OPA anhydrase activity.

Hoskin *et al.* (1984) recognized two categories of DFP hydrolyzing OPA anhydrases, Mazur-type and squid-type. The typical Mazur-type OPA anhydrase found in hog kidney is characterized as being stimulated by Mn^{2+}, hydrolyzing soman faster than DFP, and being non-tolerant of ammonium sulfate precipitation. The enzyme is found to be demeric with a molecular weight of approximately 62,000 D (Storkebaum and Witzel, 1975). Mazur-type OPA anhydrase is competitively inhibited by mipafox, a strong structural analog to DFP (Hoskin, 1985). The typical squid-type OPA anhydrase is only found in the nervous tissue, saliva and hepatopancreas of cephalopods (Hoskin and Brande, 1973; Hoskin and Prusch, 1983). In contrast to the Mazur-type OPA anhydrase, squid-type OPA anhydrase is unaffected or slightly inhibited by Mn^{2+}, hydrolyzes DFP faster than soman, can be purified using ammonium sulfate, and has a molecular weight of approximately 23–30,000 D. DFP hydrolysis by squid-type OPA anhydrase is not inhibited by mipafox (Hoskin, 1985).

Numerous investigations have found high OPA anhydrase activity in vertebrate liver and kidney tissue homogenates (Mounter *et al.*, 1955a,b; Mounter, 1955; Storkebaum and Witzel, 1975; Chemnitius *et al.*, 1983). In several cases this activity was augmented by Mn^{2+} or Co^{2+} (Mounter *et al.*, 1955a,b; Mounter, 1955). For example, Chemnitius *et al.* (1983) investigated phosphorylphosphatase activity in various organs from several mammalian species, chicken hens, trout and carp. The use of a spectrophotometric assay for paraoxonase and the fluoride ion assay for DFPase allowed them to differentiate these two activities. Most organisms had paraoxonase activity of only 1% or less of the DFPase activity. Chickens exhibited only low activities toward both substrates. The DFP and paraoxonase activities had different distributions within the monkey and the hog. This fact indicated that there may be two distinct enzymes responsible for the hydrolysis of DFP and paraoxon. However, single- and mixed-substrate experiments on bovine liver homogenate indicated some overlap in specificity for the two substrates, and the authors suggest that the two substrates might be hydrolyzed at different active sites of the same enzyme (Chemnitius *et al.*, 1983).

Little *et al.* (1989) studied the OPA anhydrase properties of the soluble fraction from a rat liver homogenate. In this case, they removed the microsomal fraction by supercentrifugation at 205,000 *g*, and considered the post-microsomal supernatant solution to be the "soluble fraction". Eighty-five percent of the soman–hydrolytic activity was in the soluble fraction, 15% in the particulate. This is in agreement with Hoskin and Prusch (1983), who stated that 80–90% of the DFPase activity of homogenate preparations from rat kidney, liver, and brain, and from squid is present in the soluble fraction. Hydrolysis rates were sarin > soman > tabun > DFP, with DFP activity in order of magnitude less than tabun. Gel filtration by HPLC indicated that all hydrolytic activities eluted at the same point, corresponding to a molecular weight of approximately 40 kD. Mn^{2+}, Co^{2+} and Mg^{2+} stimulated the activity of sarin and soman hydrolysis, with Mg^{2+} having the greatest effect. None of these affected tabun hydrolysis, but Mn^{2+} and Mg^{2+} did stimulate DFPase activity. In tests with sarin and soman, concentrations above 5 mM Mg^{2+} correlated with significant increases in spontaneous hydrolysis. Little *et al.* (1989) stated that this may be due to Mg^{2+} catalysis, similar to the theories of Epstein and Mosher (1968) and Epstein (1970).

A large portion of the A-esterase and arylesterase activities in mammalian sera is associated with the lipoprotein fraction (Mackness *et al.*, 1985; Mackness and Walker, 1981, 1983, 1988). The lipoprotein fraction of sheep serum appears to contain the majority of paraxonase activity (Mackness and Walker, 1981), but pirimiphos–methyloxonase activity was nearly equally divided between lipoprotein and non-lipoprotein fractions. The paraoxonase activity was completely inhibited by EGTA (ethyleneglycol-bis-(β-amino-ethyl-ether)N,N′-tetra-acetic acid), but the inhibition was fully reversed by $CaCl_2$, indicating the requirement of Ca^{2+} as a cofactor (Mackness and Walker, 1981). Human serum somanase activity was inhibited by two other chelating agents, 8-hydroxy quinoline and EDTA (ethylenediamine tetraacetic acid) (De Bisschop *et al.*, 1987). Similar to the sheep serum, paraoxonase and DDVP-hydrolyzing activities of EDTA-treated human serum returned after incubation with Ca^{2+} (De Bisschop *et al.*, 1987; Traverso *et al.*, 1989).

Avian species have been utilized as one means of testing the effects of organophosphates (OPs) on nontarget species, particularly since birds are generally more sensitive than mammals to these compounds, and are representative of the species that may be exposed in agricultural applications. Mallard ducklings (Fleming, 1981), Japanese quail (Dieter and Ludke, 1978; Westlake *et al.*, 1981), and starlings (Grue *et al.*, 1981; Grue and Hunter, 1984) have been used in testing the effects of organophosphorus compounds.

There are several reasons for using the chicken (*Gallus domesticus*) in an initial characterization of avian OPA anhydrases. Chickens are readily available to researchers, are cost efficient and easily maintained, and provide more than adequate amounts of tissue for multipe analyses on one individual. Once general parameters and distribution of OPA anhydrases in this species have been established, researchers can conduct comparative studies, much as the hog kidney DFPase functions as a comparative mammalian OPA anhydrase. Because it is a common species, and is available to most researchers, an extensive body of literature exists on the anatomy and physiology of this species.

This study demonstrates an activity in *G. domesticus* able to hydrolyze the DFP at rates comparable to other eucaryotic sources. The activity is found in the liver and kidney, is inhibited by mipafox, and has a molecular weight in the range of 80,000 D. In many characteristics the enzyme is similar to that found in mammalian tissues.

MATERIALS AND METHODS

Chemicals

Diisopropylfluorophosphate (DFP) was supplied by Sigma D-0878, lot no. 129F-0390. N,N′-diisopropylphosphorodiamidofluoridate (mipafox) and *Tetrahymena* OPA anhydrase were kindly supplied by M. V. Haley, Chemical Research Development and Engineering Center, Aberdeen Proving Ground, MD.

Chicken tissues

Chicken tissues were kindly made available by the Draper Valley Farms processing plant in Mount Vernon, Washington. Chickens were Arbor Acre (female)–Peterson (male) hybrids, and were 46 days old at the time of processing. Prior to processing, each bird was weighed for comparisons of live body weight. The birds were then tagged and placed on an automated processing line where they were stunned, decapitated, bled, and had their feathers removed. At this point, the tagged birds were removed from the line and maintained on crushed ice until the tissues were removed. They were cut open by a processing supervisor and inspected by the resident Department of Agriculture inspector for disease and physiological abnormalities. After the birds had passed inspection the organs were removed by dissection, with special care taken to leave associated organs intact and unremoved.

The organs were double-bagged in freezer storage bags and placed in a cooler containing dry ice and crushed ice. The samples were frozen in a –15°C freezer upon return to Western Washington University.

Preparation of homogenates

Homogenization was carried out in an 80 mM Tris (Trizma base (Sigma)) buffer in 0.15 M NaCl, pH 7.2 to 7.4 (hereafter called TSB). In preparation for homogenization, the organs were thawed and rinsed with TSB to remove blood, and were blotted dry. To avoid loss of enzyme activity organs and homogenates were maintained on ice at all times. Individual preparations were made of each liver and kidney from each animal.

Organs were homogenized in a 50 ml Potter–Elvejhem homogenizer in one volume of TSB (1:1 (w/v)) with three passes at the highest rpm, and an additional 0.5 volume of TSB was used to rinse the homogenate into a beaker. Homogenates were centrifuged using a Sorvall RC-5 Superspeed refrigerated centrifuge. An initial centrifugation at 10,000 *g* for 10 min failed to produce a distinct pellet. Thereafter, the initial centrifugation was done at 15,000 *g* for 20 min. The supernatant solution was carefully removed with a Pasteur pipette and centrifuged again at 20,900 *g* for 20 min. The first pellet was resuspended in 0.1% Triton X-100 in TSB to solubilize any membrane bound enzymes, and brought up to the volume of the original homogenate. This mixture was frozen in aliquots and stored at −15°C for possible future investigations. This preparation was used for subsequent tests, and is designated as homogenate throughout the remainder of this paper. The second pellet was also resuspended in 0.1% Triton X-100, brought to one fourth the original homogenate volume, and frozen.

Assay and characterization of OPA anhydrase activity

Hydrolysis of the substrates DFP and mipafox releasing fluoride ions into solution was measured by following the change in fluoride concentration. Fluoride concentration was measured with an Orion Model 96-09 fluoride electrode in combination with an Orion SA720, Orion 901, or Fisher Accumet Model 925 ionanalyzer. Concentration values were recorded at one minute intervals by hand or with a Brother M-1109 Dot Matrix Printer. Reaction temperature was controlled by carrying out the assays in a 10 ml water-jacketed vessel, connected to a temperature-controlled circulating water bath. Prior to the assays the meter was calibrated with 10^{-4} and 10^{-5} M NaF standards.

The general procedure for each assay has been outlined by Landis *et al.* (1986). Prior to each set of assays at a given set of parameters, spontaneous hydrolysis of the substrate was recorded until readings had stabilized for 5–8 min. This determination of spontaneous hydrolysis was used for the calculation of activity for this and other enzyme trials at these parameters. Following equilibration, 100 μl of the homogenate were added to the substrate, and the change in concentration of F^- was followed through at least 5 minutes of linear change. Triplicate assays were performed to insure reliability of results, and standard deviation was calculated to indicate variability. *Tetrahymena* DFPase, from a partially purified homogenate, served as a positive control in several tests.

Soluble protein concentration in the homogenates and fractions was determined by the method of Bradford (Bradford, 1976), using bovine serum albumin (BSA, Sigma) as the calibration protein.

pH Optima

pH Optimum tests were conducted using kidney homogenate supernatant solution from male #2 (hereafter designated as KS2) and liver homogenate supernatant solution from male #10 (LS10). The assays were carried out at 23°C, with solutions of Hoskin buffer adjusted to the appropriate pH with 1 N HCl. DFP-buffer solutions were prepared with these buffers immediately prior to use. Rates of spontaneous hydrolysis and enzyme activity were tested at pH 4.0, 5.0, 6.0, 7.0, 8.0, 8.5, 9.0, and 9.5.

Temperature optima

Temperature optima for the KS2 and LS10 homogenates were determined at pH 7.4, and temperatures of 10, 20, 30, 40, 50, 55, and 60°C. DFP-buffer solutions were allowed to equilibrate to the assay temperature for 5 minutes prior to the addition of the enzyme solution.

Denaturation test

Homogenates were heated at 65°C for 30 min, cooled to room temperature, and tested for hydrolytic activity on the DFP substrate. The specific activity was compared with the activity of homogenates that had not been heat-treated.

Column chromatography

Molecular size column chromatography was carried out on liver and kidney homogenates. Substantial amounts of the LS10 homogenate remained after optimal tests, but insufficient quantities of KS2 were left. Another male kidney homogenate with a similar level of activity, KS12, was used for column chromatography and subsequent experiments.

A 2.1 cm × 65 cm glass column packed with Sephacryl S-300 HR was utilized for the chromatography. Calibration of the column was accomplished by several protein markers (Boehringer Mannheim) and blue dextran in 0.05 M potassium phosphate buffer, pH 7.5. Multiple calibrations were performed and several markers were duplicated as cross-references to determine reproducibility. The column was washed with 500–1000 ml of Hoskin buffer between each fractionation. The composition of each set of calibration mixtures is presented in Table 1. The void volume was 96 ml. Flow rate during calibration was 0.55–0.66 ml/min; it was 0.59–0.66 during fractionation of the homogenates.

Two ml fractions were collected with a Gilson FC-80 Micro Fractionator. Blue dextran eluted first off the column, and served to indicate the void volume. Fractions were collected through the elution of cytochrome C. Relative protein distribution among fractions was determined by measuring absorbance at 280 nm with quartz cuvettes and a Perkin-Elmer Lambda 3B UV/Vis spectrophotometer. This method was used to indicate the elution profile of the

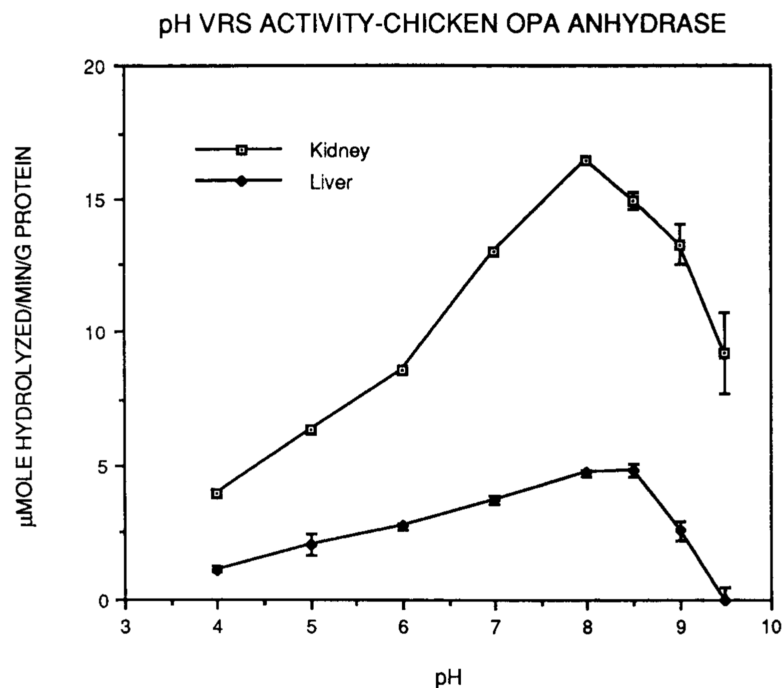

Fig. 2. Activity versus pH of the chicken OPA anhydrase. The pH range at which activity can be demonstrated is large, from pH 4 to 9. The optimum pH lies at 8.0 to 8.5. Again, the kidney homogenate demonstrates a much higher activity than that of the liver derived tissue.

successful. The rate of the reaction was so rapid at pH 8 and 40°C that at higher DFP concentrations, the production of F^- ions exceeded the calibrated range of the probe/meter. For this reason there was insufficient data for higher substrate concentrations. A second attempt at estimating the K_m and V_{max} was made, with a 1:8 (v/v) dilution of the homogenate. Once again, the enzyme did not appear to be ap-

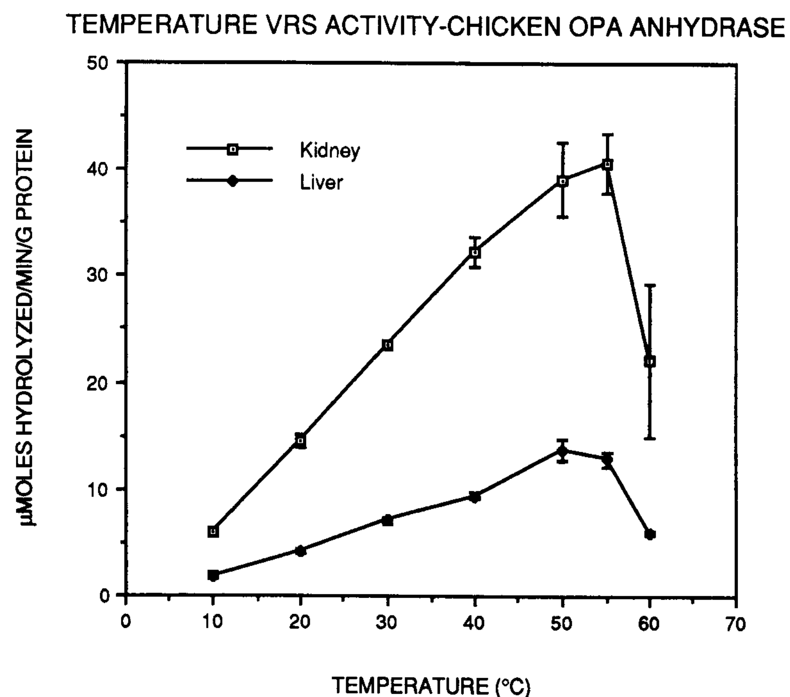

Fig. 3. Activity versus temperature of the chicken OPA anhydrase. A temperature of 50 to 55°C was optimal for the activities derived from the kidney and liver tissues. Interestingly, these optimal temperatures are much higher than the typical physiological temperature of a bird, approximately 40°C.

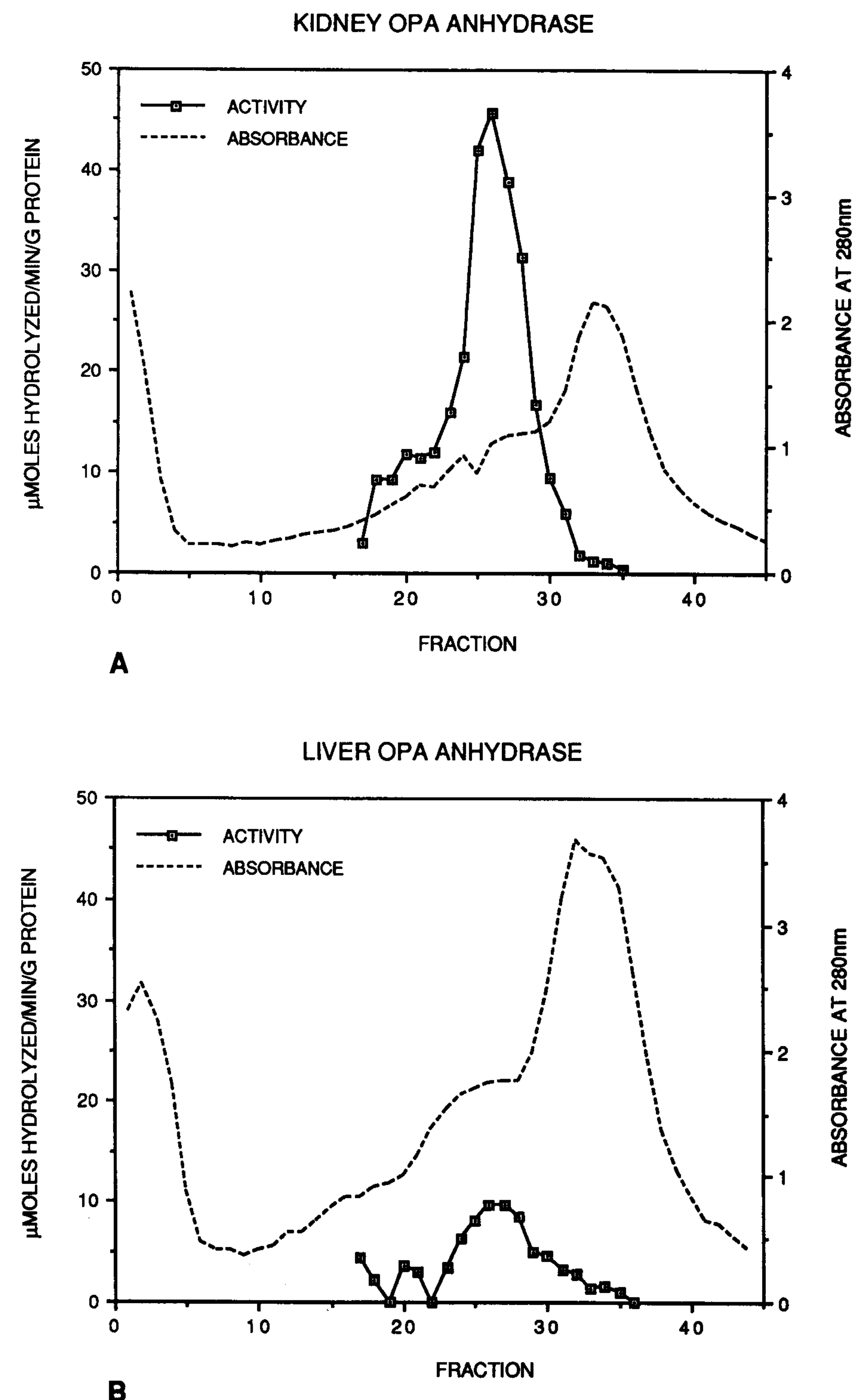

Fig. 4. Column chromatography of the chicken OPA anhydrases. The peak of the specific activity from kidney homogenate (A) corresponds to a molecular weight of 93,000 D. Specific activity of the partially purified liver homogenate (B) is of the same approximate molecular weight, a range of between 82,000 and 93,000 D. Even when partially purified, the specific activity of the liver homogenate is much lower than that of the kidney.

Table 3. Comparison of *G. domesticus* OPA anhydrase activities with other eucaryotic sources

Phylum/Class *Genus*	Mn^{2+} stimulation	Mipafox inhibition	Substrate preference	MW (Dalton)
Aves				
Chicken kidney and liver	+for DFP	+		82–93,000
Mammalia				
Hog kidney (Mazur Type)	+for DFP and soman	+	Soman > DFP	62,000[1] 30,000 (unit)
Protista/*Tetrahymena*[2]	−for DFP* +for soman	+	Soman = DFP	67–96,000 multiple
Mollusca/ *Loligo*[3] Cephalopoda (squid-type)	−	−	DFP > soman	23–30,000 monomeric
Mollusca/ Pelecypoda *Mytilus*[4]	−or DFP	−		24–32,000 24–32,000†

*One of the purified Tetrahymena activities is stimulated by Mn^{2+}.
†For mipafox hydrolyzing OPA anhydrase.

proaching substrate saturation with DFP concentrations in the limits of the probe calibration. Therefore, a third attempt was made, utilizing pooled fractions. Since kidney and liver chromatographies eluted the active enzyme at the same point, fractions 24–27 were pooled for both tissues. Fractions from KS12-2 and LS10-1 were highest in activity, and provided the material for pooled fraction tests. Hemoglobin and some of the other extraneous proteins were excluded by the gel chromatography; the pooled fractions were thereby partially purified and would permit a more accurate initial estimation of the liver and kidney enzyme kinetics. An additional measure was taken to lower the assay temperature to 25°C, at pH 8.0, in order to keep activity within the calibration range of the probe.

The pooled fractions were tested with 1, 2, 3, 5, 8, and 10 mM DFP concentrations. It appears that the enzyme(s) from both sources behave similarly, as activities are parallel throughout the substrate range, and begin to plateau above 5 mM DFP. Values for K_m and V_{max} from Lineweaver–Burke, Hanes–Woolf, and Woolf–Augustinsson–Hofstee plots are presented in Table 3. Interestingly, the Hanes–Woolf estimation gives the highest values, and Lineweaver–Burke is always lowest in estimations of K_m and V_{max}. From this data it appears that the kidney enzyme has a V_{max} in the range of 70.6 to 86.1 μmol F^-/min/g protein, and a K_m of 5.2 to 6.2 mM DFP. The pooled liver fraction, in fitting with its overall lower specific activity, has a V_{max} between 31.7 and 35.4 μmol F^-/min/g protein, and a K_m of 2.1 to 3.3 mM DFP.

Incubation of the homogenates with 0.1 mM EDTA reduced kidney activity by 9% and liver specific activity by 22%. In comparison, the activity of the DFPase control from *T. thermophila* was reduced by 25%. Mn^{2+} stimulates activity of all three samples, with *Tetrahymena* DFPase showing the greatest increase in hydrolysis. Its activity increased 260%, while kidney activity was augmented by only 20%, and the liver by 30%.

Both kidney and liver homogenates showed a limited capacity to hydrolyze mipafox. Figure 5 shows the results of substrate tests with kidney and liver homogenates and the positive control, *Tetrahymena* DFPase. Mipafox activity was 21% of DFP activity for *Tetrahymena* DFPase, 20% for kidney homogenate, and 17% for liver homogenate. This activity was further reduced in the partially purified, pooled fractions, such that the kidney's activity is only 2% of activity on the DFP substrate activity, and the liver drops to 11%.

Mipafox drastically inhibits DFP-hydrolzying activity in mixed-substrate tests for all of the OPA anhydrases tested. Similar to the *Tetrahymena* DFPase (71% inhibition), the kidney and liver homogenates were inhibited by 70% and 69%, respectively. The pooled fractions were both inhibited to an even greater degree, approximately 91%.

DISCUSSION

Based on the results of several tests, it was apparent that both liver and kidney homogenates from the male chicken contain at least one DFP-hydrolyzing OPA anhydrase. Heat-denaturation of an enzyme would presumably destroy the active site of the enzyme, reducing or greatly limiting activity. This was documented by a lack of activity in the heat-treated homogenates. Temperature, pH, and kinetics tests also provided characteristics of an enzyme-catalyzed hydrolysis of the substrate, rather than a generalized protein interaction.

In similar preparations of the liver and kidney homogenates, the kidney exhibited 2 or 3 times greater specific activity throughout all experiments. The vertebrate liver is known to serve as a primary detoxication site (Spies and Gandolfi, 1986) with high activities of various enzyme systems such as the cytochrome P-450 mixed function oxidases. However, the kidney of many vertebrate species (e.g. hog and rat) is also a source of high OPA anhydrase activity (Mounter, 1955; Mounter *et al.*, 1955b). In this respect, avian species may have a slight physiological advantage since birds are able to direct blood from the gut to the kidneys via the coccygeomesenteric vein (Walker, 1983). Mammals do not have this circulatory pathway, and nearly all orally ingested compounds are routed to the liver.

The DFPase activity of 15.2 μmol F^-/min/g protein (kidney homogenate) and 5.69 μmol F^-/min/g protein (liver homogenate) is considerably higher than reported by Chemnitius *et al.* (1983) for the hen: 2.9 μmol F^-/min/g protein, and 1.1 μmol F^-/min/g protein for kidney and liver, respectively. These differences may be attributable to procedures and assay parameters, as well as age and sex-related

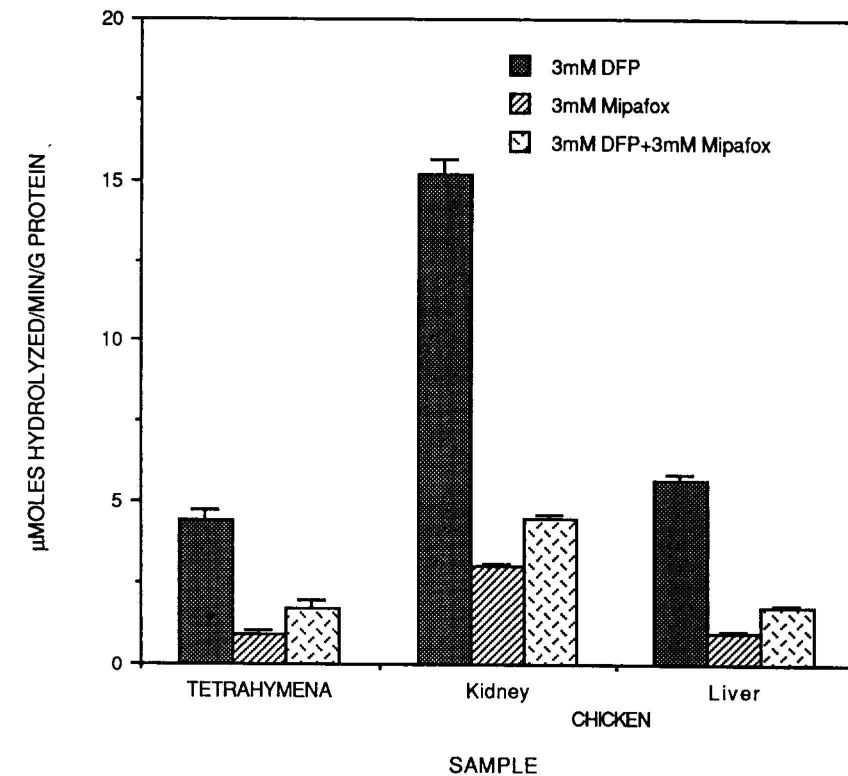

Fig. 5. Mipafox inhibition of DFP hydrolysis. As is typical with many mammalian and many other eucaryotic organisms, the OPA anhydrase is inhibited by mipafox. The patterns of inhibition for both the kidney and liver homogenates is similar to that of the *Tetrahymena* control.

differences (Walker, 1980). The pH of the two assays was slightly different (7.3 vs 8.0), Chemnitius *et al.* (1983) and utilized a higher concentration of DFP (110 mM DFP vs 3 mM DFP), but assay temperatures were the same. Differences may also be augmented by the buffer solution. Chemnitius *et al.* (1983) used a phosphate–carbonate buffer with 0.,137 M NaCl and 0.003 M KCl. In these experiments, Hoskin buffer was used to facilitate comparisons of results with the squid type OPA anhydrase and the positive control, *T. thermophila* DFPase. Landis *et al.* (1986) tested a homogenate of *T. themophila* in 0 and 500 mM NaCl, and Hoskin buffer. In comparison to 0 mM NaCl, the rate was 8% higher in 500 mM NaCl, but 150% higher in Hoskin buffer. This pattern is similar to that of purified hog kidney DFPase (Storkebaum and Witzel, 1975). The activity of the hog kidney OPA anhydrase did not change from 0 to 0.6 M NaCl, but increased between 0.6 and 1 M NaCl. Beyond 1 M NaCl the hog DFPase started to precipitate with a loss of activity.

Both of the chicken tissue homogenates exhibited singular peaks in pH experiments, establishing the optimum pH for activity at 8.0 to 8.5. The enzyme appeared to be stable over the time frame of the assay (15–25 min) at this and all other tested pHs. This optimum is in agreement with other pH optima for squid type OPA anhydrase (pH 7.5–8.5, Hoskin and Long, 1972), and A-esterase activity in the lipoprotein fraction of sheep serum (pH 8.0–8.5, Mackness and Walker, 1981). Traverso *et al.* (1989) reported that human serum A-esterase activity continues to increase with pH across the range of pH 6 through 10.

The temperature optimum for sustained activity of the enzyme is 50°C, although a slight, time-dependent loss of activity was evident in a few assays. This is in accord with the body temperature of avian species that is typically 40°C. *In vitro* optimum parameters for enzymes do not usually reflect the *in vivo* conditions necessary for long-term stability of biological systems. Time-dependent denaturation of the enzyme is apparent at 55°C, and almost all activity is destroyed by heating the enzyme at 65°C for 30 min. Extracts of *T. thermophila* behaved similarly, loosing 90% of activity when heated at 65°C for 30 min (Landis *et al.*, 1985). In contrast, Storkebaum and Witzel (1975) were able to heat hog kidney DFPase at 60°C for 40 min with only a 25% loss of activity. They theorized that the hog kidney enzyme was stabilized in part by the presence of Mn^{2+}, intentionally included in the media for that purpose. This cation was not provided by the buffers used to prepare and test the chicken tissues. In addition to heat denaturation of the active site, the chicken homogenate OPA anhydrases would be subject to the action of proteases that had been eliminated as the hog kidney DFPase was purified to near homogeneity.

Chromatography indicated a molecular weight of 82,100 to 93,300 D for the active OPA anhydrase in kidney and liver homogenates. The dilution volumes for the calibration markers were confirmed by a second fractionation, and chromatography of the two homogenates were repeated with similar results. The gel chromatography affected only a slight purification of enzyme from either source, with a recovery of 20–25% of activity, and there are no protein peaks to correlate with the DFPase activity peaks.

It is difficult to determine if the active enzyme has also dissociated into subunits. Chow and Ecobichon (1973) isolated two distinct forms of hepatic esterases, 56,000 D and 240,000 D. The higher molecular weight form could be dissociated by a combination of acidic pH and high salt concentration into 56,000 D units. Miller *et al.* (1980) purified a monomeric carboxylesterase of 66,100 D and an oligomeric carboxylesterase of 209,000 D from rabbit liver. The oligomer dissociated into an active monomer subunit at high dilutions and by treatment at pH 4.35 or with 1.0 M KCl. Extreme pH (≤ 3 or ≥ 9.5) dissociates hog kidney DFPase, as does NaCl greater than 1.0 M (Storkebaum and Witzel, 1975). Neither of these conditions were met in the homogenization or elution buffers. The molecular weight of the chicken OPA anhydrase appears to be within the range of Mazur type enzymes and well below that of the serum A-esterase (Table 5).

By any method of estimation, kinetic parameters of the two tissue enzymes were different from each other: V_{max} is 70.6 to 86.1 μmol F^- min/g protein for kidney, and 31.7 to 35.4 μmol F^- min/g protein for the liver. K_m estimates for the two were 2.1 to 3.3 mM DFP for the kidney, and 5.2 to 6.2 mM DFP for the liver. It was hoped that the lower assay temperature (25°C), and the use of an enzyme that was partially purified but also diluted by the fractionation process, would allow the testing of a sufficiently broad range of DFP concentrations and present a better estimation of the enzyme kinetics. Two important factors must be taken into account when considering these estimates of the kinetics. First, only slightly purified enzymes were tested. A great deal of the differences between the kidney and liver DFPases may be attributed to a higher concentration of non-active proteins in the liver fraction, which affect the calculation of specific activities and kinetics. As has been mentioned previously (see Introduction), inhibitors, ions and proteases in whole cell homogenates interact with the enzyme of interest in ways that cannot be controlled or accounted for. Thus, the lack of a purified enzyme precludes anything but initial characterizations of kinetic parameters, and allows only the most general comparisons to the literature.

A second consideration is the capacity of experimental substrate concentrations to encompass the K_m. The most reliable estimations of kinetics will be accomplished if substrate concentrations extend well below and above the K_m (Neame and Richards, 1972; Segel, 1976). Experimental substrate concentrations covered a range from 1 mM to 10 mM DFP; concentrations of DFP lower than 1 mM were beyond the detection limits of the probe. Concentrations of DFP above 10 mM were not utilized because the resultant activity was beyond the calibrated range of the probe. The estimated K_m for the kidney enzyme is within the lower range of these substrate concentrations. Thus, substrate concentrations did encompass the K_m, but tests of activity at substrate saturation were not permissable with the present calibration and enzyme concentration.

The OPA anhydrase of both tissues shows a slight stimulation in activity after incubation in 1 mM Mn^{2+}, and this ion produced a significant increase in hydrolysis by the positive control, *Tetrahymena* DFPase. The effect of Mn^{2+} on *Tetrahymena* DFPase in these experiments with the reconstituted partially purified material exhibits a stimulation in contrast to the crude homogenates originally investigated by Landis *et al.* (1985). They reported that DFP hydrolysis was unstimulated or slightly inhibited by 1 mM Mn^{2+}. However soman hydrolysis by *Tetrahymena* DFPase was greatly stimulated by Mn^{2+}.

Tests with the chelating agent EDTA revealed slight reductions in activity of the *Tetrahymena* DFPase and both chicken homogenates. Again, the results for the *Tetrahymena* DFPase are in opposition to a previous report (Landis *et al.*, 1985), in which EDTA halted DFPase activity. In this case there may be a simple explanation of the conflicting results. When EDTA is dissolved, the pH of the solution is lowered significantly, with a resultant attenuation of enzyme activity. In the experiments on the chicken enzymes, the pH of the EDTA solution was corrected prior to its use. However, no pH correction methods have been revealed in a limited review of the literature. This may be due to the brevity of most accounts of experimental procedures. If such corrections are not made, it is very well possible that a loss of activity due to low pH will instead be attributed to the chelation of metal ions. This is probably the case in the literature on *Tetrahymena* DFPase (Landis *et al.*, 1985).

EDTA selectively chelates divalent cations. If the chicken OPA anhydrase is a Mn^{2+} or Ca^{2+} requiring enzyme, it would seem probable that EDTA would reduce activity to a greater degree than observed. The possibility exists that the tissue homogenate contained sufficient levels of the ions that a 0.1 mM EDTA solution was not able to remove these ions from both the solution and the enzyme. One possible approach to this problem would be to increase EDTA concentration, or dilute the ions in the initial preparation. For their investigations, Little *et al.* (1989) diluted rat liver homogenate to 5% (w/v) prior to centrifugation for the soluble fraction. Little and coauthors theorized that this approach reduced the exogenous Mg^{2+} to a concentration of only about 0.01 mM Mg^{2+} in the final soluble fraction, permitting a better estimation of activity in the absence of Mg^{2+}.

Procedures like these, in combination with the use of chelating factors, could clarify the understanding of OPA anhydrase metal ion requirements. If all solutions were corrected for pH, the methods employed by De Bisschop *et al.* (1987), Traverso *et al.* (1989), and the experiments by Hoskin (1990) provide results that should be used in future comparisons of OPA anhydrases. Hoskin (1990) avoided the addition of cations in preparing squid OPA anhydrase so he could selectively test for activity with Ca^{2+}, Mg^{2+}, and Mn^{2+} and several chelating agents of different specificity. The requirements for a specific cation could be revealed by incubating the enzyme with a chelator, followed by the metal ion. Theoretically, activity following chelation would be recovered to a greater degree by a required ion than by a stimulating cation, as became apparent with Ca^{2+} (required), and Mg^{2+} (stimulating ion) (Hoskin, 1990).

Single substrate tests revealed that mipafox is a poor substrate for the OPA anhydrase of either chicken tissue. Limited capacity to hydrolyze this structural analog of DFP has also been demonstrated for hog kidney DFPase and *Tetrahymena* DFPase (Landis *et al.*, 1989), indicating their similarity in this respect. The positive control, *Tetrahymena* DFPase, exhibited activity similar to published results (Landis *et al.*, 1989). In the pooled fractions mipafox-hydrolyzing activity approached the level of spontaneous hydrolysis, in contrast to the dramatically higher specific activity toward DFP in the fractions. Limited amounts of mipafox prevented a screening of all the fractions for mipafox-hydrolyzing activity. It may be that purification of the DFPase excluded the mipafox activity, or diluted it.

While DFPase activity was partially purified by the gel chromatography, it also appeared to be more sensitive to inhibition by mipafox. Mixed-substrate tests with 3 mM DFP and 3 mM mipafox show that mipafox is a powerful inhibitor of DFP activity in the homogenates and pooled fractions. The results for the *Tetrahymena* DFPase are similar to that reported previously and both the chicken and *Tetrahymena* OPA anhydrases are similar to the Mazur type hog kidney DFPase (Landis *et al.*, 1989). Beyond general comparisons to OPA anhydrases from other sources, it is difficult to draw other conclusions from the results of the mixed-substrate tests.

In summary, initial characterization of the DFPase from chicken kidney and liver indicates the activity is probably the result of the same enzyme in both tissues. This enzyme has a low level of mipafox-hydrolyzing activity, and the DFPase activity is strongly inhibited by mipafox. The enzyme is slightly stimulated by Mn^{2+}, and shows a slight loss of activity when incubated with EDTA. Gel column chromatography indicates that the chicken OPA anhydrase has a molecular weight of 82,000 to 93,000 D. These initial characteristics point to a similarity to the Mazur type enzymes. A more purified enzyme and further substrate specificity tests could clarify the relationship of the identified OPA anhydrase with other such enzymes.

Avian sera appears to have very low or undetectable OPA anhydrase activity on several organophosphate substrates (Brealey *et al.*, 1980; Machin *et al.*, 1976). The inability to hydrolyze these compounds has been implicated as a cause for avian species' greater susceptibilities to organophosphate poisoning (Brealey *et al.*, 1980; Chemnitius *et al.*, 1983; and Machin *et al.*, 1976). Little is known about the presence or specificity of OPA anhydrases in most species of birds, even though the starling and bobwhite quail are utilized extensively for field studies on the effects of organophosphates. The information presented in this paper is an initial characterization of avian OPA anhydrases that could be used for comparative investigations of detoxication enzymes in other avian and vertebrate species.

In this issue it is reported that DFP hydrolyzing OPA anhydrase activities have also been identified from bobwhite quail, mallard and stilt (Landis and Shough, 1992). Activities are higher in the kidney than the liver and resemble the enzymes characterized in the chicken. Temperature and pH ranges and kinetics are similar to chicken OPA anhydrase. It does not appear that the OPA anhydrase in chicken is phylogenetically isolated. Although the literature reports low or non-detectable levels of OPA anhydrase activity in birds, this may be due to the substrate specificity of the enzymes being narrow and more typical of the OPA anhydrases in other eucaryotes.

Classification of the OPA anhydrases is in need of further refinement, and it may be necessary to develop a system that accounts for phylogenetic differences (Noellgen, 1991). Isolation of a natural substrate(s) for the OPA anhydrases would facilitate several aspects of this research, and until such compounds are discovered the natural relationships between phyla or species and OPA anhydrase activity will not come to light. Unless natural organophosphate substrates are determined, OPA anhydrase activity must be considered a secondary activity of a diverse and broadly distributed group of enzymes. Naturally occurring organophosphates have been discovered, such as 2-aminoethylphosphoric acid (AEP) (Hilderbrand and Henderson, 1983), but they have not been utilized as substrates. AEP is incorporated into lipids and proteins, as well as in its free form. The biological function of this phosphate is not clear. Halogenated compounds approximating the P–F bonds of DFP and soman have not been discovered, though there are a diverse number of halogenated organics. The majority of these halometabolites discovered so far are found in the marine environment. Halogenation is carried out by bacteria, fungi, algae, higher plants, and to a smaller degree, animals (Neidleman and Geigert, 1986), with the general characteristic of increasing the biological activity of the final compound. One of the fluorometabolites, fluoroacetate, is highly toxic, and is produced by several higher plants. Testing these halogenated compounds as substrates for the OPA anhydrases is an avenue of investigation that should be explored.

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