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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-diisopropylphosphorodi-mido-fluoridate (mipaflox).

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 their 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-methylthyl)-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 methylxon ([*O*,*O*-diethyl-*O*-[2-(1-methylthyl)-4-pyrimidinyl 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 its 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 pirimiphosmethylxon 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...
to use it to indicate paroxonase 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 by being stimulated by Mg and inhibited by Mn. The squid-type OPA anhydrase found in somatic cells is much more resistant to DFP, and being non-tolerant of ammonium sulfate precipitation. The enzyme is found to be deermic with a molecular weight of 62,000 D (Storkbeern 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, the squid-type anhydrase is unaffected or slightly inhibited by Mn, hydrolyses DFP faster than soman, can be purified using ammonium sulfate, and has a lower molecular weight of approximately 23-36,000 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; Storkbeern and Witzel, 1975; Cheminius et al., 1984). Later studies have shown that soman, human serum anhidrase activity was augmented by Mn or Co (Mounter et al., 1955a, b; Mounter, 1955). For example, Cheminius et al. (1983) investigated phosphorylphosphatase activity in liver and kidney homogenates from mammalian species, chicken hens, trout and carp. The use of a spectrophotometric assay for paroxonase and the fluoride ion assay for DFPase allowed them to differentiate between the two. Postmortem studies indicated that paroxonase activity of only 1% or less of the DFPase activity. Chickens exhibited low activity or very low activity in the kidney, particularly in the liver, which is more reactive than the kidney. In guinea pig, human serum anidrase activity was determined in serum to be much more sensitive to paroxonase activity of the same enzyme (Cheminius et al., 1983).

Little et al. (1989) studied the OPA anhydrase activity of the solvent fraction from a rat liver homogenate. In this case, they removed the microsomai fraction by supercentrifugation at 205,000 g, and considered the post-microsomal supernatant "solvent fraction"" matrix. Eighty-five percent of the soman-hydrolitic 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 "solvent fraction" matrix. In the solvent fractions, the activity was higher in the weight of the fraction at 80,000 D. In many mammalian species, the enzyme is similar to that found in mammalian tissues.

**MATERIALS AND METHODS**

**Chemicals**

Dipropylphosphorothionate (DFP) was supplied by Sigma D-087, lot no. 129F-0300, N,N-dipropylphosphophosphate (DPP) was provided by the Research Development and Engineering Center, Aberdeen Proving Ground, MD.

**Chicken tissue**

Chicken tissues were kindly made available by the Drapper Valley Poultry Plant of Mount Vernon, Washington. Chickens were Arbor Acre (female)-Pekin (male) hybrids, and were 46 days old at the time of processing. Pekin chickens the average weight of live body weight. The chickens were then tagged and placed on an automated processing line where they were stunned, de-feathered, and eviscerated. At this point, the tagged birds were removed from the line and maintained on crushed ice until the tissues were removed. The animals were processed by a USDA federal inspector and a USDA federal inspector and were removed by the resident Department of Agriculture inspector for disease and physiolog al 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 to Western Washington University.

**Preparation of homogenates**

Homogenization was carried out in 80 mM Tris (Tris base) was buffered to pH 7.4 with 0.1 M NaCl, pH 7.2 to 7.4 (hereafter called TSB). For homogenization, the organs were thawed and rinsed with TSB to remove blood and to prevent tissue injury. To avoid loss of enzime activity organs and homogenates were maintained on ice at all times.

**Assay**

Acetylcholineste was incubated with RC-Super speed refrigerated centrifuge. An initial centrifugation at 10,000 g for 10 minutes failed to produce a distinct pellet. The hydrolysis of OPA was done at a temperature of 37°C for 20 minutes. The supernatant was carefully removed with a Pasteur pipette and centrifuged again at 9,000 g for 20 minutes. The fluorescence in 0.1% Triton X-100 in TSB was solubilized to determine enzyme activity.

**Column chromatography**

The columns were accomplished by using one of the following: Betonlb (5 μm) and Superdex 200 HR. Fractions of 10 ml were collected at 1 ml/minute. The column was washed with 500-1000 ml of buffer from each fractionation. The composition of each set of chromatograms was determined using a Perkin-Elmer Model 600 Micro Fractionator. The Fluoride elution was monitored by using a Waters Model 490A Micro Fractionator. The fraction was eluted first of the column, and served to indicate the void volume. Fractions were collected and their fluorescence values were recorded at one minute intervals by hand or with a Badger 1110 910 Matrix. Reaction was cooled to 0°C with a HPLC on a 30 cm, 5 μm, 4.6 mm column, and the mobile phase was a linear gradient of target mobile phase was a linear gradient of 50-0% methanol. The reaction was run at 2°C.

**Reagents**

**Fluorescein isothiocyanate (FITC)**

FITC was purchased from Sigma Chemical Company, St. Louis, Missouri. The solution was prepared by dissolving 5 mg of FITC in 1 ml of dimethyl sulfoxide (DMSO).

**Materials**

**Analytical grade chemicals**

The following chemicals were purchased from Sigma Chemical Company, St. Louis, Missouri: Fluorescein isothiocyanate (FITC), fluorescein (FIT), and fluorescein diacetate (FDA).

**Buffers**

The following buffers were used throughout the experiments:

- **pH 7.4**: 0.1 M potassium phosphate buffer, pH 7.4
- **pH 7.0**: 0.1 M potassium phosphate buffer, pH 7.0
- **pH 6.0**: 0.1 M potassium phosphate buffer, pH 6.0
- **pH 5.0**: 0.1 M potassium phosphate buffer, pH 5.0
- **pH 4.0**: 0.1 M potassium phosphate buffer, pH 4.0

**References**

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 1:10 (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 Da. 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 Da. Even when partially purified, the specific activity of the liver homogenate is much lower than that of the kidney.
Table 3. Comparison of G. domes tici OPA anhydrase activities with other eucaryotic sources

<table>
<thead>
<tr>
<th>Phylum/Class Genus</th>
<th>Mn²⁺ stimulation</th>
<th>Mipaflox inhibition</th>
<th>Substrate preference</th>
<th>MW (Daltons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aves</td>
<td>for DFP</td>
<td>+</td>
<td>82-93,000</td>
<td></td>
</tr>
<tr>
<td>Mammalia</td>
<td>for DFP</td>
<td>+</td>
<td>Soman &gt; DFP</td>
<td>62,000</td>
</tr>
<tr>
<td>Hog kidney (Mauror Type)</td>
<td>for DFP and soman</td>
<td>+</td>
<td>Soman &gt; DFP</td>
<td>30,000</td>
</tr>
<tr>
<td>Protilis (Tetrahymena)</td>
<td>for DFP⁴</td>
<td>+</td>
<td>Soman &gt; DFP</td>
<td>67-96,000</td>
</tr>
<tr>
<td>Mollicus/Loligo⁴</td>
<td>for DFP</td>
<td>+</td>
<td>DFP &gt; soman</td>
<td>23-30,000</td>
</tr>
<tr>
<td>Ophrutila (squalid-type)</td>
<td>for DFP</td>
<td>-</td>
<td>Monosomic</td>
<td></td>
</tr>
<tr>
<td>Mollicus</td>
<td>for DFP</td>
<td>-</td>
<td>24-32,000</td>
<td></td>
</tr>
<tr>
<td>Petelypida Mytilus⁴</td>
<td>for DFP</td>
<td>-</td>
<td>24-32,000</td>
<td></td>
</tr>
</tbody>
</table>

*A One of the purified Tetrahymena activities is stimulated by Mn²⁺.

**For mipaflox 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 chromatograms 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 measurement was taken to establish a correlation between 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 in similar proportions. 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 total and free from Lineweaver-Burke, Hanes-Woolf, and Woolf-Augustinsson-Hoestee plots are presented in Table 3. Interestingly, the Hanes-Woolf estimation gives the highest values, and Lineweaver-Burke is always lowest in estimations of Kₐ and Vₘₐₓ. From this data it appears that the kidney enzyme has a Vₘₐₓ in the range of 70.6 to 86.1 μMol F⁻¹/min/g protein, and a Kₐ of 5.2 to 6.2 mM for liver fraction. In fitting, the enzyme exhibited 2 or 3 timer greater specificity activity throughout all experiments. The vertebrate liver is known to serve as a primary degrading homogenates. Temperature, pH, and kinetics tests also provided characteristics of an enzyme-catalyzed hydrolysis of the substrate, rather than a generalized protein interaction.

In homogenates of the liver and kidney homogenates, the kidney exhibited 2 or 3 timer greater specificity activity throughout all experiments. The vertebrate liver is known to serve as a primary degrading homogenates. Temperature, pH, and kinetics tests also provided characteristics of an enzyme-catalyzed hydrolysis of the substrate, rather than a generalized protein interaction. The patterns of inhibition for both the kidney and liver homogenates are similar to that of the Tetrahymena control.

**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-denatured homogenates. Temperature, pH, and kinetics tests also provided characteristics of an enzyme-catalyzed hydrolysis of the substrate, rather than a generalized protein interaction.

The pattern is similar to that of purified hog kidney DPase (Storkenbaum 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 DPase started to precipitate with a loss of activity.

Both of the chicken tissue homogenates exhibited strikingly similar 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, losing 90% of activity when heated at 65°C for 30 min (Landis et al., 1985). In contrast, Storkenbaum and Witzel (1975) were able to heat hog kidney DPase 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 Mn²⁺, intentionally added in the media for that purpose. This calcium 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 DPase 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 chromogram of the gel filtration of OPA activity by a second fractionation, and chromatography of the two homogenates were repeated with similar results. The gel chromatography afforded only a slight purification of the enzyme. The recovery of enzyme activity was 20-25% of activity, and there are no protein peaks to correlate with the DF-Pase activity peaks. It is difficult to determine if the active enzyme has also been eluted in this last fractionation, but it is possible that the homogenates of Hepatocytes (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 acetic acid and high salt concentration into 56,000 D units. Miller et al. (1980) purified a monomeric carboxyesterase of 66,100 D and an oligomeric enzyme of 1,860,000 D. 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 (≤ 5 or ≥ 9.5) dissociates hog kidney DF-Pase, as does NaCl greater than 1.0 M (Storkenbaum and Witzel, 1975). Neither of these conditions were met in the homogenization or elution buffers. The molecular weight of the chicken OPA anhydrase is approximately the same as the Mazur type enzymes and well below that of the serum A-esterase (Table 5).

By any method of estimation, kinetic parameters of the mammary gland that were determined from each enzyme, v_e is to 76.0 ± 8.6 μM F - min g protein for kidney, and 37.0 ± 35.4 μM F - min g protein for liver. K_e for the two were 2.1 ± 3.0 mm DF-Pase, while K_e for the liver was 1.1 ± 0.3 mm DF-Pase. The higher activities may be lower significantly, with a resultant attenuation of enzyme activity. In the experiments on the chicken enzymes, the pH of the DF-Pase 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, the results of DF-Pase assays will be lower due to low pH will instead be attributed to the chelation of metal ions. This is probably the case in the literature on Tetrahymena DF-Pase (Landis et al., 1985), which EDTA halted DF-Pase activity. In this case there may be a simple explanation of the conflicting results. While EDTA may be the method of choice for low pH, the dfp is strongly bound by EDTA, and a more purifying of enzyme preparations is necessary. A great deal of the differences between the kidney and liver DF-Pases may be attributed to a higher concentration of non-active proteins in the liver fraction, which is the result of the dilution of active enzyme in biological activity.

The enzyme has a low level of mipafos, hydroxylating activity, and the DF-Pase activity is strongly inhibited by mipafos. The enzyme is slightly soluble in distilled water, but it has a slight loss of activity when, after being incubated, DF-Pase. 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 and other enzymes.

Avian sera appears to have very low or undetectable OPA anhydrase activity on several organophosphate substrates (Aldridge et al., 1980; and Machin et al., 1982). The inability to hydrolyze these compounds has been implicated as a cause for avian species’ greater susceptibilities to organophosphate poisoning. These compounds include a variety of organophosphate compounds and have been used in OPA anhydrase assay in many species of birds, even though the stimulating and bovine sera are utilized extensively in 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 to investigate the detoxification enzymes in other avian and vertebrate species.

In this issue is reported that DF hydrolyzing activity in OPA anhydrase actives have also been identified from bovine and mallard and still (Landis and Shough, 1982). Activities are higher in the kidney than in the liver and resemble the enzymes characterized in the chicken. Temperature and pH range 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 in birds, this may be due to the substrate specificity of the enzymes being narrow and more typical of the OPA anhydrase in other eutherians.

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, 1981). 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 organophosphates substrates are determined, OPA anhydrase activity could be considered to be phylogenetically different and more distributed and broadly distributed group of enzymes. Naturally occurring organophosphates have been discovered, such as 2-aminoethylphosphonic acid (AEP) and dibenzylphosphonate, 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 phosphophate is not known. The hydrolyzing activity of 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 halogenated organics are found in the marine environment. Halogenation is carried out by bacteria, fungi, algae, higher plants, and to a smaller degree, animals. The major unusual characteristic of increasing the biological activity of the final compound. One of the fluorometabolites, fluoracetate, is highly toxic, and it is oxidized by the mammalian liver. The halogenated compounds as substrates for the OPA anhydrases is an avenue of investigation that should be explored.

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