A Method for the Characterization of Two Similar B-Esterases Present in the Chicken Central Nervous System

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It has previously been shown that in the chicken central nervous tissue there are several esterases that will hydrolyse PPA and PPP* (Poulsen & Aldridge, 1964). These substrates are hydrolysed by enzymes that are inhibited by low concentrations of phenyl saligenin phosphate. This compound is neurotoxic and causes ataxia and selective damage to certain tracts in the spinal cord of chickens, and it is probable that it is the major neurotoxic metabolite produced *in vivo* from tri-*o*-tolyl phosphate. Since the possibility exists that there is a correlation between this type of neurotoxicity and the inhibition of esterases other than the cholinesterases (Aldridge, 1954*a*), it was essential to have a method for determining the esterases hydrolysing PPA and PPP. This would enable us to determine the degree of inhibition produced *in vivo* by both neurotoxic and non-neurotoxic organophosphorus compounds.

From a study of the kinetics of inhibition of these esterases by DFP, the two major esterases hydrolysing PPA and PPP have been characterized. The information derived from these kinetic studies has been confirmed on enzyme preparations purified by chromatography on DEAE-cellulose.

MATERIALS AND METHODS

Special chemicals and reagents. PPA and PPP were synthesized, purified and analysed as described by Poulsen & Aldridge (1964). DFP was from Allen and Hanbury Ltd.; *NN'*-di-isopropyl phosphorodiamidic fluoride (mipafox) was given by Dr G. S. Hartley (Fisons Pest Control Ltd.); phenyl saligenin phosphate was given by Dr H. Coates (Albright and Wilson Ltd.); Triton X-100 was from C. Lennig and Co. Ltd.

Animals. All chickens used in the present studies were of the cross-breed Rhode Island Red $\times$ Light Sussex aged 6–12 months.

Studies with the manometric technique for esterase determination. For all measurements of the relationship between concentration of inhibitor and inhibition, a 10% or 20% (w/v) homogenate of brain or spinal cord respectively was used. Details of the manometric technique for esterase

determination are given by Poulsen & Aldridge (1964). The following procedure was used in the experiments with inhibitor: Portions (2 ml.) of homogenate and various concentrations of solutions of inhibitor were separately warmed to 37°. At 20 sec. intervals 0.4 ml. of warmed inhibitor was added to each of the homogenates and the mixture kept at 37°. After exactly 30 min., samples were withdrawn and added to ice-cold substrate in the main compartment of Warburg flasks. The flasks were then gassed quickly with CO₂ + N₂ (5:95) and residual activity was determined as described by Poulsen & Aldridge (1964).

Preparation of a dry powder from chicken brain. The brains from 12 chicken heads packed and stored in ice were homogenized in 600 ml. of acetone at –18°. Acetone was removed by filtration through a coarse sintered funnel. The residue was then washed successively twice with 250 ml. each of acetone, ethanol and chloroform and four times with ether. The resulting residue was dried in air, ground fine in a pestle and mortar and stored in a desiccator at room temperature. The yield was 5.30 g. A centrifuged extract of this powder in 0.005% Triton X-100 liberated 17.9 and 85 μ moles of phenol/min./g. of original powder at 37° from PPA and PPP respectively (*P/A*, ratio 4.8), determined by the colorimetric method (see below).

Fractionation on diethylaminoethylcellulose. DEAE-cellulose (Whatman DE 50) was pretreated and washed as described by Peterson & Sober (1962). A suspension of the washed cellulose was poured into a column, washed with water, 0.2M-potassium phosphate buffer, pH 6.8, and water, and then equilibrated with 5 mM-potassium phosphate buffer, pH 6.8. Extracts of the brain powder in unbuffered 0.005% Triton X-100 were run on the column and various fractions eluted with increasing concentration of phosphate buffer or finally with the ionic strength increased by NaCl. The fractionation was carried out at 2–4°.

Colorimetric determination of esterase activity. The method depends on the colorimetric determination of the liberated phenol by the method of Gottlieb & March (1946). The suspensions of PPA and PPP were made by shaking 50 mg. in 50 ml. of unbuffered 0.04% Triton X-100 with 1 mm. glass beads. To 0.8 ml. of 0.2M-sodium phosphate buffer, pH 7.4, was added 2 ml. of substrate suspension and, after being warmed to the required temperature, 0.2 ml. of enzyme solution. After 10 min., 1 ml. of 0.05% 4-aminoantipyrine and 1.0 ml. of 0.4% potassium ferricyanide were added. After leaving for 5 min. at room temperature for colour development, 3 ml. of dimethylformamide was added to solubilize the excess of substrate. The colour was measured at 510 m μ . The above reagents were prepared and stored as described by Aldridge (1954*b*).

The liberation of phenol was linear with respect to time and enzyme concentration, with both substrates. The origi-

* Abbreviations: PPA, phenyl phenylacetate; PPP, phenyl 3-phenylpropionate; *P/A* ratio, the ratio of the rates of hydrolysis of PPP and PPA; DFP, di-isopropyl phosphorofluoridate.

nal extract used in the experiment described in Fig. 3 gave P/A ratios 4.6 and 4.7 for the manometric and colorimetric techniques respectively. For the combined fractions 4-8, the corresponding P/A ratios were 0.18 and 0.16. In absolute units, the activity by the manometric technique is approx. 60% of that determined by the colorimetric method. The results obtained by these two methods can therefore be directly compared and this has been confirmed for experiments with inhibitors (see Table 2).

Protein determination. Protein was determined directly in fractions eluted from the DEAE-cellulose by the method of Lowry, Rosebrough, Farr & Randall (1951), with albumin as standard.

RESULTS

Inhibition of the hydrolysis of phenyl phenylacetate and phenyl 3-phenylpropionate by di-isopropyl phosphorofluoridate. It is now well established that the inhibition of esterases by organophosphorus compounds is in principle the same type of reaction as that involved in the hydrolysis of substrates (Aldridge, 1956). The kinetics of the inhibitory process are first-order and show the characteristics of a bimolecular reaction with one component in excess (Jansen, Fellows-Nutting & Balls, 1948; Aldridge, 1950; Aldridge & Davison, 1952):

$$k = \frac{1}{Ct} \ln \left(\frac{100}{b} \right)$$

where k is the bimolecular rate constant ($\text{l. mole}^{-1} \text{ min.}^{-1}$), C the concentration of inhibitor (M), t the time (min.) and b the enzyme activity as a percentage of the control. With a fixed time of reaction a single enzyme will show a straight line passing through the origin, when $\log b$ is plotted against concentration of inhibitor.

Poulsen & Aldridge (1964) showed that, as the concentration of DFP is increased, P/A ratio increases to a constant value of about 10. This indicates that an enzyme is present that hydrolyses PPP 10 times as fast as PPA. In Fig. 1 are shown the results of the hydrolysis of PPA and PPP obtained after pretreatment for 30 min. with various concentrations of DFP. When $\log$ (percentage activity) is plotted against concentration of DFP, a straight line is obtained for concentrations above $2 \mu\text{M}$. This is true for the hydrolysis of PPA and PPP, and both give lines with the same slope. These results confirm the findings (Poulsen & Aldridge, 1964) that an enzyme or enzymes is removed by incubation with low concentration (below $2 \mu\text{M}$) of DFP and that the activity remaining against PPA and PPP is due to one enzyme. Calculation from the results in Fig. 1 confirms that this enzyme hydrolyses PPP approximately 10 times as fast as PPA.

Further examination of Fig. 1 shows that when the calculated best straight line for PPP hydrolysis

is extended back to zero concentration of DFP the line passed through 100% activity (calculated value $2.006 = 101.4\%$). However, the line for PPA hydrolysis when extended to zero concentration of DFP does not pass through the origin but cuts the vertical axis at the calculated value of 1.755 (56.9%). These results indicate that the enzyme that is most sensitive to DFP hydrolyses PPA much faster than PPP, and about one-half of the activity against PPA is due to this enzyme(s).

Such an experiment has been repeated five times and the relevant values have been calculated and are shown in Table 1. The bimolecular rate constants for the inhibition of PPA and PPP hydrolysis by DFP are the same. The lines for the inhibition of PPP hydrolysis intersect the abscissa at 100% activity, but those for PPA hydrolysis intersect at a mean value of 57% activity. Over the range of concentrations of DFP when the relationship between $\log$ (percentage activity) and concentration is linear (Fig. 1), the mean P/A ratio for the five experiments in Table 1 is 9.75 ± 0.15 (21); this is the P/A ratio for the esterase less sensitive to DFP. When the possible errors in the results are taken into account it is not possible to say whether the P/A ratio for the other enzyme is less than 0.3.

One experiment for spinal cord (Fig. 2) shows exactly the same characteristics. The increase in inhibition with increasing concentrations of DFP is the same for the hydrolysis of both PPP and PPA. The bimolecular rate constant calculated from these results is $1.09 \times 10^4 \text{ l. mole}^{-1} \text{ min.}^{-1}$ compared with $5.8 \times 10^3 \text{ l. mole}^{-1} \text{ min.}^{-1}$ when brain was examined.

Fig. 1. Inhibition by DFP of esterases of chicken brain. The activity was determined after incubation of the homogenate with DFP for 30 min. at 37° . Details of technique are given in the Materials and Methods section. ●, Hydrolysis of PPA; ○, hydrolysis of PPP. The lines drawn are the calculated regression line.

Table 1. *Kinetics of inhibition by di-isopropyl phosphorofluoridate of esterases in chicken-brain homogenate*

Activity was determined after incubation with various concentrations of DFP for 30 min. at 37°. Experimental conditions are given in the Materials and Methods section. The rate constants were the slope of the regression line through five points (cf. Fig. 1). From the slope $\pm$ s.e. the points where the lines cut the vertical axis were calculated.

Expt. no.	10 ⁻³ × Rate constant (l. mole ⁻¹ min. ⁻¹)		log (Percentage activity) when concn. of DFP is 0	
	PPA	PPP	PPA	PPP
1	5.21	5.55	1.735 $\pm$ 0.059	2.019 $\pm$ 0.059
2	5.72	6.29	1.750 $\pm$ 0.018	2.023 $\pm$ 0.003
3	5.78	5.42	1.742 $\pm$ 0.049	1.970 $\pm$ 0.058
4	6.15	6.55	1.776 $\pm$ 0.025	1.982 $\pm$ 0.023
5	5.78	5.51	1.755 $\pm$ 0.029	2.006 $\pm$ 0.013
Mean	5.73 $\pm$ 0.17	5.86 $\pm$ 0.23	1.752 $\pm$ 0.007 (57% activity)	2.000 $\pm$ 0.01 (100% activity)

Fig. 2. Inhibition by DFP of esterases of chicken spinal cord. The activity was determined after incubation of the homogenate with DFP for 30 min. at 37°. Details of technique are given in the Materials and Methods section. ●, Hydrolysis of PPA; ○, hydrolysis of PPP. The lines drawn are the calculated regression line.

This difference is probably due to variation in the condition of the two experiments, e.g. the concentration of the tissue was 20% (w/v) for spinal cord instead of 10% (w/v) for brain. The mean P/A ratio derived from these results is 9.6 ± 0.25 (4). Again extension of the lines to the zero concentration of DFP indicates an enzyme hydrolysing PPA much faster than PPP.

Separation of the esterases on diethylaminoethyl-cellulose. This part of the study was made much easier by the finding that a dried powder of chicken brain may be prepared that contains most of the original esterase activity. In initial experiments with DEAE-cellulose the first fraction eluted with 5 mM-phosphate buffer, pH 6.8, hydrolysed PPA much faster than PPP. Increases in ionic strength, either by increasing the concentration of phosphate buffer or by adding sodium chloride, eluted frac-

Fig. 3. Fractionation of esterases of chicken brains. The column (3 cm. $\times$ 12 cm.) was packed with DEAE-cellulose pretreated as described in the Materials and Methods section. An extract of brain powder (27 ml. containing 485 mg. of protein and activity against PPA and PPP of 38.6 and 183 μ moles of phenol liberated/min. respectively) was run on to the column. After the dead space, the next ten 5 ml. fractions contained 35% of activity against PPA, 1.1% of that against PPP and 21% of the protein. ●, Hydrolysis of PPA; ○, hydrolysis of PPP; ▲, P/A ratio.

tions that hydrolysed PPP faster than PPA. In Fig. 3 are the results of an experiment designed to produce enough of the enzyme hydrolysing PPA faster than PPP to study its properties. The P/A ratio varies from 0.05 to 0.28; this indicates that this is not a pure fraction. In another experiment (with less loading of the column) specifically designed to obtain the P/A ratio, it was found to be 0.05. The hydrolysis of PPA by fractions 4–8 (of Fig. 3) have been examined for their sensitivity to DFP and mipafox. The results are given in Fig. 4. A straight line is obtained on plotting log (percentage activity) against concentration of inhibitor, and with both inhibitors the lines when extended to zero inhibition concentration pass through the

origin. This indicates that a single enzyme is responsible for this activity.

We conclude therefore that the major hydrolytic activity of chicken brain against PPP and PPA is due to two enzymes, one (enzyme X) hydrolysing PPA faster than PPP (P/A ratio 0.05) and the other (enzyme Y) hydrolysing PPP much faster

than PPA (P/A ratio 9.75). It is therefore a simple calculation to derive for any specimen of chicken brain or spinal cord the activity of enzyme X and enzyme Y against PPA and PPP respectively.

Let P and P_r be the total activity and the activity due to enzyme Y against PPP respectively, and A and A_x be the total activity and the activity due to enzyme X against PPA respectively. Thus:

$$P = P_r + 0.05A_x$$

$$\text{and} \quad A = 0.1026P_r + A_x$$

$$\text{Whence:} \quad P_r = 1.005P - 0.0503A$$

$$\text{and} \quad A_x = 1.005A - 0.103P$$

By using this method of calculation, from previous experiments of the inhibition of PPA and PPP hydrolysis by DFP and mipafox (cf. Table 3 or Poulsen & Aldridge, 1964); the pI_{50} for the inhibition of enzyme X by each inhibitor was derived. In Table 2 is shown the excellent agreement between these results and those obtained by direct measurement on the fractionated enzyme.

DISCUSSION

Esterases have been subdivided into various fractions on the basis of their reaction with organophosphorus compounds (Aldridge, 1953). The A-esterases are those enzymes that are not inhibited by organophosphorus compounds (this group has been subdivided into A- and C-esterases, these being enzymes that hydrolyse and do not hydrolyse the inhibitors respectively; Bergmann, Segal & Rimón, 1957). Esterases that are inhibited by organophosphorus compounds are B-esterases. The two enzymes hydrolysing PPA and PPP present in the chicken central nervous system are both B-esterases. By a determination of the degree of inhibition of the hydrolysis of two substrates after treatment with various concentrations of an organophosphorus compound it can be established whether the hydrolyses are due to more than one enzyme (Aldridge, 1961; Poulsen & Aldridge, 1964). The present paper describes a method whereby characteristics of each B-esterase in a mixture of two may be established. The esterases have been defined by the ratio of the rates of hydrolysis of the two substrates PPP and PPA, and it has been demonstrated how these may be derived from the relationship between activity against each substrate and concentration of organophosphorus compound (in this case DFP).

However, this analysis will not differentiate enzymes with the same sensitivity to organophosphorus compounds, nor could small amounts (say

Fig. 4. Inhibition of fractionated esterase by DFP (●) and mipafox (○). The enzyme (combined fractions 4–8, 20–40 ml. of effluent) was incubated with inhibitor for 5 min. at 37° before determination of the residual activity by the colorimetric technique.

Table 2. Inhibition of esterases of chicken brain by di-isopropyl phosphorofluoridate and mipafox

The fractionated enzyme was the combined fractions 4–8 from the experiment shown in Fig. 3. Details of technique are given in the Materials and Methods section.

	Concn. giving 50 % inhibition in 30 min. (μM)	
	DFP	Mipafox
Calculated from results obtained with 10% brain homogenate, the manometric technique and 30 min. incubation with DFP (cf. Table 3 of Poulsen & Aldridge, 1964)	0.35	80
Direct determination on enzyme fractionated on DEAE-cellulose, by the colorimetric technique and 5 min. incubation with DFP	0.49	106
Direct determination on enzyme fractionated on DEAE-cellulose, by the manometric technique and 30 min. incubation with DFP	0.56	—

5 %) of activity against either PPP or PPA due to other enzymes be detected. Indeed, from preliminary experiments with fractionation on DEAE-cellulose we have indications that small amounts of such esterases may be present.

SUMMARY

1. A method is described whereby from the relationship between concentration of di-isopropyl phosphorofluoridate (DFP) and esteratic activity of chicken brain against phenyl phenylacetate and phenyl 3-phenylpropionate, the ratio of the rates of hydrolysis of the two substrates by the two major esterase components may be calculated.

2. The results have been confirmed by a qualitative fractionation on DEAE-cellulose.

3. Chicken brain contains two esterases: one much more sensitive to DFP and *NN'*-di-isopropyl phosphorodiamidic fluoride (mipafox) and hydrolysing the phenylacetate faster than the phenylpropionate (phenylpropionate/phenylacetate ratio, 0.05) and the other hydrolysing the phenylpropionate faster than the phenylacetate (phenylpropionate/phenylacetate ratio, 9.75).

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REFERENCES

- Aldridge, W. N. (1950). *Biochem. J.* **46**, 451.
 Aldridge, W. N. (1953). *Biochem. J.* **53**, 110.
 Aldridge, W. N. (1954a). *Biochem. J.* **56**, 185.
 Aldridge, W. N. (1954b). *Biochem. J.* **57**, 692.
 Aldridge, W. N. (1956). *Rep. Progr. Chem.* **53**, 294.
 Aldridge, W. N. (1961). In *Biochemists' Handbook*, p. 275. Ed. by Long, C. London: E. and F. N. Spon Ltd.
 Aldridge, W. N. & Davison, A. N. (1952). *Biochem. J.* **51**, 62.
 Bergmann, F., Segal, R. & Rimon, S. (1957). *Biochem. J.* **67**, 481.
 Gottlieb, S. & March, P. B. (1946). *Analyt. Chem.* **18**, 16.
 Jansen, E. F., Fellows-Nutting, M. D. & Balls, A. K. (1948). *J. biol. Chem.* **175**, 975.
 Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, B. J. (1951). *J. biol. Chem.* **193**, 265.
 Peterson, E. A. & Sober, H. A. (1962). In *Methods in Enzymology*, vol. 5, p. 6. Ed. by Colowick, S. P. & Kaplan, N. O. New York and London: Academic Press Inc.
 Poulsen, E. & Aldridge, W. N. (1964). *Biochem. J.* **90**, 182.

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Reduced Nicotinamide-Adenine Dinucleotide as a Rate-Limiting Factor in Gluconeogenesis

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In previous experiments it was noted that in slices of kidney cortex gluconeogenesis was slower from oxaloacetate than from fumarate or malate (Krebs, Bennett, de Gasquet, Gascoyne & Yoshida, 1963), and that in pigeon-liver homogenates gluconeogenesis from pyruvate was slower than from lactate (Krebs, Dierks & Gascoyne, 1964). These findings are somewhat unexpected because oxaloacetate and pyruvate are intermediates in carbohydrate formation from fumarate (or malate) and lactate respectively, and as intermediates they should react at least as rapidly as the starting materials. The present paper is concerned with the clarification of this apparent discrepancy. It is shown that oxaloacetate and pyruvate, when added at substrate-level concentrations, cause a major fall in the concentration of NADH_2 , which is required in gluconeogenesis for the reduction of diphospho-

glycerate to triose phosphate. The fall in the concentration of NADH_2 is due to its rapid interaction with oxaloacetate and pyruvate to form malate and lactate respectively, and this can account for the decreased rate of gluconeogenesis. The inference, then, is that the added oxo acids successfully compete with diphosphoglycerate for the available NADH_2 .

EXPERIMENTAL

Analytical procedures. The four forms of nicotinamide nucleotides (NAD , NADH_2 , NADP and NADPH_2) were determined in duplicate by the method of Bassham, Birt, Hems & Loening (1959), with the following modifications suggested by Dr K. Burton (see also Lindall & Lazarow, 1964). The alkaline extract of the tissue after adjustment to pH 8.2 (see Fig. 1 of Bassham *et al.* 1959) contained, apart from NADH_2 and NADPH_2 , variable amounts of NAD and NADP owing to autoxidation of the reduced forms during