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# ORGANOPHOSPHATE INHIBITORS: THE REACTIONS OF BIS(*p*-NITROPHENYL) METHYL PHOSPHATE WITH LIVER CARBOXYLESTERASES AND α-CHYMOTRYPSIN

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## Summary

Bis(*p*-nitrophenyl) methyl phosphate (BNMP) has been tested as a spectrophotometric titrant for a group of serine hydrolases. Bis(*p*-nitrophenyl) methyl phosphate reacts rapidly with liver carboxylesterases from chicken, sheep, and horse, and more slowly with α-chymotrypsin, releasing 2 mol of *p*-nitrophenol per active site titrated, and producing a phosphorylated enzyme very stable to dephosphorylation. However, pig liver carboxylesterase produces 2.2 mol of *p*-nitrophenol per active site titrated. Reaction of pig and chicken liver carboxylesterases with bis(*p*-nitrophenyl) [$^{3}$H]methyl [$^{32}$P]phosphate clarified this difference. One molecule of the chicken enzyme reacts with one molecule of bis(*p*-nitrophenyl) methyl phosphate, releasing both *p*-nitrophenol residues, and resulting in an inhibited enzyme with one phosphorus atom and one methyl group covalently bound. Pig enzyme reacts rapidly, forming (presumably) methyl *p*-nitrophenyl phosphoryl-carboxylesterase. This further reacts, concurrently producing methyl phosphoryl-carboxylesterase plus *p*-nitrophenol, or free enzyme plus methyl *p*-nitrophenyl phosphate, in the ratio of about 5 : 1 at pH 7.55. The free enzyme produced undergoes further reaction with bis(*p*-nitrophenyl) methyl phosphate until all the carboxylesterase is inhibited.

## Introduction

The serine esterases, notably acetylcholinesterases, cholinesterases and car-

Abbreviations: DFP, diisopropyl phosphorofluoridate; BNMP, bis(*p*-nitrophenyl) methyl phosphate; DIP-, diisopropylphosphoryl.

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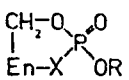
boxylesterases, and the serine proteinases such as α-chymotrypsin are so classified largely because they react stoichiometrically with organophosphate inhibitors to yield inactive derivatives in which a particular serine residue is phosphorylated. Subsequent to the initial reaction with the serine, one of the substituent groups on the phosphorus can be displaced in an intramolecular reaction catalyzed by a group in the active site of the enzyme [1]. This reaction has been referred to as ageing, and is characterized by the fact that its product is not reactivated by treatment with good nucleophiles, in contrast to the initial phosphorylated enzyme species. Ageing appears to occur by different mechanisms, depending on the enzyme and the nature of the substituents on the phosphorus atom. With alkyl substituents the mechanistic studies which have been performed [2,3] support the loss of alkyl groups as carbonium ions, catalyzed by a group with $pK_a^1$ values of approx. 6, active in the protonated form [2]. This mechanism may not apply, however, to simple alkyl substituents (e.g. methyl, ethyl) for which the carbonium ions are not stabilized. This type of ageing reaction has been observed with acetylcholinesterase and cholinesterase derivatives but not with α-chymotrypsin derivatives. With aryl substituents, P-O bond cleavage occurs, catalyzed by a group with $pK_a^1$ values 6—7, active in the basic form. This type of ageing reaction has been observed with electric eel acetylcholinesterase inhibited with diphenyl phosphorochloridate [4] and with several substituted phenyl methylphosphonochloridates [5]. These studies have been limited, partly at least by the small quantity and doubtful purity of available enzyme, to the demonstration that ageing occurs, and in some cases, to a study of the rate of ageing. More extensive studies of the mechanism of this reaction have been performed with α-chymotrypsin derivatives. Lee and Turnbull [6], in a pioneering study, showed that ageing of diphenyl phosphoryl-chymotrypsin is accompanied by the loss of one molecule of phenol per molecule of enzyme. Bender and Wedler [7] have since performed a detailed investigation of the reaction of α-chymotrypsin with Tris(*p*-nitrophenyl) phosphate. Their results support the nucleophilic attack of a group in the enzyme, possibly the imidazole group of his-57, on bis(*p*-nitrophenyl) phosphoryl-enzyme to give a cross-linked intermediate (I).



I (R = *p*-nitrophenyl)

Carboxylesterases have not previously been examined with respect to the ageing reaction. Our study of the reactions of liver carboxylesterases with bis(*p*-nitrophenyl) methyl phosphate (BNMP) was undertaken, as part of a general study of carboxylesterases, for the following reasons:

(i) Carboxylesterases can be obtained in pure form in amounts sufficient for studies of the stoichiometry and mechanism of the ageing reaction, studies previously restricted to α-chymotrypsin [8,9].

(ii) Carboxylesterases resemble acetylcholinesterases both in molecular size and in catalytic efficiency much more closely than does α-chymotrypsin [10].

(iii) Carboxylesterases in animals are important enzymes in the metabo-

lism of organophosphate insecticides [11], and hence, a detailed study of all aspects of their reactions with organophosphate inhibitors is important in attempts to design more specific inhibitors.

(iv) Evidence has been obtained indicating the involvement of a histidine residue in carboxylesterase catalysis [12]; thus, it is possible that an intermediate similar to I could be formed in the reaction in question, thereby cross-linking active-site serine and histidine residues.

(v) BNMP was selected because the nitrophenyl groups are sufficiently activated to be displaced readily, but not so reactive that rapid displacement by solvent could occur; because of the desirable spectral characteristics of the *p*-nitrophenyl group; and because the system should be less complex than if Tris(*p*-nitrophenyl) phosphate were used.

Initially we examined BNMP as a potential titrant for the carboxylesterases, considering that loss of both *p*-nitrophenyl residues from one BNMP molecule during phosphorylation of a carboxylesterase molecule would render BNMP twice as sensitive a titrant as paraoxon. In addition, the methyl phosphoryl-carboxylesterase was expected (as an "aged" phosphoryl-enzyme) to be more stable towards dephosphorylation than the diethylphosphoryl-carboxylesterase produced by titration with paraoxon. Although, as shown in this report, BNMP fulfilled our expectations as a titrant for α-chymotrypsin, and liver carboxylesterases from chicken, horse and sheep, it is our observation of an unexpected reaction of pig liver carboxylesterase with BNMP which we wish to emphasize here. In this work, the experimental approach has been concerned mainly with the determination of the stoichiometry of the reaction, both in terms of *p*-nitrophenol release, and by using BNMP labelled with $^{32}P$ and with $^{3}H$ in the methyl group. The interesting difference in stoichiometry between the reactions of pig liver carboxylesterase and other carboxylesterases with BNMP has been clarified using the labelled inhibitor.

## Materials and Methods

Liver carboxylesterases from pig [8], chicken [9], sheep [9], and horse [9] were purified in this laboratory as detailed elsewhere. Pig liver carboxylesterase was chromatographed through Sephadex G-200 (Pharmacia, Uppsala) and dialyzed against 0.05 M Tris · HCl buffer, 0.15 M KCl, pH 7.55, immediately before use. When assayed against ethyl butyrate at 38°C, it had a specific activity of 9.17—9.34 (mkat/l)/$A_{280\,nm}$ compared with the highest reported [8] value of 9.67. Chicken liver carboxylesterase was chromatographed through Sephadex G-100 in the same buffer prior to use, and had a specific activity, measured against ethyl butyrate, of 3.83—3.92 (mkat/l)/$A_{280\,nm}$, compared with a reported [9] maximum of 4.00. The horse enzyme was stored as a suspension in 85% saturated $(NH_4)_2SO_4$ for 7 months, after purification to maximum specific activity [9]. After dialysis against 0.01 M Tris · HCl buffer, pH 7.6, titration of the enzyme with paraoxon gave an equivalent weight of 74 410, indicating that the enzyme was approx. 93% active. Sheep liver carboxylesterase was precipitated from 85% $(NH_4)_2SO_4$, and redissolved in and dialysed against 0.05 M phosphate buffer, pH 7.54. The specific activity was 4.45 (mkat/l)/$A_{280\,nm}$, compared with a reported [9] maximum of 4.50.

α-Chymotrypsin, thrice crystallized, was obtained from Worthington Biochemical Corp. and was prepared as a 10 mg/ml solution in 0.01 M Tris · HCl buffer, 0.05 M NaCl, pH 7.5, immediately before use. The concentration of α-chymotrypsin solutions was determined by titration with *p*-nitrophenyl acetate [13]. Twice crystallized pepsin from Worthington was prepared as an 8 mg/ml solution in 0.01 M HCl. After removal of some insoluble material by centrifugation, the pH of the supernatant was adjusted to 2.00 with 0.10 M HCl. Venom phosphodiesterase (type V from Sigma Chemical Co.; the crude dried venom of *Bothrops atrox*) was dissolved ( 2 mg/ml) in 0.02 M Tris · HCl buffer, pH 7.55.

[$^{32}$P]DFP was obtained from the Radiochemical Centre, Amersham, as a sterile solution (approx. 2.7 mM) with a specific radioactivity of approx. 74 Ci/mol, in propylene glycol. Aliquots of this solution were added directly to solutions of enzyme to be inhibited. Paraoxon, obtained from Albright and Wilson, was purified by passing a chloroform solution through a column of neutral alumina, activity grade I (Woelm). After removal of chloroform, a stock solution ($1.00 \cdot 10^{-2}$ M) in acetonitrile was prepared; the material was 99.1% pure by release of *p*-nitrophenol upon alkaline hydrolysis. Sodium bis(*p*-nitrophenyl) phosphate and disodium *p*-nitrophenylphosphate were obtained from Sigma and Koch-Light respectively, and were used without further treatment. Methyl dihydrogen phosphate was obtained by chromatography of methyl bis(cyclohexylammonium) phosphate (prepared by the method of Kirby [14]) through Dowex 50W-X2 in water. *p*-Nitrophenyl methyl hydrogen phosphate was prepared from BNMP by the method of Moffatt and Khorana [15], and was crystallized from benzene; m.p. 119—122°C. ref. 15: 123.5—124.5°C. Unlabelled Tris(*p*-nitrophenyl) phosphate from Aldrich Chemical Co. was recrystallized from hot acetone; m.p. 154—157°C, ref. 15: 155—156°C. Tris(*p*-nitrophenyl) [$^{32}$P]phosphate was obtained from the Radiochemical Centre, with a specific radioactivity of 13.2 Ci/mol. Unlabelled dry methanol was redistilled from magnesium methoxide. [*Me*-$^{3}$H]methanol was supplied by the Radiochemical Centre, with a specific radioactivity of 128 Ci/mol. Dichloromethane from Baker Chemical Co. was dried over Linde 3A molecular sieves.

Unlabelled BNMP was prepared as reported previously [16] and crystallized from chloroform/carbon tetrachloride; m.p. 143°C, ref. 15: 142—143°C. [$^{3}$H,$^{32}$P]BNMP was synthesized by the slow addition of sodium [$^{3}$H]methoxide in dichloromethane to an equimolar amount of Tris(*p*-nitrophenyl) [$^{32}$P]phosphate in dichloromethane, as follows. A solution of Tris(*p*-nitrophenyl) [$^{32}$P]phosphate (179 mg) and unlabelled triester (320 mg) in 25 ml dichloromethane, was placed in a 50-ml conical flask fitted with a pressure-equalizing, stoppered, dropping funnel. Sodium [$^{3}$H]methoxide was prepared by reacting 25.8 mg sodium with 300 μl dry unlabelled methanol and then transferring 33.7 μl of [$^{3}$H]methanol to the resulting sodium methoxide at −180°C on a vacuum line (mercury diffusion pump). The sodium [$^{3}$H]methoxide in excess methanol was washed into the funnel with dichloromethane (5.0 ml in four washes). The sodium methoxide was added dropwise to the solution of Tris(*p*-nitrophenyl) phosphate at 0°C over 6 h. The reaction mixture was then taken to dryness in a 100-ml spherical flask on the vacuum line. The dry orange residue was extracted with dichloromethane (one 20-ml and two 5-ml washes) and the insoluble orange residue was filtered off. The filtrate

was dried ($Na_2SO_4$) and evaporated at reduced pressure. The resultant creamy solid was recrystallized twice by dissolving in refluxing ethanol (10 ml, 8 ml) and cooling to −10°C. The white crystals after filtering and drying had a low melting point (126—133°C, cf. ref. 15: 142—143°C). After dissolving the crystals in chloroform (10 ml) and washing the solution with 0.05 molal borate, pH 9.2 (2 × 5 ml) and water (5 ml), the solution was dried ($Na_2SO_4$), evaporated, and the resultant white solid recrystallized several times from either ethanol or chloroform/carbon tetrachloride (5 : 12, v/v)as described above*. The purity of the crystalline BNMP was judged by the amount of *p*-nitrophenol released on mild alkaline hydrolysis (0.1 M NaOH at 25°C quantitatively removes one equivalent of *p*-nitrophenol from BNMP in about 5 min); by constancy of the ratio of $^3H/^{32}P$ measured by liquid scintillation counting (see below); and by constancy of the ratio $^{32}P$/*p*-nitrophenol released. The BNMP used in the enzyme studies described here was finally crystallized from chloroform/carbon tetrachloride, m.p. 138.5—139.5°C. Mild alkaline hydrolysis released 0.9825 ± 0.0005 equivalents of *p*-nitrophenol. Further recrystallization from chloroform/carbon tetrachloride did not enhance the purity of the BNMP, the two ratios of which varied by less than 0.015% in each case. A single solution of 5.36 mg BNMP/10 ml acetonitrile (Eastman Kodak, Spectro grade) (BNMP Solution I) and a 10-fold dilution of this solution in acetonitrile (BNMP Solution II) were analysed for purity, and were used in all enzyme inhibitions and in the preparation of standards for scintillation counting.

Buffers were prepared using analytical grade reagents and Spectro grade acetonitrile. pH measurements were made on a Radiometer pH meter 4c at 25°C, standardized according to Bates [17], and are accurate to ±0.01 pH unit.

All spectrophotometric measurements were performed in a Cary 14 recording spectrophotometer equipped with 0—0.1 and 0—1.0 absorbance slide wires, and a cell compartment maintained at 25 ± 0.1°C. To minimize evaporation cells were covered with closely-fitting Teflon lids. In a typical experiment, an aliquot (usually 135 μl) of BNMP in acetonitrile was added to 3 ml enzyme solution and 200 μl acetonitrile, equilibrated at 25°C. The increase in absorbance at 400 nm was followed for a few minutes until the trace was linear; the linear portion of the trace was extrapolated to zero time and from this, the concentration of *p*-nitrophenol released during the initial fast reaction between enzyme and inhibitor was determined. The molar absorption coefficient of *p*-nitrophenol was determined under the conditions of each experiment.

The activity of carboxylesterases was measured by adding an aliquot of enzyme to a cell containing 3.0 ml aqueous phenyl butyrate solution (1.96 mM) and 0.20 ml phosphate buffer, 0.8 M, pH 7.50, equilibrated at 25°C. The increase in absorbance at 270 nm was observed ($\Delta\epsilon$ = 1510). Protein concentrations (mg/ml) were estimated by measuring the ultraviolet spectra, and dividing the absorbance at 280 nm by 1.341 (pig [18]) or 1.215 (chicken [18]).

* Purification of BNMP by crystallization was impeded when solid BNMP, in the process of dissolution, was heated significantly higher than approx. 95°C. Such heating caused the formation of a highly insoluble by-product with a $^3H/^{32}P$ ratio the same as that for BNMP. The material was presumed to contain pyro- or polyphosphate derivatives of BNMP, and this explanation was consistent with chromatography of samples of the labelled BNMP on Sephadex G-25.

Scintillation counting was carried out using a Beckman model LS 250 or a Nuclear Chicago Mark I model 6080 liquid scintillation counter. Routinely, 1 ml of aqueous solution was added to 15 ml of scintillation fluid containing Triton X-100/toluene/Liquifluor scintillator (35 : 48 : 2, by vol.) or toluene (containing 4 g Omnifluor scintillator per litre)/Triton X-100 (10 : 7, v/v). Internal standards were prepared from the supplied [$^{32}$P]DFP solution or standard [$^{3}$H]toluene (New England Nuclear). Radioactive peptides were located on paper chromatograms by radioautography on Kodak "Kodirex" X-ray film.

Products from reactions of esterases with organophosphorus inhibitors were separated by chromatography on Sephadex G-25 (Pharmacia) equilibrated at 25°C with Tris · HCl buffer, with or without acetonitrile, as described in the text. The volume of eluted fractions was measured by weight, and fractions were monitored for radioactivity and absorbance at 280 nm. The elution volumes of methanol, BNMP, methyl *p*-nitrophenyl phosphate, bis(*p*-nitrophenyl) phosphate, methyl phosphate, *p*-nitrophenyl phosphate and orthophosphate were measured using samples of the authentic compounds. Methanol in eluant fractions was measured by a colorimetric method following oxidation [19]; methyl phosphate was detected semi-quantitatively by the precipitate which formed on adding 2 drops of saturated aqueous $LaCl_3$ to 1 ml of eluant; orthophosphate was measured by the method of Fiske and SubbaRow [20]; all other compounds were measured by their absorbance at 280 nm. In one experiment, dialysis was used rather than Sephadex chromatography, to separate excess inhibitor and products from inhibited enzyme. However, after several changes of dialysis buffer, significant amounts of radioactive compounds remained not covalently bound to enzyme, as shown by Sephadex chromatography. Radioactive reaction products suspected to be either methyl *p*-nitrophenyl phosphate or bis(*p*-nitrophenyl) phosphate were digested with phosphodiesterase to which sufficient of the authentic compound had been added so that the digestion could be monitored spectrophotometrically. In these digestions, carried out at 38°C in a stoppered cuvette, the diester (190 $\mu$M) in 0.05 M Tris · HCl buffer adjusted to pH 8.1 was mixed with magnesium acetate (30 mM) and venom diesterase (0.333 mg/ml) and the absorbance at 400 nm measured periodically.

Radioautographs of peptic digests of esterases inhibited with [$^{32}$P]DFP or [$^{3}$H, $^{32}$P]BNMP were prepared as follows. Samples of both pig and chicken carboxylesterases were inhibited completely with excess [$^{32}$P]DFP or [$^{3}$H, $^{32}$P]BNMP in 0.05 M Tris · HCl buffer, 0.15 M KCl, pH 7.55, then dialysed free of excess inhibitor, firstly against 10% (v/v) aqueous acetonitrile, and then against water. The protein solutions were evaporated to dryness, redissolved in water (to 4.2 mg/ml), adjusted to pH 2.0 with 0.101 M HCl, and digested with pepsin (0.32 mg/ml) at 38°C. Aliquots (2—10 nCi) were spotted on to Whatman 3 MM chromatography paper at various intervals (5, 10, 20, 60 and 360 min) after which the papers were chromatographed in descending butanol/acetic acid/water (40 : 6 : 15, by vol.) for 12.5 h, dried and radioautographed (2 days).

## Results

*Inhibition of carboxylesterases and α-chymotrypsin with unlabelled BNMP*

In preliminary experiments, the reaction of BNMP with four purified liver carboxylesterases was studied. On addition of the inhibitor to each enzyme, a rapid release of *p*-nitrophenol occurred (approx. 95% complete within 6 s). For the pig enzyme, the tail of the "burst" (approx. 5%) was found to represent a first-order reaction with $k_{obs}$ $2.23 \cdot 10^{-2}$ $s^{-1}$ *. This burst was followed by a very slow zero-order increase in absorbance at 400 nm. The stoichiometry of *p*-nitrophenol released by each enzyme in the burst phase is given in Table I. Included in Table I is the result of a similar experiment with α-chymotrypsin. In this case, the release of *p*-nitrophenol was found to be first order, with $k_{obs}$ $6.11 \cdot 10^{-3}$ $s^{-1}$.

When a sample of pig liver carboxylesterase which had been reacted with an excess of BNMP was assayed with phenyl butyrate, no activity could be detected. No reactivation of the inhibited enzyme was observed when a sample was dialysed against 0.075 M acetate buffer (pH 5.02) containing 0.25 M NaCl, 4 mM cysteine and 20% (v/v) acetonitrile, for 48 h at 25°C. Similarly, when 0.25 M hydroxylamine was included in the dialysis buffer, no reactivation could be detected. In both cases acetonitrile was omitted after the first 4 h of dialysis.

In order to explain the unexpected ratio of 2.23 ± 0.03 obtained for different highly purified samples of the pig enzyme, independently prepared, the reactions of the pig and chicken enzymes with [$^{3}$H, $^{32}$P]BNMP were examined in detail.

TABLE I

INHIBITION OF HORSE, SHEEP, CHICKEN AND PIG LIVER CARBOXYLESTERASES AND α-CHYMOTRYPSIN BY BNMP

Enzyme molarities were determined by titration with paraoxon (carboxylesterases) or *p*-nitrophenyl acetate (α-chymotrypsin) under the conditions of BNMP titrations.

| Enzyme | $[E]_0$ (μM) | $[BNMP]_0$ (μM) | Buffer | Stoichiometry of *p*-nitrophenol release |
|---|---|---|---|---|
| α-Chymotrypsin | 20.23 | 97.6 | 0.008 M Tris·HCl, 0.04 M NaCl, pH 7.50, 20% (v/v) acetonitrile | 2.00 |
| Carboxylesterase | | | | |
| Horse | 12.53 | 101.9 | 0.008 M Tris·HCl, pH 7.60 | 2.04 |
| Sheep | 18.14 | 108.0 | 0.008 M Tris·HCl, 0.04 M KCl, pH 7.28, 20% (v/v) acetonitrile | 2.00 |
| Chicken | 4.23 | 80.0 | 0.009 M Tris·HCl, pH 7.60, 10% (v/v) acetonitrile | 2.00 |
| Pig | 2.08 | 94.6 | 0.008 M Tris·HCl, 0.04 M NaCl, pH 7.50, 20% (v/v) acetonitrile | 2.20 |

* Similarly, the last 4% of the burst in paraoxon titration of the pig enzyme appeared to occur much more slowly than predicted from the 95% completion of the burst in the 6-s before observation commenced.

TABLE II

STOICHIOMETRY OF THE REACTION OF [$^{3}$H, $^{32}$P]BNMP WITH CHICKEN AND PIG LIVER CARBOXYLESTERASES

Titrations with BNMP and paraoxon were performed in 0.05 M Tris·HCl buffer, 0.15 M KCl, pH 7.55, 10.14% (v/v) acetonitrile. $[E]_0$ (chicken) = 6.489 μM; $[E]_0$ (pig) = 5.692 μM; $[BNMP]_0$ = 43.85 μM; $[paraoxon]_0$ = 150.5 μM.

| Enzyme | Equivalent weight (g protein/mol $^{32}$P)* | Equivalent weight (BNMP titration)** | Equivalent weight (paraoxon titration) | $^{3}$H/$^{32}$P of inhibited enzyme | $^{3}$H/$^{32}$P of BNMP standard |
|---|---|---|---|---|---|
| Chicken | 67940 | 69551 | 70851 | 2.726 | 2.861 |
| Pig | 69708 | 63778 | 70672 | 2.513 | 2.861 |

* Corrected for residual activity of the inhibited enzymes: chicken, 1.35% of original; pig, 1.03%.
** Based on the release of *p*-nitrophenol from BNMP in the 'burst', assuming the release of 2 mol *p*-nitrophenol per mol enzyme.

*Inhibition of chicken and pig enzymes with labelled BNMP ($[I]_0 > [E]_0$)*

Samples of chicken and pig liver carboxylesterases were reacted with excess labelled BNMP. Aliquots (3.35 ml) of the BNMP-inhibited enzymes were chromatographed on Sephadex G-25 (2.5 cm × 15 cm) equilibrated with 0.05 M Tris · HCl buffer, pH 7.60, 10% (v/v) acetonitrile, to remove unbound radioactivity from the inhibited enzymes. The inhibited, chromatographed enzymes were assayed for protein, residual activity and radioactivity ($^{32}$P and $^{3}$H). From these results, the equivalent weights of the enzymes in the reaction with BNMP were calculated (g protein/mol $^{32}$P) and are given in Table II, together with the equivalent weights obtained by titration with paraoxon. The $^{3}$H/$^{32}$P ratios in the inhibited enzymes and in the BNMP standards, and the equivalent weights determined by release of *p*-nitrophenol from BNMP are also included.

Aliquots (1 ml) of the inhibited, chromatographed enzymes were stored in sealed vials at 25°C for 14 days, then rechromatographed on Sephadex G-25 (1.1 cm × 14 cm) equilibrated with 0.05 M Tris · HCl buffer, pH 7.62, 10% (v/v) acetonitrile. No unbound radioactivity was detected in fractions eluted after the enzyme. The $^{3}$H/$^{32}$P ratios of the rechromatographed enzymes (relative to the ratio for BNMP) were within 2% of the ratios measured after the first chromatography. No reactivation of inhibited enzymes was detected during this period.

*Inhibition of chicken and pig enzymes with BNMP ($[E]_0 > [I]_0$)*

Aliquots of the diluted stock solution of labelled BNMP in acetonitrile (Solution II) were allowed to react with an approximately 2-fold excess of both chicken and pig liver carboxylesterases. A duplicate aliqout of the same stock solution of BNMP was hydrolysed in 0.1 M NaOH, 10% (v/v) acetonitrile, and from the release of *p*-nitrophenol the molarity of the BNMP solution was obtained. Hence, the stoichiometry of release of *p*-nitrophenol from BNMP by excess enzyme (mol *p*-nitrophenol/mol BNMP) was determined (Table III). Based on the release of *p*-nitrophenol in alkali, the concentration of BNMP in

TABLE III

REACTION OF BNMP WITH LIVER CARBOXYLESTERASES ($[E]_0 > [I]_0$)

Reactions were performed in 0.05 M Tris·HCl buffer, 0.15 M KCl, pH 7.55, 10% (v/v) acetonitrile.

| Enzyme | $[E]_0$ ($\mu$M) | $[BNMP]_0$* ($\mu$M) | Mol of *p*-nitrophenol per mol of BNMP | [Inhibited enzyme]** ($\mu$M) | Mol of BNMP/mol of inhibited enzyme** | [Inhibited enzyme]*** ($\mu$M) | Mol of BNMP/mol of inhibited enzyme*** |
|---|---|---|---|---|---|---|---|
| [$^3$H, $^{32}$P] BNMP | | | | | | | |
| Chicken | 12.58 | 5.706 | 2.02 | | | | |
| Pig | 10.65 | 5.706 | 1.817 | | | | |
| Unlabelled BNMP | | | | | | | |
| Chicken | 13.18 | 5.784 | 1.96 | 6.01 | 0.962 | 5.79 | 0.998 |
| Pig | 11.12 | 5.784 | 1.80 | 4.78 | 1.210 | 4.914 | 1.178 |

* BNMP concentrations determined by measuring *p*-nitrophenol release.

** The concentration of inhibited enzyme was determined by paraoxon titrations of enzyme before and after treatment with BNMP.

*** The concentration of inhibited enzyme was calculated from the residual enzyme activity (%) after inhibition, and the initial enzyme concentration determined by paraoxon titration.

stock Solution II was 93.4% of the calculated value. The concentration of BNMP in stock Solution I when prepared 10 days prior, was 98.25%, indicating that considerable breakdown of BNMP occurred in the acetonitrile solution. In order to determine whether the results obtained using Solution II of BNMP were affected by the significant level of impurity in the solution, this experiment ($[E]_0 > [I]_0$) was repeated, using a freshly prepared solution of unlabelled BNMP which was 99.7% pure on the basis of *p*-nitrophenol release. In addition, titration with paraoxon (before and after reaction of enzyme with BNMP) was used to determine the number of molecules of enzyme inhibited per molecule of BNMP. A second estimate of the concentration of inhibited enzyme was obtained by assay of the enzyme against phenyl butyrate, before and after inhibition. The results are summarized in Table III. The experiments using labelled and unlabelled BNMP give the same stoichiometry of *p*-nitrophenol released per BNMP added, showing that impurities present in the solution of labelled BNMP due to the breakdown noted above do not affect the result.

*Identification of reaction products*

The products of the reaction of labelled BNMP with excess enzyme were analyzed. Reaction mixtures (3.335 ml) prepared as described above ($[E]_0 > [I]_0$) were chromatographed on Sephadex G-25 (2 cm × 14 cm) equilibrated with 0.05 M Tris · HCl buffer, pH 7.55, and the eluted fractions were monitored for radioactivity and absorbance at 280 nm (Figs 1 and 2). In Fig. 1, Peak 1 corresponds to inhibited enzyme. Peak II was identified as methyl *p*-nitrophenyl phosphate as follows. An aliquot (10 $\mu$l) of a solution of unlabelled methyl *p*-nitrophenyl phosphate (11.4 mM) was added to 1 ml of material from Peak II and the mixture was chromatographed on Sephadex G-25 (1.1 cm × 47 cm) equilibrated with 0.05 M Tris · HCl, pH 7.62, 10% (v/v) acetonitrile. The eluted peaks of radioactivity and absorbance at 280 nm were

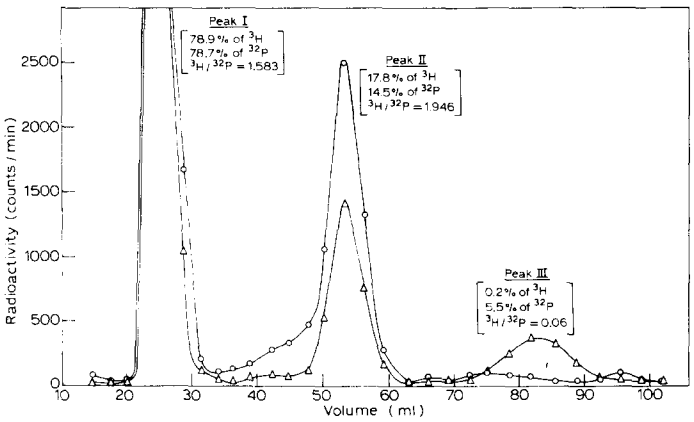



Fig. 1. Gel filtration of the reaction products of excess pig liver carboxylesterase with BNMP. Enzyme (10.65 $\mu$M) was reacted with BNMP (5.72 $\mu$M) in 0.045 M Tris · HCl buffer, pH 7.55, 0.135 M in KCl, and 10% (v/v) acetonitrile. The reaction mixture (3.335 ml) was chromatographed on a Sephadex G-25 column (2 cm X 14 cm; 0.05 M Tris · HCl buffer, pH 7.55; flow rate approx. 1.25 ml/min). Peak I was identified as inhibited enzyme by $A_{280\ nm}$ and elution volume. Radioactivity, in cpm, of 1-ml aliquots: ○———○, $^3H$; △———△, $^{32}P$.

coincident. A mixture of Peak II and methyl *p*-nitrophenyl phosphate was digested partially with snake venom phosphodiesterase. The reaction was monitored spectrophotometrically until approximately half of the unlabelled methyl *p*-nitrophenyl phosphate had been hydrolysed. An aliquot (1 ml) was then chromatographed on Sephadex G-25 (Fig. 3). The results demonstrate that after diesterase digestion, about 50% of the total radioactivity was eluted in the same position as methyl phosphate.

Peak III was conclusively identified as bis(*p*-nitrophenyl) phosphate, by co-chromatography and by phosphodiesterase digestion in the same manner.

Considering Fig. 2, Peak I again corresponds to inhibited enzyme. From their elution volumes, Peaks III and IV are probably methyl *p*-nitrophenyl

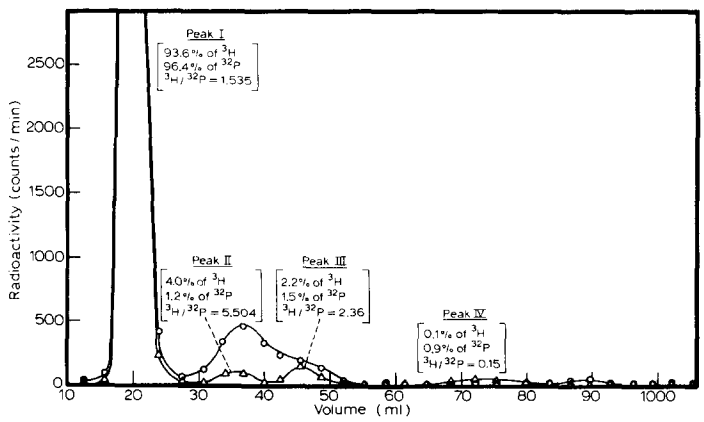



Fig. 2. Gel filtration of the reaction products of excess chicken liver carboxylesterase with BNMP. Enzyme (12.58 $\mu$M) was reacted with BNMP and chromatographed on Sephadex G-25, as described for Fig. 1. ○———○, $^3H$ radioactivity; △———△, $^{32}P$ radioactivity.

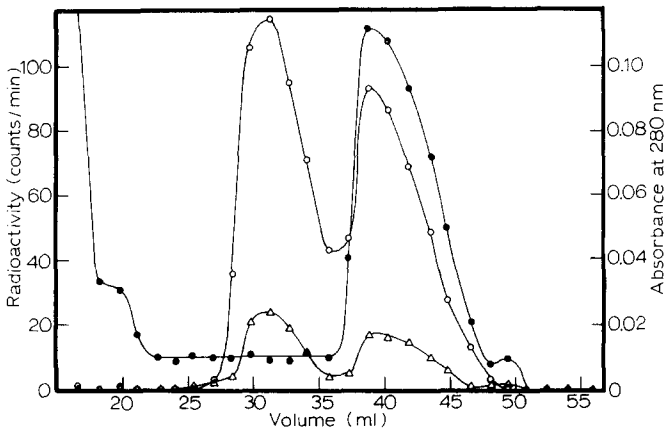



Fig. 3. Gel filtration of Peak II, Fig. 1, after partial digestion by phosphodiesterase. A 2.2-ml aliquot of pooled Peak II fractions adjusted to pH 8.1 with 1 M KOH was digested in a reaction mixture (3.0 ml) containing magnesium acetate (30 mM), phosphodiesterase (1.0 mg), and unlabelled methyl *p*-nitrophenyl phosphate (190 μM) at 38°C, monitored at 400 nm. After approx. 50% hydrolysis, 1 ml of reaction mixture was chromatographed on a Sephadex G-25 column (1.1 cm × 47 cm; 0.05 M Tris · HCl buffer, pH 7.62). Radioactivity, in cpm, of 1-ml aliquots: ○———○, $^{3}H$; △———△, $^{32}P$. $A_{280\ nm}$, ●———●.

phosphate and bis(*p*-nitrophenyl) phosphate, respectively. Peak II, predominantly labelled with $^{3}H$, has a different elution volume from methanol, and has not been identified. The total radioactivity in Peaks II—IV corresponds to 3.6% of the $^{32}P$ and 6.3% of the $^{3}H$. When a sample of BNMP (Solution II) was chromatographed under these conditions, radioactivity was eluted from the column in the same regions as Peaks II and III; further, the impurities present in Solution II quantitatively account for the radioactivity present in these peaks. BNMP has the same elution volume as Peak IV [bis(*p*-nitrophenyl) phosphate].

Bis(*p*-nitrophenyl) phosphate (Peak III of Fig. 1, Peak IV of Fig. 2) is present in both reaction mixtures, but in much greater amount in the case of the pig enzyme. Bis(*p*-nitrophenyl) phosphate was shown to be present in the solutions of labelled BNMP by Sephadex chromatography after alkaline hydrolysis. Alkaline hydrolysis of unlabelled BNMP gave no bis(*p*-nitrophenyl) phosphate. Quantitatively, the amount of bis(*p*-nitrophenyl) phosphate present as an impurity was sufficient to account for all of that shown in Fig. 1, even allowing for the slow reaction of bis(*p*-nitrophenyl) phosphate with the pig enzyme. The rates of reaction of pig and chicken liver carboxylesterases with bis(*p*-nitrophenyl) phosphate were determined independently, at similar concentrations to those present in the reaction mixtures. The chicken enzyme reacts about 8-fold faster than the pig enzyme with bis(*p*-nitrophenyl) phosphate under these conditions, accounting for the much lower amount of bis(*p*-nitrophenyl) phosphate remaining in the chicken reaction mixtures (Fig. 2). This reaction with bis(*p*-nitrophenyl) phosphate also quantitatively accounts for the significantly lower $^{3}H/^{32}P$ ratio of the inhibited chicken enzyme (Peak I, Fig. 2), compared with the value recorded in Table II for the inhibited chicken enzyme, and the value for the inhibited pig enzyme (Peak I, Fig. 1). The reason for the low ratio for the inhibited pig enzyme recorded in Table II, and also observed in a similar experiment, is not clear, but may

indicate that some demethylation has occurred. With $[I]_0 > [E]_0$, as in Table II, no reaction would be expected with bis(*p*-nitrophenyl) phosphate, since its reaction with the enzyme is very much slower than that of BNMP. On the time scale, and at the concentrations present in these experiments, there is negligible reaction between either pig or chicken liver carboxylesterase and methyl *p*-nitrophenyl phosphate. As stated previously, the small amount (1.5% of counts) of (presumed) methyl *p*-nitrophenyl phosphate present in the chicken reaction mixture was probably present as an impurity in Solution II of BNMP. However, $\geqslant$ 90% of the methyl *p*-nitrophenyl phosphate present in the pig reaction mixture (Peak II, Fig. 1) must have arisen from the enzymatic reaction.

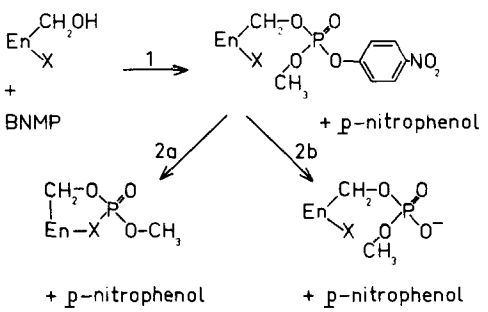
*Peptic digestion of chicken and pig liver carboxylesterases inhibited with BNMP*

Radioautographs of the peptic digests of chicken and pig liver carboxylesterases inhibited with BNMP or DFP showed the following results.

(i) One major radioactive peptide, resistant to further peptic digestion, was produced from both chicken and pig enzymes inhibited with BNMP. (ii) The $R_F$ values of these peptides (0.037, chicken; 0.025, pig) are much lower than the $R_F$ values of the corresponding peptides from the DIP-enzymes.

## Discussion

A model for the reactions of BNMP with liver carboxylesterases, based on previous observations with α-chymotrypsin and acetylcholinesterases discussed in Introduction, is shown in Scheme I. The data of Table I showing the release

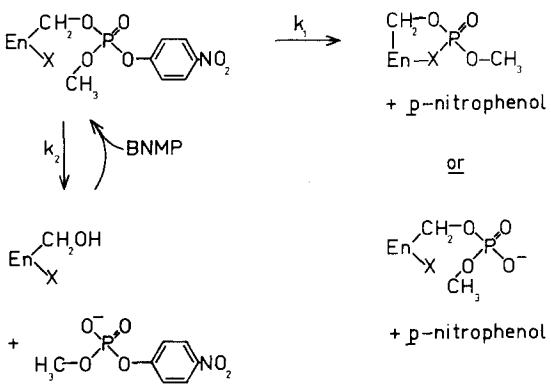



Scheme I

of 2 molecules of *p*-nitrophenol per active site (2.2 for the pig enzyme, vide infra) are consistent with such a scheme. The release of *p*-nitrophenol occurs very rapidly, being virtually over within 10 s. Therefore, to achieve a kinetic separation of the steps in which the *p*-nitrophenol is released, rapid reaction techniques would be required. This contrasts with the release of *p*-nitrophenol from bis(*p*-nitrophenyl) phosphoryl-chymotrypsin [7] and methyl *p*-nitrophenyl phosphoryl-chymotrypsin which occurs sufficiently slowly to allow its measurement by standard methods. The product of the reaction of the liver carboxylesterases with BNMP does not reactivate spotaneously, nor on treatment with hydroxylamine, and therefore can be considered to be an "aged"

enzyme. In this regard, bis(*p*-nitrophenyl) methylphosphonate and *p*-nitropheny! methylphosphonochloridate react with eel acetylcholinesterase, very rapidly producing an aged enzyme [5].

For the liver carboxylesterases from horse, sheep and chicken, two molecules of *p*-nitrophenol are released per active site within experimental error. However, for pig liver carboxylesterase, with $[I]_0 > [E]_0$, a ratio of 2.23 ± 0.03 molecules of *p*-nitrophenol per active site was reproducibly found, using different enzyme preparations. Possible explanations for this difference include: (i) the presence of two enzymes in the pig liver carboxylesterase preparations, the predominant enzyme reacting with paraoxon, and both enzymes reacting with BNMP; (ii) the reaction of more than one molecule of inhibitor with some active sites; (iii) competition between dephosphorylation and ageing, as shown in Scheme II, for the pig enzyme but not for the other carboxylesterases.




Scheme II

The first and second possibilities are ruled out by the equivalent weight determinations in Table II. If either of these possibilities were correct, an equivalent weight of 63 800 should have been found for bound $^{32}P$ rather than the experimental value of 69 700, which compares well with the equivalent weight of 70 700 for the paraoxon titration. The third possibility was checked by performing the experiments with $[E]_0 > [I]_0$. This explanation for the release of 2.23 mol of *p*-nitrophenol per active site titrated requires a ratio of the first-order rate constants $k_1/k_2$ (Scheme II) of 4.35. This ratio predicts that, with $[E]_0 > [I]_0$, 1.81 mol of *p*-nitrophenol will be released per mol of BNMP added. Table III shows that this value was indeed obtained. Scheme II, together with the ratio of 4.35 for $k_1/k_2$, also predicts that methyl *p*-nitrophenyl phosphate will be produced in the proportion of 1 mol of methyl *p*-nitrophenyl phosphate per 4.35 mol of enzyme inhibited. Methyl *p*-nitrophenyl phosphate has been unambiguously demonstrated in the reaction products from BNMP and pig liver carboxylesterase, and shown to have arisen via an enzymatic reaction. The data of Fig. 1 yield a ratio of 78.7/14.5, i.e. 5.4, for the ratio of mol of enzyme inhibited/mol of methyl *p*-nitrophenyl phosphate produced. Therefore, all the data obtained both qualitatively and quantitatively support Scheme II as the explanation for the results obtained with pig

liver carboxylesterase. In this connexion Fife et al. [21], in studying the reaction of bis(*p*-nitrophenyl) carbonate with α-chymotrypsin, observed a stoichiometry of 2.9 mol *p*-nitrophenol released per active site inhibited. This observation is consistent with a reaction sequence analogous to Scheme II.

According to Scheme II, $\approx 0.19$ ($= k_1/(k_1 + k_2)$) of the methyl *p*-nitrophenyl phosphoryl-carboxylesterase (pig liver) dephosphorylates before the second nitrophenyl group is displaced. The rate constant for this dephosphorylation must be greater than or equal to 0.1 $s^{-1}$ ($t_{½} \leqslant 5$ s). This rate is orders of magnitude faster than rates of reactivation (dephosphorylation) previously reported [1]. An attractive possibility to account for the partitioning of methyl *p*-nitrophenyl phosphoryl-enzyme between dephosphorylation and ageing is that in the initial reaction, the two enantiomeric forms of methyl *p*-nitrophenyl phosphoryl-enzyme are formed in the ratio of approx. 5 : 1. The predominant enantiomer would then age much more rapidly than it dephosphorylated, whereas for the minor enantiomer, dephosphorylation would be greatly enhanced with respect to ageing. In support of this explanation are the very large differences in the rates of ageing between enantiomeric forms of phosphorylated enzymes. For example, R and S forms of cyclopentyl methylphosphonylacetylcholinesterase differ in ageing rates by a factor of $> 3700$ [22].

The preliminary results from radioautography of peptic digests of BNMP-inhibited pig and chicken esterases are consistent with either of the products of Reaction 2a or 2b, Scheme I. Cross-linking (2a) would result in larger peptic peptides than the octapeptide from the DIP-enzymes, and hence smaller $R_F$ values in butanol/acetic acid/water. Alternatively, Reaction 2b results in an additional negative charge on the peptic peptides, which should also produce a lower $R_F$. Providing any cross-linked product is stable, distinction between products of Reactions 2a and 2b could be made by characterizing the radioactive peptic peptides, and this we intend to do.

A significant difference has been documented between the reactions of BNMP with carboxylesterases from closely related animal species. This type of difference is important when considering the degree of selectivity which could be built into organophosphorus insecticides. Additional experiments suggested by the foregoing results include study of the reaction of pig liver carboxylesterase with other inhibitors with two good leaving groups, to see how the ratio of 2.23 observed with BNMP may vary. Preliminary experiments with bis(*p*-nitrophenyl) benzyl phosphate indicate that it reacts more slowly with carboxylesterases, which could make kinetic and mechanistic studies more feasible.

Although the kinetics of the reaction of BNMP with pig and chicken liver carboxylesterases have not been analysed, the observation of a slow tail in the burst (which accounts for approx. 4% of the total reaction) indicates that the analysis will be complex. In this regard, the reactions of bis(*p*-nitrophenyl) phosphate with pig and chicken liver carboxylesterases (ref. 23; Hamilton, S. and Inkerman, P., unpublished), and the reaction of *p*-nitrophenyl dimethylcarbamate with pig liver carboxylesterase [24] are also found to be complex and apparently biphasic*. Clearly, the explanation of the kinetics of these titrations

* The equivalent weights determined by bis(*p*-nitrophenyl) phosphate and *p*-nitrophenyl dimethylcarbamate are the same as those determined by paraoxon titration.

awaits further investigation. However, we have demonstrated conclusively that the determination of different equivalent weights (based on *p*-nitrophenol release) for pig liver carboxylesterase using BNMP and paraoxon (Table I) does not result from the presence of different types of active site in the pig liver carboxylesterase preparation.

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