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Rapid Aging of Neurotoxic Esterase after Inhibition by Di-isopropyl Phosphorofluoridate

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1. It was proposed [Johnson (1974) *J. Neurochem.* **23**, 785–789] that an essential step in the genesis of delayed neuropathy caused by some organophosphorus esters was aging of phosphorylated neurotoxic esterase, involving generation of a charged monosubstituted phosphoric acid residue on the protein. 2. Neurotoxic esterase of hen brain was inhibited with di-isopropyl phosphorofluoridate either unlabelled or mixed-labelled with ^{3}H and ^{32}P. 3. Reactivation of inhibited enzyme by KF was possible only immediately after a brief inhibition: aging at pH 8.0 and 37°C occurred with a half-life of about 2–4 min. 4. When the radiolabelled enzyme was studied no loss of label was observed during the expected aging period, but a change in the nature of the bound radioisotopes occurred (half-life = 3.25 min). 5. Alkaline hydrolysis of labelled enzyme liberated di-isopropyl phosphate at early times after labelling, but increasing amounts of monoisopropyl phosphate plus a volatile tritiated compound (possibly propan-2-ol) at later times. 6. Treatment of labelled enzyme with KF released di-isopropyl phosphate and caused reactivation of enzyme to similar degrees. It is concluded that the chemical change from di-isopropyl phosphoryl-enzyme to mono-isopropyl phosphoryl-enzyme and the loss of reactivatibility are related. 7. The rate of aging is similar at pH 5.2, 6.5 and 8. Aging is unaffected by addition of reduced glutathione and imidazole at pH 5.2 or 8, and none of the transferred ^{3}H is trapped by these reagents. The mechanism of aging must be different from the better-known dealkylation aging of the cholinesterases.

OP esters such as di-isopropyl phosphorofluoridate (DiPF) react with AChE according to the general Scheme 1, and it seems likely that each of the steps illustrated can occur with other organophosphorus-sensitive esterases (see Aldridge & Reiner, 1972).

Reaction (3) (reactivation) may occur spontaneously and may, in some cases, be catalysed by agents such as fluoride salts and some nucleophilic oximes. After inhibition an alternative reaction is (4), which involves loss of the R group and generation of a charged substituent on the enzyme. This reaction has been most studied with the cholinesterases. For these enzymes it is clear that once reaction (4) occurs, reactivation in the manner of reaction (3) is no longer feasible. The enzyme is said to have aged (i.e. lost its responsiveness to reactivators) as a result of the group loss.

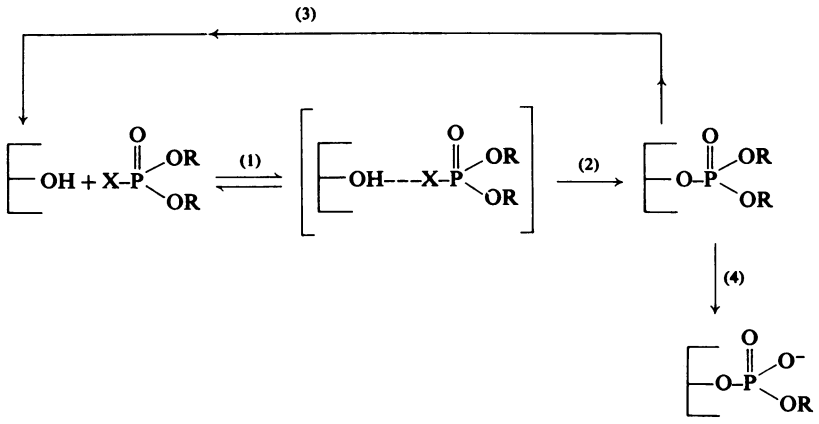
DiPF and some other OP esters cause a delayed neuropathy in man and many experimental animals apart from small rodents (reviewed by Johnson, 1975). This effect is quite separate from acute anticholinesterase effects, and the initial biochemical event is inhibition of a distinct esterase (NTE) in nervous tissue (Johnson, 1969*b*, 1970). Structure/activity studies led to the proposition that, in the genesis of neuropathy, reaction (4) is a necessary further step after inhibition of NTE (Johnson, 1974). A preliminary claim (Johnson, 1976) that this reaction had been demonstrated depended on a manufacturer's stated value for the specific radioactivity of an OP ester. We have recently had cause to doubt that value and have therefore examined the hypothesis more thoroughly. We find that group loss from, and aging of, DiPF-inhibited NTE occurs rapidly *in vitro*, but that the process differs from the aging of DiP–AChE in that the fate of the isopropyl group is different.

Abbreviations used: OP, organophosphorus; DiPA, DiPF and DiP, the acid, fluoridate and derived group respectively of di-isopropyl phosphoric acid; MiPA and MiP, the analogues based on monoisopropyl phosphoric acid; AChE, acetylcholinesterase; ChE, cholinesterase; NTE, neurotoxic esterase.

Materials and Methods

Chemicals

Trypsin (type I), chymotrypsin (type II) and horse serum cholinesterase (type IV) were from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. Acetylthiocholine iodide and citric acid



Scheme 1. *Steps in the interaction of AChE with an OP inhibitor*
(1) Formation of Michaelis complex; (2) phosphorylation of enzyme; (3) reactivation; (4) aging. R is usually one of a variety of alkyl or aryl groups and X is a more labile group commonly referred to as the leaving group.

were from B.D.H., Poole, Dorset, U.K. Soluene 350 and Instagel scintillant were from Packard Instruments, Caversham, Reading, U.K., and 5,5′-dithiobis-(2-nitrobenzoic acid) was from Aldrich Chemicals, Gillingham, Dorset, U.K. Other reagents and inhibitors were obtained as described before (Johnson, 1969*a*,*b*, 1974, 1977). 1,3-^{3}H- and ^{32}P-labelled DiPF were supplied dissolved in propylene glycol from The Radiochemical Centre, Amersham, Bucks., U.K. These were mixed in varying proportions according to the decay of ^{32}P to obtain a ratio of ^{3}H/^{32}P in the reagent of 5–10 and a chemical concentration of about 2mM. The labelled compounds contained small amounts (<0.1%) of impurities that reacted rapidly with brain homogenate and also with purified chymotrypsin; if these reactions (see below for method) were carried out with mixtures of ^{3}H- and ^{32}P-labelled DiPF, then the ratio ^{3}H/^{32}P of the earliest-bound radioactivity differed from that of the bulk of radioactivity bound after the initial 0.1% of reagent had reacted. The ratio of the earliest-bound radioactivity also differed from that of the bulk reagent and varied with each of three different ^{32}P-labelled preparations that were mixed with samples of one ^{3}H-labelled preparation. In each case we found that most of the impurity was inactivated by diluting the mixed propylene glycol stocks (2 vol.) with Tris/citrate pH6.0 buffer (1 vol.) and incubating for 20–40h at 37°C. The ^{3}H/^{32}P ratio of labelled NTE was then the same as that of labelled purified enzymes (chymotrypsin, trypsin or cholinesterase: see below). With different batches of labelled DiPF the radioisotopic ratio for labelled proteins was 107–119% of the ratio in the reagent.

A carrier mixture of unlabelled DiPA and MiPA was prepared by dropwise addition of propan-2-ol (1.95g = 0.033mol) to $POCl_3$ (3.34g = 0.022mol) in a tall glass tube (20cm × 3cm). The mixture was heated at 100°C for 30min and water (10ml) was added cautiously. NH_3 (about 3ml of 18M) was added cautiously to adjust the pH to 10–11. Portions of this carrier solution were added to products released from labelled enzymes by alkali.

Buffers

For NTE studies, 50mM-Tris/0.2mM-EDTA was neutralized to pH8.0 with HCl (about 12M) or else was adjusted to the desired pH (5.2, 6.0 or 8.0) by admixture with necessary amounts of 50mM-citric acid/0.2mM-EDTA: these buffers are subsequently identified only by pH. For study of AChE, solutions of KH_2PO_4 and Na_2HPO_4 (each 100mM) were mixed to give the desired pH.

(1) *Studies involving hydrolysis of substrate by NTE*

(*a*) *Background.* NTE is one of a number of esterases that are present in brain tissue and preferentially hydrolyse phenyl phenylacetate or phenyl valerate. The selective assay of NTE (Johnson, 1977) depends on: (*a*) the initial inhibition in a pair of tissue samples of all esterases sensitive to paraoxon (phosphoric acid diethyl 4-nitrophenyl ester); (*b*) the selective inhibition of NTE among the remaining esterases by mipafox [*NN*′-bis(1-methylethyl)phosphorodiamidic fluoride]; (*c*) comparison of the hydrolysis of phenyl valerate in the samples treated with or without mipafox.

In simple screening of inhibitors for effects on NTE they are included in the preincubation with paraoxon and mipafox. That preincubation, however, is standardized at 20min and pH8 and the ongoing inhibition of the mixture of esterases is then halted by addition of substrate. To detect rapid aging of NTE it was necessary to complete the inhibition with test compounds in 1–2min and then to allow reactivation or aging to occur without the complication of further inhibition. For this a revised procedure was developed: (1) pairs of samples were treated with paraoxon (or benzenesulphonyl fluoride: see below) with and without mipafox as above; (2) inhibition was slowed by cooling and diluting the mixture; (3) the remaining active particle-bound enzymes, including NTE, were sedimented from the inhibitor solution by centrifugation and resuspended in buffer free of inhibitor; (4) inhibition, aging and reactivation studies with the preinhibited pairs were then carried out under any condition of time, pH etc. that was convenient.

A confusing factor in preliminary studies was that paraoxon-sensitive enzymes that were inhibited in step (1) above were partially reactivated along with NTE during some reactivation procedures. It was known (M. K. Johnson, unpublished work) that these irrelevant esterases were also sensitive to benzenesulphonyl fluoride, and we found that the sulphonated enzymes were not reactivated under our treatments, so that this compound was then used in preference to paraoxon in step (1). The detailed preparation of such preinhibited tissue pairs is given below.

(*b*) *Preparation of pairs of tissue samples for NTE assay.* Homogenates of whole hen brain (3–3.5g/35ml) were made in pH8 buffer as before (Johnson, 1969*b*). Blood cells, some nuclei and large debris were sedimented by centrifugation in an HS 18 MSE centrifuge at $750g_{av.}$ for 10min. The supernatant was decanted, diluted to 35ml with pH8 buffer and 2×16ml samples were warmed to 37°C in plastic centrifuge tubes. Benzenesulphonyl fluoride (200μl of 20mM stock in acetone) was added. After 20min incubation at 37°C either pH6 buffer or mipafox (400μl of 10mM stock in pH6 buffer) was added and incubation was continued for a further 5min. The tubes were cooled in ice and centrifuged at $30000g_{av.}$ for 20min. The supernatants were discarded and the differentially preinhibited particles were resuspended in a small volume of ice-cold Tris/citrate buffer, pH5.2, to give preparations that were routinely referred to as P and M: these were identical except that only P contained active NTE. The difference between the abilities of P and M either to hydrolyse phenyl n-valerate or to bind labelled DiPF was a measure of the NTE activity.

(*c*) *Inhibition of NTE.* Samples of P and M (5ml, each containing particles from about 700mg of brain) were incubated at 37°C and pH5.2 and inhibitor (50μl in acetone to give a chosen final concentration) was added; uninhibited controls were treated with acetone only.

(*d*) *Aging of NTE.* After 2min inhibition a portion (2ml) was removed from each sample and was mixed with Tris/citrate buffer (48ml) at 37°C and a chosen pH: this 25-fold dilution step decreased inhibition to a negligible rate during the next 5min. One set of samples of diluted inhibited preparations P and M was at once put through the reactivation procedure (see below). The delay in mixing and transferring was 30–40s. These samples had minimal opportunity to age and were used as a point of reference for a further set that were processed identically after standing for 5min in the aging medium.

(*e*) *Reactivation of NTE.* Two samples (10ml each) of dilute inhibited preparation P and two of M were removed from the aging medium. One of each was transferred to Tris/citrate buffer (25ml at 37°C and pH5.2) and the other to the same buffer that also contained KF (200mM final) and each was mixed thoroughly.

Reactivation was slowed after 10min by cooling the tube contents to 0–5°C. The treated particles were sedimented by centrifugation at 2–5°C at $30000g_{av.}$ for 60min (three times the period used in their initial isolation) and were then resuspended in pH8 buffer (10ml).

(*f*) *Assay of NTE.* Esteratic activity of samples was measured by the simplified procedure of Johnson (1977) with phenyl n-valerate as substrate, and the NTE activity was derived from the difference in activity of processed P and M samples. Activity was determined of both control and inhibited samples that had been put through the complete procedure above and was calculated as percentage of activity of uninhibited sample that had been diluted 25-fold with cold buffer and then assayed immediately without any intermediate aging or reactivation.

Reactivation was assessed by comparing the activities found in samples treated with and without KF. Aging was assessed by comparing the amount of reactivation found in samples processed at 40 and 340s after halting of inhibition.

(2) *Inhibition, aging, reactivation and assay of hen brain AChE*

Samples of inhibited AChE free of inhibitor were prepared as follows. Homogenates were freed from cellular debris as in (1*b*) above and the supernatant was divided into two portions. These were incubated for 5min at 37°C with di-isopropyl 4-nitrophenyl phosphate [20μM, $\geq 10\times I_{50}$ (the negative log of the concentration of the inhibitor that causes 50% inhibition); Davison, 1953] or with buffer for controls and were then diluted and centrifuged as in

(1*b*) above to remove inhibitor. The control and inhibited particles from one brain were each resuspended in 100mM-sodium/potassium phosphate buffer, pH7.4 (10ml), and incubated at 37°C. At various times portions (1ml) were removed and incubated for 90min at 37°C with 1ml of either buffer, pyridine-2-aldoxime methanesulphonate (10mM final concentration) or KF (300mM final concentration) in buffer. Reactivation was slowed by addition of ice-cold buffer (38ml) and the particles were sedimented to free them from reactivator by centrifugation at 30000$g_{av.}$ for 60min and were resuspended in buffer (10–20ml). Portions (0.5ml) of the treated particles were assayed for AChE by the method of Ellman *et al.* (1961).

(3) *Studies involving radiochemical labelling of the active site of NTE*

The principle of the differential labelling procedure is identical with that of the differential substrate-hydrolysis procedure described in (1) above (see also Johnson, 1969*a*).

(*a*) *Labelling of NTE.* Suspensions (P and M) of particles preinhibited with paraoxon or paraoxon+mipafox were prepared as above in Tris/citrate pH5.2 buffer. Suspensions (3–5ml) were prewarmed to 37°C in wide-necked jars and labelled DiPF in buffer/propylene glycol was added to give a final concentration of 58μM (for 1min labelling reactions) or 34μM (for $1\frac{1}{2}$–$1\frac{3}{4}$min reaction) with the concentration of propylene glycol $\leqslant$1% (v/v). After 1–$1\frac{3}{4}$min reaction a 20-fold excess of warm (37°C) buffer of chosen pH was added and the whole mixture was swirled vigorously. In most experiments, the added buffer contained a mixture of 4-nitrophenyl di-n-pentyl phosphate (100μM) and 2,2-dichlorovinyl diphenyl phosphate (100μM), which had been prepared just before use by addition of stock concentrated solutions (30mM) in acetone to the buffer. The combined effects of dilution of the radioactive inhibitor and competition for labelling sites by these unlabelled inhibitors effectively halted the labelling of NTE and decreased to an acceptable low rate (equal in both P and M) the ongoing labelling of other sites.

(*b*) *Aging of labelled NTE.* At various times after dilution, samples (12–15ml, equivalent to particles from 300–350mg of original brain) were transferred to stoppered tubes containing ice-cold acetone/diethyl ether (2:1, v/v; 30–35ml) and the contents were mixed and left in ice for at least 10min.

(*c*) *Counting of labelled NTE.* As described by Johnson (1969*a*), precipitated protein was sedimented and washed three times to remove unbound inhibitor and the absence of non-covalent binding at zero time was confirmed. After removal of the final supernatant wash fluid, the residue was dissolved in Soluene 350 (3ml) and transferred quantitatively to scintillation vials with 3×5ml of Instagel. Acetic acid (0.1ml) was added to quench chemiluminescence and the radioactivity of ^{3}H and ^{32}P was determined in a Philips scintillation counter with channels set so that not greater than 1% of each radioisotopic count was recorded in the complementary channel. ^{3}H-counting efficiency was 20–25% and that for ^{32}P 92–94%, and corrections were made by using an external-standard channels-ratio method. Samples were counted for radioactivity for 40min and gathered counts were over 20000, with negligible counting error.

(*d*) *Reactivation of labelled NTE.* (1) Labelling of P and M samples was carried out as above. (2) Further labelling was slowed by dilution as above except that no unlabelled OP compounds were added. For these experiments the dilution buffer pH was 5.2. (3) The dilute labelled samples were allowed to age for various times. (4) For each chosen time, portions of the aged samples were precipitated with acetone/diethyl ether and processed for counting as above (sections 3*b* and 3*c*). (5) Coincidental with (4), further portions of the aged samples (18ml) were transferred to screw-thread polycarbonate centrifuge tubes containing reactivator (12ml of 1.5M-KF in pH5.2 buffer, final pH6.0) at 37°C. After 10min incubation to reactivate unaged NTE the tubes were cooled, filled to capacity (35ml) with pH5.2 buffer, sealed with aluminium screw caps and centrifuged at 40000rev./min (117000$g_{av.}$) for 40min in an 8×35 titanium rotor of an MSE Superspeed 65 centrifuge; the pelleted material was washed once by resuspension in pH5.2 buffer (35ml), resedimented and finally resuspended in pH8 buffer (18.5ml). (6) From each suspension of reactivated tissue one portion (15ml) was precipitated, washed and counted for radioactivity as above and duplicate 1ml portions were assayed for phenyl valerate hydrolase activity. (7) The uninhibited NTE rate was determined with samples put through the complete procedure (steps 1–6), but omitting DiPF.

(4) *Radiolabelling of other enzymes*

The general procedure was the same as for labelling NTE, but different buffers and concentrations of DiPF were used and reaction was quenched directly without any delay; no aging was expected during the few minutes of reaction.

(*a*) *Trypsin and chymotrypsin.* The buffer was 50mM-Tris containing 15mM-$CaCl_2$ and adjusted to pH7.6 with HCl. Enzyme stock solutions (2mg of trypsin/ml or 1mg of chymotrypsin/ml in 1mM-HCl) were kept at 2°C. Portions (15–50μl) were transferred as required to buffer (2ml) at 37°C and

labelled DiPF (20μl of 2.2mM) was added immediately. After 3½min a portion (3ml) of ice-cold brain tissue to act as protein carrier was added and was followed immediately by cold acetone/diethyl ether as usual to quench the reaction. The amount of carrier was not critical and portions of preparations P and M derived from about 200mg of brain were used. Preparation of samples for radioactivity counting was as before.

(*b*) *Cholinesterase.* The buffer was obtained by mixing 100mM-Na_2HPO_4, 100mM-KH_2PO_4 and 100mM-Tris (adjusted to pH7.4 with HCl) in proportions 4:1:15 (by vol.). Use of the usual phosphate buffer (100mM) led to intractable pellets that could not be redispersed in the final washing procedure. Fresh enzyme solution (0.1–0.3ml of 1mg/ml in buffer) was used and the general procedure was as for trypsin.

(*c*) *Radiochemical study of aging of hen brain cholinesterase.* This was determined by a differential process similar to that for NTE, except that different inhibitors were used. It was necessary to separate the particles from inhibitor after labelling because the long aging period meant that dilution procedures to halt ongoing labelling were ineffective. The initial supernatant derived from homogenate (6.4g of brain) was incubated with benzenesulphonyl fluoride (50μM) plus phenylmethanesulphonyl fluoride (20μM) for 20min at 37°C to remove many irrelevant phosphorylation sites: these concentrations caused negligible inhibition of AChE or ChE (see also Myers & Kemp, 1954); the suspension was then cooled and centrifuged at 30000$g_{av.}$ for 20min. The preinhibited particles were resuspended in Tris/phosphate buffer, pH7.4 (12ml), and samples (2ml) were incubated for 10min at 37°C in screw-cap centrifuge tubes (35ml capacity) with 0.1ml buffer with or without eserine (10μM final concentration). Mixed-label DiPF (25μl, giving 25μM final concentration) was added to each sample and incubation was continued for 1½min, which (based on the published I_{50}; Davison, 1953) was estimated to give complete labelling of ChE and 80–90% labelling of AChE in the sample without eserine. Reaction was halted by addition of ice-cold Tris/phosphate buffer (32ml) containing paraoxon (67μM). Paired tubes were centrifuged at 117000$g_{av.}$ for 40min at 3–5°C; the sedimented particles were washed by resuspension and re-centrifugation in buffer and were finally suspended in buffer (13ml) and incubated at 37°C to permit aging to proceed. Duplicate samples (2×2ml) were withdrawn from each of the pair at 0, 5 and 18h after commencement of aging, mixed with water (3ml) and precipitated with acetone/diethyl ether (2:1 v/v; 13ml). One of each duplicate was counted for radioactivity and one was hydrolysed etc. exactly as described below for NTE. In each case the measured counts were over 10000.

(5) *Characterization of radioactivity bound to protein*

Bound counts were liberated by alkaline hydrolysis and then were partitioned between acid and chloroform as follows.

(*a*) *Hydrolysis.* Duplicate labelled samples were processed (as in section 3*c*) to the point of readiness for solution in Soluene. One sample was then dissolved in Soluene etc. and counted for radioactivity, and the duplicate was dissolved in 0.1M-NaOH (6ml) and incubated at 90°C for 60min. Mixed DiPA+MiPA carrier solution (0.1ml) was added.

(*b*) *Concentration.* After cooling the tubes, 13M-NH_3 (0.2ml) was added followed by 1M-formic acid (2.0ml) and 2ml of water. The pH was 6–8 and protein was precipitated by addition of acetone/ether (2:1, v/v; 30ml). After sedimentation of the protein as in section 3(*c*) the supernatant was reserved and the pellet was washed once as usual before being dissolved in Soluene and counted for radioactivity. The concentration of radioactivity in the supernatant was too low to measure and it was therefore concentrated to a dry (slightly sticky) residue by rotary evaporation at 30–40°C. In this step any volatile labelled compounds were removed. The residue was carefully dissolved in 0.1M-NH_3 (3ml) and treated as in either section 5(*c*) or section 5(*d*).

(*c*) *Direct counting.* A large sample (1–1.5ml) of the 3ml solution in dilute NH_3 was taken without further processing for counting; Soluene 350 (1.5ml), Instagel (15ml) and acetic acid (0.1ml) were added. The efficiency, with these samples, of 40min counts was reproducible within 2%.

(*d*) *Partition followed by counting.* From the 3ml solution in section 5(*b*) a sample (0.3ml) was taken for counting in Instagel as in section 5(*c*). The remainder was transferred to a small separating funnel having a Teflon key and the flask was rinsed into the funnel with successive portions of NH_3 (2×1ml of 0.1M) and HCl (1ml of concentrated, about 12M). The acid solution (5.7ml) then contained HCl (about 2M) plus a considerable quantity of ammonium formate carried through from after the hydrolysis step: the salts were found to increase the partition ratio of DiPA, which was transferred into chloroform (3×20ml) by repeated extraction. The chloroform extracts were pooled and evaporated to dryness in a round-bottomed flask with a rotary evaporator at 30°C. The residue was dissolved in 0.1M-NH_3 (1ml) followed by Instagel (17ml). The flask contents were transferred to a counting vial and acetic acid (0.1ml) was added. Rinsing of the flask contents with NH_3 and Instagel was repeated twice and these rinsings were also counted for radioactivity, since it was found that only 90% of the counts were transferred in the first rinsing. A portion (1ml) of the acid solution remaining after chloroform extraction was also counted for radio-

activity. All samples were counted for 40min. The precision of counting of the chloroform-extracted radioactivity was better than 2%, but the counting precision for the small aqueous samples was less and is discussed in the Results section.

(6) *Assessment of alkaline hydrolysis of labelled NTE and of the subsequent partitioning*

It seemed likely that aging of DiPF-inhibited NTE would involve conversion of DiP–enzyme into MiP–enzyme. To characterize these products if they were formed some model compounds were prepared, hydrolysed and processed as described above. The models were (i) a mixture of ^{3}H- and ^{32}P-labelled DiPF, and (ii)–(iv) mixed-labelled proteins for which the aging properties were well known so that the nature of the hydrolysis products was not in doubt. The enzymes were (ii) chymotrypsin labelled with DiPF at pH7.6 (known not to age under these conditions; Jansz *et al.*, 1959), (iii) horse serum ChE labelled with DiPF and then quenched immediately, and (iv) ChE treated as in (iii) but allowed to age for 22h at 45°C and pH6.0 before quenching; in this last case extensive aging with formation of MiP–ChE would be expected to occur (Berends *et al.*, 1959). Treatment of the labelled proteins with alkali (see section 5*a*) released all the bound radioactivity. For (i)–(iii) the ^{32}P of the hydrolysate was expected to be exclusively in the form of DiPA. The observed extraction of isotope into chloroform by method (5*d*) was over 98% and the ratio $^{3}H/^{32}P$ in the extract was 96–105% of the ratio of radioisotopes originally bound to protein (four experiments). With aged horse serum ChE (iv) 79–81% (two experiments) of ^{32}P remained in the acid phase with $^{3}H/^{32}P = 50\%$ of the ratio for unaged enzyme and 13% was extracted into chloroform with $^{3}H/^{32}P = 109\%$ of the ratio for unaged enzyme (this rather high ratio depends on only 4000 gathered counts). These results together show that nearly complete segregation of DiPA (into chloroform) and MiPA (into acid) was achieved by the extraction.

Results

Substrate-hydrolysis studies

Use of KF as reactivator in aging studies. Preliminary studies (unpublished work, B. Clothier) showed that a number of nucleophiles (including KF and some oximes known to reactivate inhibited AChE) were capable of reactivating an inhibited (diethylphosphinyl) NTE that could not age, but none were outstandingly active. KF seemed suitable for studies that would eventually include bulky inhibitor molecules offering steric hindrance to the action of larger nucleophiles: it is also stable and very soluble. KF has not been much studied as a reactivator and we did not know whether it would reactivate aged inhibited enzymes. Heilbronn (1965) showed that fluoride would not reactivate soman-inhibited AChE, but would reactivate sarin-inhibited AChE. It seemed likely, but was not proved, that the difference was due to aging in the first case. Fig. 1 shows a time-dependent loss of response of DiPF-inhibited AChE to reactivation by KF or pyridine-2-aldoxime methanesulphonate (a typical oxime reactivator). It was therefore presumed that aged inhibited NTE would likewise be resistant to KF reactivation. In preliminary experiments with NTE inhibited by a number of compounds and in which the time between commencement of inhibition and of reactivation was 1–2h, the only reactivation found was with phosphinylated enzyme that could not age. It therefore seemed likely that aging, where it could occur, was rapid and it was necessary to develop conditions in which inhibition could be completed quickly (see the Materials and Methods section) and in which reactivation time was not long in comparison with aging time. Table 1 shows that the rate of reactivation by KF of diethylphosphinyl–NTE increased as pH was lowered. [This same effect of pH on KF activity was noted by Heilbronn (1965) with inhibited AChE.] The lower limit of pH for NTE stability was about 5, so for convenience the successive operations of inhibition, aging and reactivation were all normally carried out at pH5.2 with only the final assay of the resuspended particles being done at pH8. The rate of

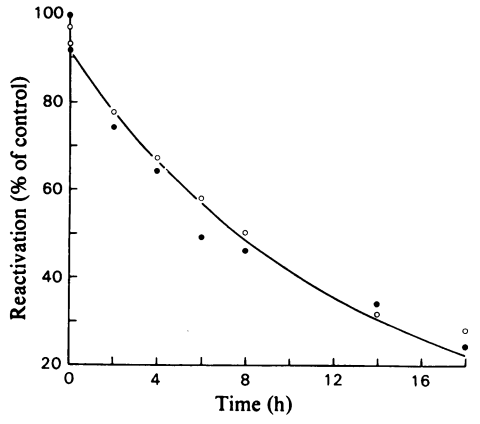


Fig. 1. *Loss of response of DiP–AChE of hen brain to reactivation by pyridine-2-aldoxime methanesulphonate* (○) *or KF* (●)

The procedure is described in the Materials and Methods section (2). Activity is calculated as a percentage of that of an uninhibited sample treated with reactivator; these treatments did not alter the AChE activity. The drawn line is the theoretical curve for a first-order reaction with $k = 0.0797\,h^{-1}$.

inhibition of NTE at pH 5.2 by a number of inhibitors is not markedly less than at pH 8.

Reactivation and aging of DiPF-inhibited NTE. Table 2 compares the effect of instituting reactivation by KF (0.2M, 10min at 37°C at pH 5.2) immediately after a 2-min inhibition followed by 25-fold dilution with the effect of the same procedure after 5 min in the dilute suspension. In the inhibited samples treated immediately with KF, NTE activity was restored to 43–50% (4–6% in samples without KF), but after 5min KF only restored it to 9–18%. For uninhibited controls recovery was 82–93% and was not significantly affected by the delay allowed for aging or by treatment with KF. Clearly a considerable decline in responsiveness of inhibited NTE to KF had occurred in 5min, whereas uninhibited NTE did not change. This change fulfils the criterion of aging. The precision of the experiments does not enable us to decide whether the change followed first-order kinetics as might be expected, but it seems that the half-life was about 2–4min.

Table 2 also shows that when inhibition and aging were carried out at several pH values and reactivation was performed at pH 5.2 then there was no marked change in the degree of aging seen in 5min. The slightly lower reactivation seen after aging in dilute pH 5.2 buffer compared with normal buffer is probably not significant.

Aging of radiochemically labelled NTE. When NTE was mixed-labelled by DiPF for 1–2min and was subsequently left for 0.6–12min at 37°C in various buffers (see the Materials and Methods section) there was no change in the bound radioactivity (typically about 9000d.p.m. of ^3H and 1200d.p.m. of ^{32}P. However, when samples that had been quenched at different times were subsequently treated with NaOH it was found that a time-dependent change had occurred that was reflected in a change in the properties of the labelled material liberated from the protein. For semantic convenience this change will be called aging even in advance of the presentation of evidence.

Table 1. *Effect of pH on reactivation of diethylphosphinyl–NTE by KF*

As described in the Materials and Methods section (1*c*–1*f*) samples P and M were inhibited for 20min at pH 5.2 with 4-nitrophenyl diethylphosphinate (500μM); samples were diluted and reactivated with KF (50mM final concentration) at various pH values before assay of NTE at pH 8. Recovery of uninhibited controls incubated with KF in the range pH 5–8 was over 90%.

pH	Reactivation (%)
9	7*
8	7
7	36
6	67
5.5	74
5.0	80
4.5	83*

* At pH 4.5 or 9 NTE was unstable, but KF afforded some protection against loss.

Table 2. *Effect of delay on ability of KF to reactivate DiPF-inhibited NTE*

As described in the Materials and Methods section (1*c*–1*f*) samples P and M were inhibited for 2min at pH 5.2 with DiPF (20μM); aging at various pH values was followed by reactivation with KF (200mM final concentration) at pH 5.2 and assay of NTE at pH 8. Where more than one value is shown, results are for separate experiments.

	Reactivation (%)			
	Started at 160s		Started at 460s	
	In buffer	In KF	In buffer	In KF
Aging buffer pH (type)				
5.2 (normal)	6, 6, 4	50, 46, 46	5, 4, 5	16, 14, 18
5.2 (dilute*)	4	43, 48	2	9, 10
6.5 (dilute*)	1, 5	38, 41	2, 3	5, 9
8.0 (dilute*)	3	38, 41	2	11, 10

* Dilution of inhibited tissue (in pH 5.2 buffer) 25-fold with dilute buffer (one-fifth of usual concentration) was sufficient to set the pH for aging as stated. It was then possible to return the pH to 5.2 for reactivation with a comparatively small volumetric dilution.

Both before and after aging no significant radioactivity remained attached to the protein after alkali treatment. When the solution of liberated radiochemicals was concentrated to dryness 93.8% (±1.0% S.E.M., $n = 37$) of the ^{32}P was recovered, but the recovery of ^3H declined from about 80% for samples quenched at the earliest times to about 50% for samples quenched after 8–14min. It appears that half the ^3H bound initially to NTE was converted rapidly into a different bound form so that it became volatile after alkali treatment and was lost in the evaporative concentration step. The change in involatile radioactivity is shown in Fig. 2 as a declining $^3H/^{32}P$ ratio. The ratio of radioisotopes initially bound to NTE has been normalized to 2.0. The drawn line is the theoretical curve for a first-order reaction with a half-life of 3.25min.

Identification of labelled compounds liberated from NTE by alkali. No attempt has been made to trap the volatile radioactivity, but the partition experiments below show that the involatile labelled compound behaved like a mixture of DiPA and MiPA. We showed (see the Materials and Methods section 6) that mixed-labelled DiPA and MiPA were completely segregated into chloroform and acid phases respec-

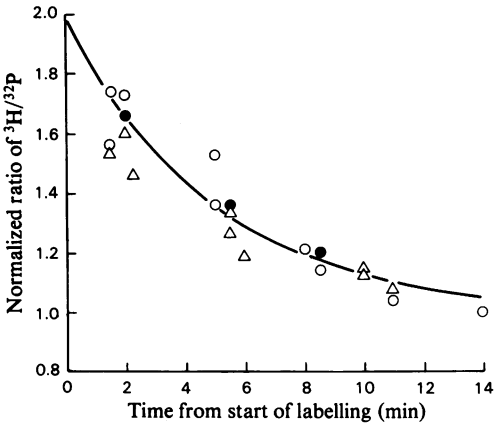


Fig. 2. *Time-course of aging of NTE inhibited by mixed-labelled DiPF*

As described in the Materials and Methods section, samples P and M were inhibited with mixed-labelled DiPF and samples were quenched at intervals thereafter. Measurement was made (counting precision $\leqslant \pm 1\%$) both of bound radioactivity and of involatile radioactivity liberated from protein by alkali [see the Materials and Methods section (5*c*)]. The ratio of $^3H/^{32}P$ bound was normalized to 2.0 and the ratio for the liberated involatile radioisotopes was then calculated by using the same factor. Time is reckoned from the beginning of the inhibition. Aging at pH5.2, ○; at 6.5, ●; at 8.0, △. The drawn line is the theoretical curve for a first-order reaction with $k = 0.213\,min^{-1}$. Half-life = 3.25 min.

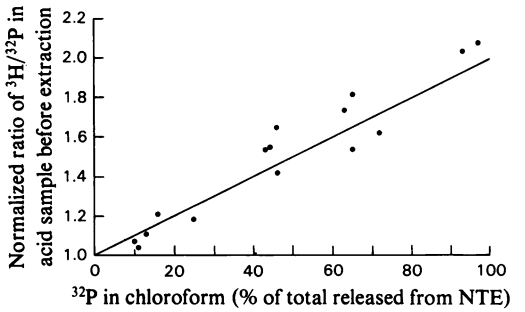


Fig. 3. *Correlation of normalized ratio of recovery of involatile radioisotopes released by alkali from NTE with percentage of released ^{32}P extracted into chloroform*

As described in the Materials and Methods section (5*d*), involatile radioactivity released from NTE was determined with limited precision (see the text) in a small portion of the acidified sample. The normalized ratio was calculated as described in the legend to Fig. 2. The remainder of the acidified sample was extracted three times with chloroform and the percentage extraction of ^{32}P was calculated. The drawn line is the relationship expected if the mixed-labelled material were only DiPA and MiPA and if these compounds were completely segregated by the procedure.

tively, by the partition process (see the Materials and Methods section 5*d*). Moreover no ^{32}P is lost when DiPA is converted into MiPA. It follows that in mixtures containing only mixed-labelled DiPA and derived MiPA the ratio $^3H/^{32}P$ in a mixture should be directly related to the percentage of ^{32}P that can be extracted into chloroform. Fig. 3 shows that such a correlation was obtained when the hydrolysis products from labelled NTE were processed as described in the Materials and Methods section 5*d*. The scatter of points is a reflection of the inprecision of radioactivity counting of the small aqueous acidic samples that were taken before and after extraction. Thus only 2000–8000 counts were obtained with these samples, depending on how much was removed by extraction. Moreover the radioactivity associated with NTE is determined by the difference between the counts for two samples (initially treated with or without mipafox) and this value was accordingly imprecise (possible error up to ±10%). Also the partition process was complex, making experimental error more likely. However, even with these limitations the mean normalized $^3H/^{32}P$ in the chloroform phase derived from variously aged samples was 1.96 (1.82–2.18, 13 experiments), whereas the ratio for acid-phase samples was 0.94–1.16 with a bias towards the higher ratio in samples containing a high proportion of chloroform-soluble counts initially. The method of counting directly the radioactivity of a large sample of hydrolysate without prior partitioning (see the Materials and Methods section 5*c*) was quicker and subject to negligible counting error. Therefore once the correlation of Fig. 3 was established only the simpler method was used routinely to follow aging.

The substrate hydrolysis and radiochemical experiments show that loss of reactivatibility of DiPF-inhibited NTE and a change from DiP–enzyme to MiP–enzyme occurred simultaneously. It is reasonable to conclude that the two events were linked and that the chemical change caused the loss of reactivatibility of the enzyme. This is confirmed by the fact (Table 3) that when mixed-labelled NTE was treated with KF the amount of NTE reactivated matched the amounts of both radioisotopes liberated from protein and that all these amounts declined with time.

During aging of inhibited NTE one tritiated isopropyl (isopropoxy?) group or radical was being transferred stoicheiometrically to some other macromolecular site from which it could subsequently be liberated in volatile form (probably as propan-2-ol) by alkaline hydrolysis. In two experiments to explore the mechanism of this transfer the aging process was

allowed to occur at pH 5.2 or 8 in the presence of GSH (1 mM) plus imidazole (1 mM) as possible trapping agents: there was no decrease in the protein-bound radioactivity at the end of aging or in the rate of aging. It seems that the transfer occurs on the protein rather than in the solution.

In contrast with the retention of the ^3H during aging of NTE, Table 4 shows that dual-labelled hen brain AChE and ChE [the enzymes sensitive to eserine (10 μM for 10 min)] lost 36% of their bound ^3H during aging for 18 h at 37°C and pH 3.4. The small concurrent loss of ^{32}P may signify spontaneous reactivation of enzymes. All remaining bound radioactivity was liberated on treatment with NaOH and none of the alkali-liberated radioactivity was volatile.

Table 3. *Time-dependent response of NTE inhibited by mixed-labelled DiPF to treatment with KF*

As described in the Materials and Methods section (3*d*), samples P and M were inhibited with mixed-labelled DiPF (37 μM) for 2 min; ongoing labelling was halted and at intervals some samples were quenched and counted, whereas duplicates were treated with KF: the reactivated NTE activity and the radioisotopes remaining bound to protein were determined. The amount of radioisotope liberated was calculated by difference. The time of commencement of reactivation was reckoned from the beginning of the inhibition. Where more than one value is shown, results are for separate experiments.

Reactivation started at (min)	Radioisotope removed (%)		Hydrolytic activity restored (% of uninhibited control)
	^3H	^{32}P	
0.6	64, 68	66, 68	69, 55
4.6	28, 28	29, 14	15, 13
13.4	11	11	Nil

Table 4. *Loss of radioisotope during aging of hen brain cholinesterases inhibited by mixed-labelled DiPF*

Labelling and aging of hen brain cholinesterases was determined in a single experiment by the differential method described in the Materials and Methods section (4*c*). Recovery of bound radioisotope was calculated as a percentage of that found bound immediately after 1½ min reaction with mixed-labelled DiPF (37 μM).

Time (h)	Radioisotope bound (%)	
	^3H	^{32}P
0	100	100
5	80	92
18	64	86

Discussion

NTE has not thus far been isolated free of other esterases and therefore its activity is always determined as the difference in activity of two samples that have had slightly different pretreatments (see the Materials and Methods section, 1*a*). Several modifications (see the Materials and Methods section, 1*b*) of the standard pretreatment (Johnson, 1969*b*) have been introduced in the present study. Preinhibited tissue free of paraoxon or mipafox was prepared to allow flexibility in times allowed for inhibition etc. of NTE. Benzenesulphonyl fluoride was used in place of paraoxon in ester-hydrolysis experiments involving reactivators to prevent significant reactivation of irrelevant esterases: such reactivation should be equal in both P and M samples, and thereby the effect cancels out, but it is nevertheless inconvenient. One further modification requires explanation. In routine assays of NTE mipafox is included in the preincubation of one sample at the same time as paraoxon or benzenesulphonyl fluoride. It does not usually matter whether the sulphonyl fluoride-sensitive esterases are actually inhibited by this compound or by the mipafox. However, if a reactivation step is introduced it is essential that in both samples P and M these inhibited enzymes should be in identical chemical form. Therefore a sequential procedure was adopted (see the Materials and Methods section, 1*b*) in which mipafox was introduced briefly into one of the paired samples only after preincubation with benzenesulphonyl fluoride had been virtually completed. By this means it was ensured that mipafox reacted only with NTE.

Neuropathy caused by organophosphorus esters in hens only occurs if (i) NTE is inhibited and (ii) the structure of the inhibitor is such that aging (reaction 4 of Scheme 1) can occur. Johnson (1974) proposed that aging was a necessary change and that the charged group that is left attached to the protein is responsible for the ultimate neurotoxic effect. It seemed likely that the chemical change would be accompanied by loss of responsiveness of the inhibited NTE to reactivation procedures (see the Introduction). For NTE inhibited by DiPF both these changes have now been shown to occur at a similar rapid rate (Tables 2 and 3 and Fig. 2). For both reactions the rate is not greatly affected by changes in pH in the range 5.2–8 (Table 2 and Fig. 2). Moreover, only the radioactivity associated with the DiP group was liberated by the reactivation procedure (Table 3). It may be concluded that aging of DiPF-inhibited NTE has been proved by two independent means.

Now that the chemical change and the loss of reactivatibility have been shown to be related for DiPF-inhibited NTE it is reasonable to use only the substrate-hydrolysis method to screen for the occurrence of the allegedly necessary chemical reaction

after inhibition of NTE by a variety of neurotoxic organophosphorus esters that are not available in radiolabelled form. Our preliminary survey (B. Clothier, unpublished work) has shown that rapid aging occurs in many cases.

It could be proposed that rather than the residual charged group it is the isopropoxy (or isopropyl) group attached to another site on the protein during aging that is the cause of the toxic effect. However, neurotoxic OP esters are known that contain groups as chemically diverse as isopropoxy-, isopropylamino-, 2-chloroethoxy-, phenoxy- and anilino-. It seems unlikely that all such groups could be transferred efficiently to a second site during aging to cause a common effect. The simpler hypothesis remains that the effect arises from the charged group that would remain at the catalytic site in each case after inhibition and aging, regardless of the nature of or the destination of the leaving group.

The transfer of half the bound ^3H to another acetone-precipitable site during aging of NTE contrasted with the simple loss of bound radioactivity found during aging of the hen brain cholinesterases (Table 4). Berends *et al.* (1959) reported that labelled propan-2-ol was found in the supernatants attained after precipitation of aged [^{14}C]DiPF-inhibited cholinesterase by trichloroacetic acid followed by centrifugation and Michel *et al.* (1967), with [^{3}H]-pinacolyl-labelled soman and eel AChE, showed that the tritiated group was released into solution by an S_N1-type mechanism during aging. Since none of the radioactivity from NTE was found in solution or was trapped by added nucleophiles such as reduced glutathione or imidazole, it is probable that the transfer is intramolecular and does not involve liberation of the group in free form into its surroundings. The insignificant effect of pH on rate of aging is a further contrast with known effects for inhibited AChE (Michel *et al.*, 1967). These two differences suggest that the mechanism of aging differs in the two cases.

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