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DEPHOSPHORYLATION *IN VIVO* OF BRAIN ACETYLCHOLINESTERASE INHIBITED BY ISOPROPYL METHYLPHOSPHONOFLUORIDATE (SARIN)

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Abstract—Intravenous injection of ^{32}P-isopropyl methylphosphonofluoridate (Sarin) into rats resulted in inhibition of brain acetylcholinesterase (AChE) activity and phosphonylation of the enzyme. Spontaneous recovery from inhibition occurred between 0·5 and 48 hr in the Sarin-intoxicated animals in correlation with comparable dephosphorylation of Sarin-derived phosphorus bound to the enzyme. Injection of 44 mg/kg of monoisonitrosoacetone (MINA) into the Sarin-intoxicated rats resulted in significant reactivation of inhibited brain AChE over and above that occurring spontaneously, accompanied by additional dephosphorylation of enzymatically bound phosphorus. Loss of phosphorus *in vivo* took place exclusively as ^{32}P-isopropyl methylphosphonic acid (^{32}P-IMPA). No significant loss of Sarin-derived ^{32}P bound to AChE as ^{32}P-methylphosphonate was apparent under these conditions.

INHIBITION of acetylcholinesterase (AChE) by organophosphorus compounds *in vitro* has been shown to take place through phosphorylation of the enzyme.[1, 2] Earlier studies of the kinetics of spontaneous recovery of the enzyme when inhibited by certain organophosphorus compounds *in vitro* are consistent with a dephosphorylation process.[2] The observation that reactivation of phosphonylated AChE by nucleophiles *in vitro* was accompanied by dephosphorylation gave further support to this interpretation.[3] However, attempts to demonstrate dephosphorylation accompanying enzyme recovery *in vivo* were inconclusive,[4] since no distinction was made between phosphorus specifically bound to AChE and that bound nonspecifically to other proteins[5] and many tissues.[6]

More recently, Harris *et al.*[7] studied the development of resistance to reactivation by oximes of rat brain AChE inhibited *in vivo* with ^{32}P-isopropyl methylphosphonofluoridate (Sarin). For each time interval, that portion of the enzyme which could be reactivated *in vitro* by 2-pyridinium aldoxime methochloride (2-PAMCl) approximated the percentage of phosphorus released as isopropyl methylphosphonic acid (^{32}P-IMPA). The amount of phosphorus retained by the enzyme after incubation with 2-PAMCl was present entirely as methylphosphonate (^{32}P-MPA) and paralleled the percentage of enzyme not reactivated ("aged" enzyme). The close correlation between the enzymatic and radioactivity measurements suggested that the method distinguished phosphorus bound to AChE from that bound to other sites. A new effort to study the relationship between dephosphorylation and the recovery of organophos-

phate-inhibited enzyme, whether occurring spontaneously or after injection of an oxime,[8] therefore appeared worthwhile.

MATERIALS AND METHODS

^{32}P-labeled Sarin was supplied by the Defense Research Board of Canada; its specific activity upon arrival was 10 mc/m-mole. The radioactive Sarin was purified according to the method previously described for ^{32}P-pinacolyl methylphosphonofluoridate (Soman).[9] The purified stock solution of labeled agent in benzene approximated 95 per cent on the basis of the radioactivity remaining in the benzene phase when repartitioned with 0·1 M Tris buffer at pH 7·4 and by its anticholinesterase potency compared to Sarin of known purity. It was stored over anhydrous sodium sulfate in the deep freeze.

Isobutyl alcohol–chloroform solvent was prepared by mixing equal volumes of the alcohol and chloroform. The solvent was saturated with acidified Tris solution prior to use. Acidified Tris solution was prepared by mixing 9 vol. of 0·01 M Tris buffer plus 1 vol. of 50% trichloroacetic acid (TCA) and sufficient 10 N HCl to yield a pH approximating 0·5.

^{32}P-labeled IMPA and MPA were then synthesized as reference compounds by hydrolysis of the purified labeled Sarin in excess NaOH and 12 N HCl respectively. The labeled phosphonic acids were characterized by their distribution between an equal volume of isobutyl alcohol chloroform (1:1) with acidified Tris solution. The mean values for the distribution coefficients between solvent and aqueous phase were determined from six trials with each acid; the distribution coefficient (D_A) for IMPA was 2·05 $\pm$ 0·10 and for MPA it was (D_B) 0·062 $\pm$ 0·004 ($P = 0{\cdot}05$). By use of the previously derived formula,[6] the mole fraction of ^{32}P-IMPA (F_A) in a mixture of ^{32}P-MPA and ^{32}P-IMPA can be calculated from:

$$F_A = \left(\frac{D_A + 1}{D_A - D_B}\right)\left(\frac{R - D_B}{R + 1}\right) \tag{1}$$

where R is the ratio of total radioactive counts in the organic phase to those in the aqueous phase. Substituting the values of D_A and D_B into equation 1 then:

$$F_A = [1{\cdot}53\ (R - 0{\cdot}062)]\ (R + 1). \tag{2}$$

INVESTIGATIONAL PROCEDURES AND RESULTS

Female rats were injected with 16 mg/kg of atropine intramuscularly 5 min prior to intravenous (i.v.) injection of 40 μg/kg of ^{32}P-Sarin (approximately 0·8 LD_{50}). At 0·5 hr after poisoning, twenty-four animals were sacrificed. The remaining rats were divided into two groups. Group I (twenty-four rats) received 44 mg/kg of monoisonitrosoacetone (MINA) i.v., since Rutland[8] had shown that treatment with this oxime reactivated Sarin-inhibited brain AChE *in vivo*. Group II (twenty-four rats) was not treated with oxime. Both groups were permitted to survive 48 hr and were then sacrificed. Unpoisoned controls were sacrificed at each time interval and processed along with experimental rats for normal brain AChE activity. In each group of animals the brains were excised, rinsed in cold 0·9% NaCl, blotted, weighed, pooled in pairs, and 10% homogenates immediately prepared in ice-cold 0·01 M borate-buffered 0·9% NaCl at pH 8·8. The homogenates were divided into two equal volumes of 8 ml

each and centrifuged at 105,000 *g* for 25 min at 2°. The supernatants were discarded. The residues were washed twice with the cold borate saline solution and centrifuged, the supernatant being discarded as before. The residue from one of the fractions of the original homogenate was suspended in 2 ml of 10^{-1} M 2-PAMCl in 0·1 M phosphate buffer, pH 7·6, while the other (control) fraction was suspended in the same volume of phosphate buffer alone. Each was incubated, with gentle agitation, for 90 min at 25°, a period sufficient for 2-PAMCl to release completely the inhibitor-derived phosphorus from unaged phosphonylated AChE.[7] The suspensions were centrifuged as above and the supernatants retained for measurement and characterization of the released radioactivity. Each residue was washed twice with successive 1-ml portions of cold 0·9% NaCl and centrifuged; the washings were pooled with the supernatant. The residues were retained in ice for further study. The supernatants were treated with sufficient 50% TCA to give a final concentration of 5%, and with 1–2 drops of concentrated HCl to an approximate pH of 0·5. The samples were centrifuged at 2000 rpm for 10 min. The supernatant was removed and shaken with an equal volume of a mixture of isobutyl alcohol and chloroform (1:1) to characterize the ^{32}P displaced as IMPA and to establish that no ^{32}P-MPA had been released by this treatment. The phases were then separated, clarified by centrifugation, and a portion was transferred to planchets, dried and counted with a Geiger–Mueller end window counter. The radioactivity due to the ^{32}P-IMPA liberated into the supernatant by 2-PAMCl incubation was calculated (after correcting for any radioactivity in the supernatant of the phosphate controls) by the formula for the mole fraction (F_A) of ^{32}P-IMPA given above.

The residues that had been retained were now washed twice more with 10 ml of ice-cold saline and centrifuged at 105,000 *g* to remove traces of 2-PAMCl, which could interfere with the assay of cholinesterase activity because of its own anti-cholinesterase properties.[10] The washed residues were now made to a 2% suspension with cold 0·3 M NaCl. The AChE activity was estimated colorimetrically by incubating 1 ml of the brain suspension for 30 min at 25° with 1 ml of 0·006 M acetyl-β-methylcholine (Mecholyl) in 0·3 M NaCl buffered to pH 7·6 with 0·05 M phosphate buffer. The remainder of the assay has been described.[11] The percentage of cholinesterase (ChE) activity reactivated was calculated from the formula suggested by Hobbiger:[12]

$$\text{Percentage of ChE reactivated} = 100 \times \frac{(\text{AChE})_T - (\text{AChE})_I}{(\text{AChE})_C - (\text{AChE})_I} \qquad (3)$$

where $(\text{AChE})_T$ is the enzyme activity of oxime-treated phosphonylated tissue, $(\text{AChE})_I$ is the enzyme activity of Sarin-inhibited tissue, and $(\text{AChE})_C$ is the enzyme activity of oxime-treated tissue prepared from unpoisoned controls.

The remainder of 2% homogenates from the 2-PAMCl treatment were centrifuged at 105,000 *g*, the supernatants were discarded, and the residues were dispersed in 2 ml of 0·1 N NaOH, then incubated at 100° for 2 hr to cleave any phosphonate bound to the brain tissue. The pH of the digest was adjusted to approximately 1·0 with 10 N HCl and TCA was added to 5% concentration. The mixture was centrifuged at 2000 rpm and the supernatant was collected. The residue was washed twice with 5% TCA adjusted to pH 0·5 with HCl and then centrifuged. The washings were added to the supernatant already collected. The solution was then shaken with a mixture of

isobutyl alcohol and chloroform (1:1); the phases were separated and centrifuged; samples were put on planchets and the radioactivity was counted as before. If we now assume that any ^{32}P-IMPA found in the residue was not bound to ChE (the evidence for this has been discussed),[7] then the ^{32}P-MPA counts are obtained from: $M = Z - I$, where M is the number of ^{32}P-MPA counts in the residue, Z is the total counts in the residue, and I is the number of ^{32}P-IMPA counts, which is also equal to $F_A Z$. Substitution for the value of F_A gives

$$M = Z[1 - 1{\cdot}53\,(R - 0{\cdot}062)/(R + 1)]. \quad (4)$$

An analysis of variance of the data obtained and the results are summarized in Table 1.

TABLE 1. CHANGES IN ACHE ACTIVITY AND ^{32}P-IMPA AND MPA IN BRAIN TISSUE OF RATS POISONED WITH ^{32}P-SARIN AND TREATED WITH MINA

Conditions studied	Time to sacrifice after poisoning with ^{32}P-Sarin (hr)	Inhibition of AChE activity ± P = 0·95 (%)	^{32}P bound (μg/g tissue wet wt. × 10^3 ± P = 0·95)		
			^{32}P-IMPA*	^{32}P-MPA$_R$†	^{32}P-IMPA$_{RNS}$†
Sarin only	0·5	67·35 (60·47 – 74·24)	5·50 (4·50 – 6·50)	0·32 (0·26 – 0·38)	1·66 (1·42 – 1·88)
Sarin only	48	48·90 (44·29 – 53·51)	0·29 (0·24 – 0·34)	3·76 (3·31 – 4·21)	1·12 (0·76 – 1·48)
Sarin followed by MINA	48	24·46 (20·70 – 28·22)	0·27 (0·21 – 0·33)	2·24 (2·03 – 2·45)	1·23 (0·81 – 1·65)

* ^{32}P-IMPA$_D$ designates the radioactivity released by 2-PAMCl *in vitro* and partitioning as IMPA.
† ^{32}P-MPA$_R$ and ^{32}P-IMPA$_{RNS}$ designate the ^{32}P remaining in the brain residues after incubation with 2-PAMCl and partitioning as MPA and IMPA respectively.

Table 1 shows a significant decrease of inhibition between 0·5 and 48 hr after poisoning with Sarin. Treatment of rats with MINA at 0·5 hr after giving Sarin led to a further lowering of enzyme inhibition. Table 1 also shows that a loss of Sarin-derived ^{32}P occurred in the absence of oxime treatment between 0·5 and 48 hr, and that additional dephosphorylation took place in the poisoned animals treated with MINA. These findings are evaluated further in the discussion below.

DISCUSSION

Reactivation of the Sarin-inhibited brain AChE with 2-PAMCl has been used to specifically displace the inhibitor from the enzyme.[7, 13, 14] In our study the Sarin-derived phosphorus released by the oxime 0·5 hr after poisoning was almost exclusively IMPA (Table 1). IMPA remaining in the brain tissue residue after incubation with 2-PAMCl is therefore taken as nonspecifically bound to sites other than AChE and is not considered in the calculation for dephosphorylation. It was found previously that the percentage of Sarin-phosphonylated enzyme not reactivated and the percentage of the phosphorus bound to AChE as ^{32}P-MPA are closely correlated,[7] permitting the inclusion of the latter in the total of ^{32}P bound to AChE.

Using the figures obtained at 0·5 hr (Table 1) and equation 5,* we find that 94·5 per cent of the phosphorus initially bound to the enzyme was IMPA.

$$100 \times \frac{^{32}\text{P-IMPA}_D}{^{32}\text{P-IMPA}_D + ^{32}\text{P-MPA}_R}. \quad (5)$$

It has been shown that 91·6 ± 13·0 per cent (P = 0·95) of the inhibited enzyme can be reactivated at 0·5 hr.[7] After 48 hr, only $0{\cdot}29 \times 10^{-3}$ μg IMPA per g of tissue was released by incubation with 2-PAMCl. This is about 5 per cent of the amount displaced at 0·5 hr. The data are consistent with our finding that little or no reactivation of AChE activity was obtained after the addition of 2-PAMCl to the brain tissues of Sarin-poisoned animals sacrificed after 48 hr.

The amount of phosphorus that spontaneously separated from the enzyme is the difference between the total amounts bound to AChE at the two time intervals studied, i.e. the difference between the sums of ^{32}P-IMPA$_D$ + ^{32}P-MPA$_R$ at 0·5 and 48 hr given in Table 1. The percentage of phosphorus spontaneously dephosphorylated = 100 × (5·82 − 4·05)/5·82 or 30·5(21·9 − 39·0) (P = 0·95).

The spontaneous decrease in inhibition of AChE activity during the same time can be calculated as follows:

$$\% \text{ decrease} = 100 \left[\frac{\% \text{ inhibition}_{0\cdot5 \text{ hr}} - \% \text{ inhibition}_{48 \text{ hr}}}{\% \text{ inhibition}_{0\cdot5 \text{ hr}}}\right]. \quad (6)$$

The value obtained is 27·5 per cent (20·5 − 34·3) (P = 0·95), which approximates the percentage of phosphorus spontaneously dephosphorylated.

In rats treated with MINA, the percentage of the specifically bound phosphorus that was dephosphorylated at 48 hr was 100 × (5·82 − 2·51)/5·82 or 56·9 (47·8 − 61·7) (P = 0·95).

The percentage decrease in inhibition of AChE activity in the rats treated with MINA was 63·7 (57·9 − 69·2) (P = 0·95). This value and the one for dephosphorylation are in substantial agreement (the 95% confidence limits overlap).

From the results obtained in our previous study,[7] 48 hr should have been sufficient for nearly complete dealkylation of the IMPA–AChE to MPA–AChE. However, the amount of ^{32}P-MPA only increased from $0{\cdot}32 \times 10^{-3}$ to $3{\cdot}76 \times 10^{-3}$ μg/g of tissue (wet weight) between 0·5 and 48 hr. This is significantly less than the decrease in ^{32}P-IMPA$_D$ ($5{\cdot}21$ μg/g × 10^{-3}) during this time (Table 1). Spontaneous release of phosphorus bound to AChE as MPA appears to be excluded by the almost 12-fold increase of this component during the time interval examined, and by the marked resistance reported for nucleophilic displacement of methyl phosphonylated AChE.[6, 7] From this, and the close correlation between release of IMPA by 2-PAMCl and reactivation of AChE *in vitro*, it is deduced that the phosphorus lost in spontaneous dephosphorylation *in vivo* derives from that bound to AChE exclusively in the alkylated form, i.e. as IMPA. This interpretation is also in harmony with our earlier finding that the rapidly dealkylating pinacolyl methylphosphonylated AChE resulting from inhibition of AChE with Soman ($T_{1/2}$ for dealkylation = 2·4 min)[6] shows little or no spontaneous recovery of enzyme inhibition for 2 days after injection of this

* ^{32}P-IMPA designates the ^{32}P released by 2-PAMCl *in vitro* and partitioning as IMPA. ^{32}P-MPA$_R$ designates the ^{32}P remaining in the brain residues after incubation with 2-PAMCl and partitioning as MPA.

organophosphate.* Sarin-derived IMPA not released by 2-PAMCl and bound to tissue components other than AChE (designated ^{32}P-IMPA$_{RNS}$ in Table 1) showed no significant decrement between 0·5 and 48 hr after poisoning, nor did it appear susceptible to displacement by MINA.

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* J. H. Fleisher, G. Bursel and L. W. Harris, unpublished observation.

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