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THE TOXIC ACTION OF SOME PHOSPHORUS ANTICHOLINESTERASES WITH CATIONIC GROUPS

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Abstract—The intravenous LD_{50} in rats and I_{50} to sheep AChE of several compounds of the type $(RO)_2PO{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}Z$ have been determined. Z represents SR, $\overset{\oplus}{S}RR'$ or, in one case, $\overset{\oplus}{N}R_3$, where the R's are alkyl groups. Special precautions had to be taken to get reliable values. The LD_{50} and I_{50} of the cationic compounds are very much lower than those of their uncharged analogues. Two of the sulphonium compounds are the most toxic phosphorus anti-AChE's reported. The results are discussed in terms of the mechanism of inhibition at the enzyme surface.

SEVERAL organophosphorus compounds have been described in which exceptionally high toxicity and anticholinesterase activity are associated with the presence of side-chain quaternary nitrogen groups. Some P:P-(fluoro)(methyl)phosphinylcholines[1] and phosphostigmines[2] are familiar examples. In this paper it is shown that some compounds with sulphonium groups in similar positions are even more toxic, estimated on a mole/kg basis, when given intravenously. They are compared with the closely related uncharged compounds from which they are derived, and with one quaternary nitrogen compound. The ten compounds studied are listed in Table 1, and can be represented by the general formula: $(RO)_2PO.S.CH_2.CH_2.Z$ where R is a methyl or ethyl group, and Z is a thioalkyl, dialkylsulphonium or trialkylammonium group.

The chemical properties of some of the compounds have been described.[3, 4] They are such that it is hard to determine accurate LD_{50}'s and I_{50}'s. The relevant features are therefore summarized here.

The thioalkyl compounds (nos. 1, 4 and 8 in Table 1) give sulphonium compounds slowly on storage and more rapidly in water.[3, 4] The sulphonium compounds are very much more toxic and active, and must be removed from the thioalkyl compounds immediately before the latter are used.

The structure of the sulphonium compounds follows from the method used to prepare them, and their behaviour during electrophoresis. In two cases, compound nos. 2 and 3, the structures have been confirmed by detailed analysis of the kinetics of reactions in which they are formed.[4] They can, however, only be got as dilute solutions. All attempts to separate them from solvent (usually water) resulted in their decomposition. Their concentrations had, therefore, to be estimated indirectly. Two methods were used. Relative concentrations of a given compound were calculated from the

capacities of different solutions to inhibit AChE. Thus if a solution must be diluted n-fold to produce 50 per cent inhibition of the enzyme under standard conditions, the concentration must be $n \times I_{50}$, whatever the I_{50} may be. Such relative concentrations are all that are needed for many kinetic studies. The only practicable way to determine absolute concentrations was by estimating the phosphorus contents of solutions. This

TABLE 1. THE COMPOUNDS ARE OF THE TYPE: $(RO)_2PO{\cdot}S{\cdot}(CH_2)_n{\cdot}Z$

No.	Compounds R	n	Z	LD_{50} (μg/kg)	I_{50} (M)	LD_{50}/I_{50}†
1	Me	2	SEt*	54,000–72,000	$6{\cdot}5 \times 10^{-5}$	4·3
2	Me	2	$\overset{+}{S}EtMe$*	52–72	$3{\cdot}9 \times 10^{-8}$	6·5
3	Me	2	$\overset{+}{S}Et{\cdot}CH_2{\cdot}CH_2{\cdot}SEt$	4–5·2	$2{\cdot}1 \times 10^{-9}$	6·8
4	Et	2	SEt	1,700–2,380	$3{\cdot}5 \times 10^{-6}$	2·2
5	Et	2	$\overset{+}{S}EtMe$*	14–18	$4{\cdot}7 \times 10^{-9}$	13
6	Et	2	$\overset{+}{S}Et_2$*	10‡	$2{\cdot}6 \times 10^{-9}$	13
7	Et	2	$\overset{+}{S}Et{\cdot}CH_2{\cdot}CH_2{\cdot}SEt$	4·7–5·8	5×10^{-10}	28
8	Et	3	SEt	12,700–28,100	$1{\cdot}8 \times 10^{-5}$	3·9
9	Et	3	$\overset{+}{S}EtMe$	290	$1{\cdot}7 \times 10^{-7}$	5·9
10	Et	2	$\overset{+}{N}Et_3$	15–21	$4{\cdot}8 \times 10^{-9}$	13

* Values from Heath and Vandekar[3].

† To calculate this ratio LD_{50}'s were expressed in moles/kg.

‡ Determined on eight rats only.

method is only valid when the only phosphorus compound present is the anticholinesterase under investigation. For a specimen of compound no. 2 this assumption has been proved correct.[3] Compound no. 2 was subjected to electrophoresis, and the profile of the peak found both from the anti-AChE activities of the different fractions (the first method) and from their phosphorus contents (the second method). The two profiles were in excellent agreement. Compound no. 2 has a half-life in water of 14·3 days at 37 °C, so one can assume that any other sulphonium compound of similar stability will be equally pure. Compound no. 3, however, is much less stable (half-life 100 min at 37 °C), and must decompose to some extent during separation and storage. The methods used to estimate the purity of this and the other unstable compound (no. 7) are described in the next section.

COMPOUNDS AND METHODS

I_{50}'s *and* LD_{50}'s

The preparation and purification of specimens suitable for the determination of I_{50}'s and LD_{50}'s are described under "Compounds".

I_{50}'s were determined by Aldridge's method.[5] Known dilutions of the anti-AChE's were incubated with washed sheep red cells for 30 min at 37 °C, and the AChE remaining determined in a Warburg apparatus using acetylcholine (ACh) as substrate. The percentage inhibition increased with the concentration of inhibitor. For the dimethyl phosphate esters (1–3) the logarithm of the fraction of AChE remaining was not related linearly to the concentration. This is always the case with such esters, as the dimethylphosphorylated enzyme produced is reactivated fairly rapidly.[3, 6] The I_{50} concentration, i.e. the concentration inhibiting half the enzyme, was calculated as described by Heath and Vandekar.[3] The remaining esters gave a straight line graph as expected. I_{50}'s were reproducible within 5 per cent.

LD_{50}'s were determined on white rats (Porton Wistar Strain) of 150–200 g wt., given the compounds by the tail vein. Compound no. 1 was given neat, and compound nos. 4 and 8 in ethanol. The dose of ethanol was always less than 300 mg/kg. The rest were given in water. Survivors were observed for 24 hr, i.e. about 18 hr after the last death. LD_{50}'s and fiducial limits were calculated from the Tables given by Weil.[7]

Compounds

The thioalkyl compounds and the quaternary nitrogen compounds were received as gifts. The purity of the specimens of compound nos. 1 and 4 had been established.[3] Compound no. 8 was assumed to be equally pure. The quaternary nitrogen compound was crystalline, and its purity was not investigated.

Preparation of sulphonium compounds

The sulphonium compounds were prepared in low yields (1–10 per cent) by the reaction of the thioalkyl compounds with methyl or ethyl iodide or 2-bromoethylthioethane in ethanol at 37 °C for several hours:

$$(RO)_2PO{\cdot}S{\cdot}(CH_2)_n{\cdot}SEt + R'X \rightarrow (RO)_2PO{\cdot}S{\cdot}(CH_2)_n{\cdot}\overset{+}{S}EtR' + \overset{\ominus}{X}$$

where R'X is the alkyl halide. This is a standard method for the preparation of sulphonium compounds. The products were separated by paper electrophoresis.[3] Each compound moved in the electric field as expected of a cation. The compounds were washed from the paper, and stored at −30°C. Their concentrations were estimated from the phosphorus content of the solutions determined as described by Heath and Vandekar.[3]

Purity of sulphonium compounds

The sulphonium compounds were contaminated with sodium acetate buffer, pH 5·3, and, of course, water. Buffer contaminants should not affect I_{50}'s or LD_{50}'s in the concentrations present. The purity of the compounds therefore refers to the relationship between the concentrations actually present, and those calculated from the phosphorus contents of the solutions. As the compounds were separated by electrophoresis, the only possible phosphorus impurities were other sulphonium compounds, and products formed during electrophoresis on that part of the strip extracted and during storage. The kinetics of decomposition can be used to show that only one sulphonium compound is present. Thus compound no. 2 decomposed in 10^{-4} N HCl according to first-order kinetics as far as the reaction was followed (about 90

per cent decomposition),[3] and the same was found for compound no. 7 in this investigation. The presence of sulphonium compounds of different stability would cause deviations from first-order kinetics. A similar test showed that compound no. 3 contained a little compound no. 2.[4] This is in accordance with the chemical properties of compound no. 1, from which compound no. 3 was prepared. Compound no. 1 forms compound no. 2 particularly readily by the reaction:

$$2(MeO)_2PO{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}SEt \rightarrow (MeO)_2PO{\cdot}S{\cdot}CH_2{\cdot}CH_2\overset{+}{S}EtMe + MeO{\cdot}\overset{\ominus}{P}O_2{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}SEt$$

Diethyl phosphate analogues undergo this reaction much more slowly.[3] It is therefore reasonable to assume that only solutions of compound no. 3 contained significant amounts of other sulphonium compounds.

Inactive phosphorus compounds were not present in compound no. 2 immediately after separation (see Introduction). Compound nos. 5, 6 and 9 were about as stable, and were therefore assumed to be pure also. Compound nos. 3 and 7 ($Z = \overset{+}{S}Et.CH_2.CH_2.SEt$) have half-lives in water of 100 and 85 min at 37 °C, respectively. (There are some incomplete experimental data and some theoretical reasons for believing that these rapid reactions are not hydrolyses but dissociation reactions:

$$-\overset{+}{S}Et{\cdot}CH_2{\cdot}CH_2{\cdot}SEt + \overset{\ominus}{X} \rightarrow -SEt + \overset{\ominus}{X}{\cdot}CH_2{\cdot}CH_2{\cdot}SEt)$$

Electrophoretic separation took several hours, so they could not have been pure. For compound no. 3 a previous investigation gives an estimate of purity.[4] From the phosphorus content and anti-AChE activity of a fresh preparation the I_{50} was calculated to be $6{\cdot}9 \times 10^{-9}$ M, assuming 100 per cent purity. Now in water compound no. 1 decomposes in two ways: according to first-order kinetics to give inert compounds, and according to second-order kinetics to give the sulphonium compound nos. 2 and 3.

The rate equation is thus:

$$dA/dt = -k_1A - k_2A^2$$

denoting the concentration of compound no. 1 by A. The k_2A^2 term can be determined from the rates at different concentrations, and hence the sum of the concentrations of compound nos. 2 and 3 calculated. The anti-AChE activities in the reaction mixtures due to compounds nos. 2 and 3 can be obtained separately, by taking advantage of the difference between their stabilities. The concentration of compound no. 2 can thus be calculated from its I_{50} and the activity present, and hence the concentration of compound no. 3 can be found by difference. This, with the anti-AChE activity of the solution gives the I_{50}. It was found to be $1{\cdot}4 \times 10^{-9}$ M. The value is not precise, as reactions involving compound no. 3 only contribute 5 per cent to the total rate constant. But this and another check show that the I_{50} is very unlikely to exceed about 2×10^{-9} M. The direct value was $6{\cdot}9 \times 10^{-9}$ M, so the purity was 30 per cent or less, 30 per cent has been assumed in this paper. No corresponding detailed analysis has been carried out for compound no. 7. During electrophoresis, however, about 75 per cent was decomposed, i.e. only 25 per cent of the anti-AChE put on the paper was recovered from it. As the paper was cut before extracting the specimen half-way

between the peak and the starting line (the rest of the paper was inactive), only inert phosphorus compounds formed in the second half of the separation should be recorded. This gives a purity of about 50 per cent, with a maximum of about 60 per cent, which has been assumed correct.

Decomposition during storage did not affect the results—for example, the anti-AChE activity of a solution of the least stable compound, compound no. 7, was determined twice, with 6 days storage at −30°C between determinations. The I_{50}'s agreed within 2·5 per cent.

Purification of thioalkyl compounds

Purification of the thioalkyl compounds immediately before use was necessary, as they contained sulphonium compounds. The formation of sulphonium compounds during purification had also to be avoided. The compounds were dissolved in chloroform or benzene, and the sulphonium compounds extracted with water. The non-aqueous solutions were then concentrated *in vacuo*. This also dried the compounds, as these solvents form azeotopes with water. To prepare specimens for LD_{50} determinations evaporation was carried out at less than 1 mm pressure at room temperature until the volume remained apparently constant. The products contained about 5 per cent of solvent, which could not be removed except by raising the temperature or greatly prolonging the evaporation, either of which led to the formation of sulphonium compounds. The compounds in the specimens were therefore estimated from determinations of phosphorus, and used within an hour, or stored at −30°C. The proportion of solvent was too small to affect LD_{50}'s. To prepare specimens for I_{50} determinations only small amounts were dissolved in the non-aqueous solvent, and the solvent was evaporated under about 15 mm pressure until the volume was reduced to 2–5 ml. Sufficient water was then added to give a solution of about 10^{-4} M, and the rest of the non-aqueous solvent evaporated. The compounds were thus never at high concentrations in aqueous solution, so avoiding the formation of sulphonium compounds, which are formed at rates proportional to the square of the concentrations of the thioalkyl compounds. The concentrations were estimated by phosphorus.

RESULTS AND DISCUSSION

The I_{50}'s and LD_{50}'s are listed in Table 1. Those for compound nos. 3 and 7 are calculated assuming 30 and 60 per cent purity, respectively. The remaining compounds were assumed to be completely pure.

The higher the purity assumed the lower the biochemical activity and toxicity recorded, with the proviso discussed in the next paragraph. For all except compound nos. 3 and 7 there is every reason to suppose that the compounds were substantially pure, and that the main errors in the values are those in the determinations of I_{50}'s and LD_{50}'s: about ±5 per cent for the I_{50}'s and those given for the LD_{50}'s. The purities of compound nos. 3 and 7 are the greatest consistent with the results. It is possible that the values are too great by a factor of 2. The ratio of LD_{50}/I_{50} is not affected by these errors, as the LD_{50} and I_{50} for each was determined on the same solution assuming the same purity.

In finding the LD_{50} or I_{50} of a sulphonium compound what was actually found was the activity of an aqueous solution containing a known concentration of combined

phosphorus. If the activity could be attributed to compounds which did not contain phosphorus, then the results would be spurious. Three types of evidence showed that the results should be attributed to phosphorus anti-AChE's.

(1) Compound nos. 1, 2 and 5 have been studied in detail.[8] The inhibited AChE's had the same stabilities as those inhibited by other dimethyl and diethyl phosphorylated-AChE's, and the intensity and duration of symptoms was related to the degree of AChE inhibition *in vivo* and the rates of re-activation of the inhibited enzymes.

(2) All the compounds induced typical cholinergic symptoms in rats. The cationic compounds induced signs faster and of shorter duration than the uncharged compounds, but there were no other differences.

(3) The LD_{50}/I_{50} ratios for all the compounds are similar, suggesting that all act in the same way.

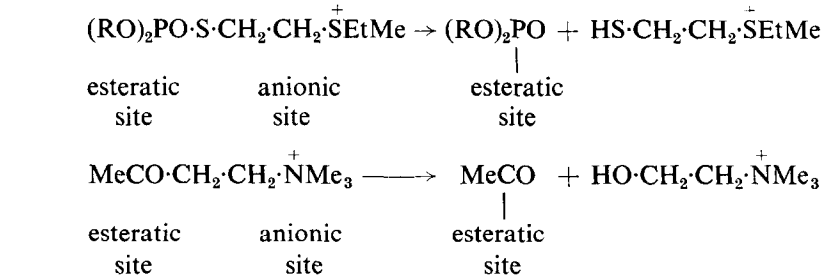
The sulphonium compounds were very much more toxic and biochemically active than the thioalkyl compounds from which they were derived. The high activity of the sulphonium compounds can be explained in the same way as the high activity of the phosphostigmines already referred to.[2] The cationic head is adsorbed on the anionic site of AChE, and greatly increases the probability that the esteratic site will be phosphorylated.

A check on this theory is available. If the rate of reaction with AChE is increased on forming a methylsulphonium derivative by a factor, f, and this factor arises solely from the stronger adsorption on the enzyme of the sulphonium compound, then the free energy of adsorption is given by $\Delta F = -RT \ln f$.[9] For the pair nos. 1 and 2, the factor is 1665, and for pair nos. 4 and 5 is 745. This gives $\Delta F = -4{\cdot}6$ kcal/mole for the first pair and $-4{\cdot}1$ kcal/mole for the second. The additional methyl group in each sulphonium compound probably contributes about $-1{\cdot}2$ kcal to these energies,[10] so the calculated energy of attraction of the positive charge on the sulphur for the anionic site is $-3{\cdot}5$ and $-2{\cdot}9$ kcal, respectively. A sulphonium ion has a pyramidal structure, and should therefore be able to approach the anionic site more closely than a quaternary nitrogen atom in, for example, acetylcholine. The energies calculated are consistent with this—in ACh the $\overset{+}{N}$–anionic site energy is rather less, about 2·1 kcal.[9, 11]

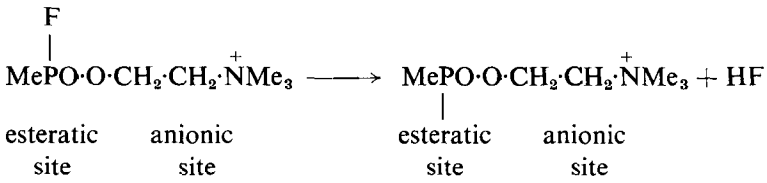
Consider now the pair nos. 8 and 9, in which the sulphur atoms are separated by a chain of three carbon atoms. For this pair $\Delta F = -2{\cdot}9$ kcal, a value not much less than for those compounds with two carbon atoms only in the chain, i.e. the increase in rate of reaction brought about by converting a compound to its methylsulphonium derivative does not vary much with chain length. The lengths of the chains measured from the central atoms in the ester group to the cationic group are 4·65 Å in ACh (carbonyl carbon to $\overset{+}{N}$), 5·75 Å in the dimethylene compounds and 7·0 Å in the trimethylene compounds. (P to $\overset{+}{S}$). The chain lengths were calculated from the bond-lengths tabulated by the Chemical Society.[12] It is obvious that the phosphorus compounds can only fit the enzyme by folding. Probably the greater flexibility of a trimethylene chain largely compensates for its greater length. This conclusion is independent of the observation that the trimethylene compounds are each less active than their dimethylene analogues, i.e. that compound no. 8 is less active than compound no. 4, and compound no. 9 than compound no. 5. Perhaps the ester bonding is more stable in the trimethylene compounds.

The only compounds of similar toxicity to some of the sulphonium compounds are some of the P:P-(fluoro)(methyl)phosphinylcholines.[1] The two groups are not as similar

as appears at first sight. Two of the sulphonium compounds have been shown to react with AChE to give dimethyl- and diethyl- phosphorylated AChE, respectively.[8] The first stage of the reaction has a marked resemblance to the first stage in the hydrolysis of ACh by AChE:



In both cases the group removed is the group adsorbed on the anionic site of the enzyme. The (fluoro)(methyl)phosphinylcholines appear to react differently:



Thus the group displaced by AChE is generally the group displaced by hydroxyl ions during alkaline hydrolysis, in these compounds the fluorine atom.[13] Also human AChE inhibited by such compounds is not re-activated *in vitro* by 2-hydroxyiminol methylpyridinium methiodide (P2-AM).[14] This result is only readily explained if the anionic site, on which P2-AM is adsorbed during re-activation[15] is shielded by a strongly adsorbed group. These considerations suggest that the adsorption of the quaternary nitrogen group on the anionic site of AChE may not contribute much to the anti-AChE activity of the (fluoro)(methyl)phosphinylcholines. It can only do so if the reaction at the esteratic site does not depend on the steric disposition of the groups. Otherwise if in ACh the adsorbed choline group is in a particularly favourable position for reaction to take place, in the phosphinylcholines the fluorine atom must be in an unfavourable position. There is some confirmation of this view. (Fluoro)-(methyl)phosphinylhomocholine is more biochemically active and toxic than its choline homologue.[1] In addition for many compounds rates of reaction with AChE are roughly correlated with rates of hydrolysis by water or by hydroxyl ions.[16] The phosphinylcholines are much less stable in water than several of the sulphonium compounds,[1, 17] so that the high activity of fluorophosphinyl compounds may be due to the readiness with which their P—F bonds are broken.

Five of the sulphonium compounds (nos. 3, 5, 6, 7 and 10) are more toxic than any non-ionic phosphorus compounds on which there are reports. The only other phosphorus compounds of comparable toxicity are also cationic. Thus the most toxic compounds are all ionic, and do not penetrate the central nervous system.[1, 2, 8] It is

obvious that action on the central nervous system is not necessary for death to ensue from cholinergic effects.

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