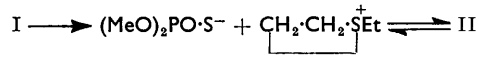
327. *Reactions of Ethylthioethyl* OO-*Dimethyl Phosphorothioates in Water.*

By D. F. Heath.

A kinetic study shows that *O*-ethylthioethyl *OO*-dimethyl phosphorothionate (I) and *S*-ethylthioethyl *OO*-dimethyl phosphorothiolate (II) decompose in water into phosphorothioate ion and, probably, a cyclic sulphonium ion:



The thiolate (II) reacts with the sulphonium intermediate to a small extent to give an ion, probably $(MeO)_2PO\cdot S\cdot CH_2\cdot CH_2\cdot \overset{+}{S}Et\cdot CH_2\cdot CH_2\cdot SEt$, which is a powerful, unstable anticholinesterase. Similar reactions take place in the absence of water. The thiolate (II) also decomposes in water by some other mechanism. It is concluded that this is direct attack by water from a comparison of its rate with the rates of hydrolysis of the sulphoxide and sulphone of (II), which cannot give cyclic sulphonium intermediates.

Some reactions in water of *S*-2-ethylthioethyl *OO*-dimethyl phosphorothiolate, $(MeO)_2PO\cdot S\cdot CH_2\cdot CH_2\cdot SEt$ (II), and *O*-2-ethylthioethyl *OO*-dimethyl phosphorothionate, $(MeO)_2PS\cdot O\cdot CH_2\cdot CH_2\cdot SEt$ (I), have been described.[1,2] 1% Suspensions of the thionate in water gave 50% yields of thiolate.[1] In solution [2] (<0·1%), the thionate gave smaller yields of thiolate, non-toxic ionic products, and less than 0·1% of a very powerful, positively charged, unstable anticholinesterase, the yield of which was increased about ten-fold by initial addition of a small amount of thiolate. The thiolate decomposed in water much more slowly than the thionate (half life 10·7 days at 37° against about 10 minutes for the thionate). The main products were non-toxic ions, but two anticholinesterases were produced. One was the methylsulphonium derivative of the thiolate, $(MeO)_2PO\cdot S\cdot CH_2\cdot CH_2\cdot \overset{+}{S}EtMe$,[2,3] which had half life in water of 14·4 days at 37°; the other had a half life of 100 min. at 37° and was assumed to be the same as that produced from the thionate. Both were formed at rates proportional to the square of the thiolate concentration. The additional yield of the unstable anticholinesterase obtained on adding thiolate to thionate was not formed from the thiolate alone, as the concentration added was only enough to account for about 1% of the increase.

I now suggest a structure for the unstable anticholinesterase, and, from study of the kinetics of its formation, suggest mechanisms for the decomposition of both the thionate and the thiolate in aqueous solution.

Results

As the reactions are acid-forming, most were carried out in 0·01N-hydrochloric acid, and those with thionate in solutions containing about 5% of ethanol to increase its rate of solution. In preliminary experiments no observable changes in rate were caused by changing the acid concentration to 0·0001N, and the effects of adding ethanol were confined to the beginnings of runs, which were not reproducible in its absence.

The unstable anticholinesterase was not separated pure, so that its concentrations could not be expressed in the usual terms. Therefore they are expressed as the number of times the solution had to be diluted to give a solution inhibiting 50% of sheep erythrocyte cholinesterase under standard conditions. The probable error in their determination was 5% except below 50 units in solutions previously extracted with chloroform to remove non-ionic products. Traces of chloroform lowered the values, and were difficult to remove completely by aeration

[1] Henglein and Schrader, *Z. Naturforsch.*, 1955, **10**, 1.
[2] Heath and Vandekar, *Biochem. J.*, 1957, **67**, 187.
[3] Heath, *Nature*, 1957, **179**, 377.

without taking so long that significant quantities of anticholinesterase decomposed in the process. Thus, below 50 units, results may be up to 5 units low.

First, it was shown that the unstable anticholinesterases from thiolate and thionate, both separately and mixed, were probably the same, and could be identified with the ethylthioethylsulphonium derivative of the thiolate, $(MeO)_2PO{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}\overset{+}{S}Et{\cdot}CH_2{\cdot}CH_2{\cdot}SEt$, which was synthesised in low yield by the action of bromoethyl ethyl sulphide on the thiolate in alcohol, and separated by electrophoresis. All the anticholinesterases were positively charged, and hydrolysed in water according to first-order kinetics at very similar rates, as shown in Table 1.

TABLE 1. *First-order constants for the hydrolysis at 21·8° of the unstable anticholinesterase.*

	From	PS	PS + PO	PO	Synthesis
10^3k (min.)	.....	1·24 ± 0·04	1·33 ± 0·02	1·32 ± 0·05	1·35 ± 0·05
,,	.....	1·26 ± 0·02	1·37 ± 0·02	1·28	—

PS, from thionate; PS + PO, from thionate–thiolate; PO, from thiolate. Errors are standard errors.

The minor differences, barely exceeding the standard errors, might be due to slight catalytic effects, as the samples contained different amounts of other ions. The reasonable assumption that the compounds are the same is justified by later kinetic work. Their structure is fixed almost unambiguously by the method of synthesis and their positive charge, the only doubt being as to which of the two sulphur atoms carries the positive charge.

The kinetic results for solutions of thionate, with and without added thiolate, are given in Tables 2—6.

Tables 2 and 3 show the concentrations of anticholinesterase obtained in typical runs. With thionate alone, the rate of formation was initially slow, and then rose rapidly to a maximum. Mixtures of thiolate and thionate however gave the anticholinesterase rapidly from the start, and a much higher concentration from a lower initial concentration of thionate. After the times shown the concentrations fell as the anticholinesterase decomposed.

TABLE 2. *Anticholinesterase from thionate alone at 21·8°.*

	Time (min.)	10	21	49	76	100	141
A	Anticholinesterase, found	4	21	129	269	318	400
	Anticholinesterase, calc.	6	26	126	241	316	409
	Time (min.)	12	20·5	40	62	95	124
B	Anticholinesterase, found	11	33	155	346	595	778
	Anticholinesterase, calc.	11	40	158	331	573	740

A, 0·314 mg. of thionate/ml.; *B*, 0·411 mg. of thionate/ml.

TABLE 3. *Anticholinesterase from thionate + thiolate at 21·8°.*

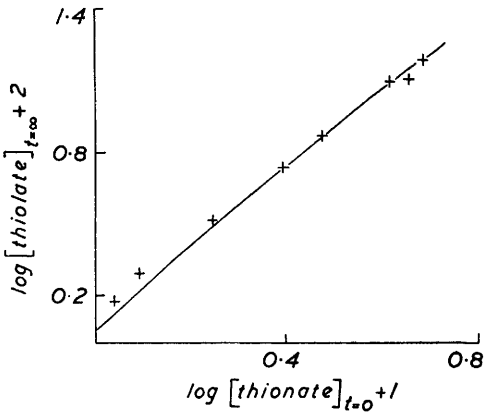
	Time (min.)	0·8	7·0	14·2	26·3	53·8	75·5	124	
A	Anticholinesterase, found...	66	707	1200	1900	3580	3800	4720	
	Anticholinesterase, calc. ...	87	701	1320	2170	3400	3980	4660	
	Time (min.)	1·2	6·2	12·2	20·0	40·0	60·0	80·4	100
B	Anticholinesterase, found...	124	699	1202	1910	3430	4000	4810	5870
	Anticholinesterase, calc. ...	135	661	1220	1850	3110	3990	4640	5260

A, 0·231 mg. of thionate/ml. + 0·333 mg. of thiolate/ml.; *B*, 0·205 mg. of thionate/ml. + 0·395 mg. of thiolate/ml.

Table 4 shows the maximum concentrations of the unstable anticholinesterase given by various concentrations of the thionate alone at 21·8°. These maximum concentrations are apparently proportional to $P_0^{2\cdot42}$, where P_0 is the initial concentration of the thionate.

Table 5 shows that, for thionate–thiolate, the concentration of anticholinesterase produced in the first 14 minutes was proportional to the thiolate concentration, when the initial thionate concentration was constant. At this time the conversion of thionate to thiolate had not gone far enough to affect the results.

Within the error on the thiolate determinations (about 5%) a log–log plot of the final concentrations of thiolate produced in runs with various initial concentrations of thionate is linear (see Figure): log [thiolate]$_\infty$ = 1·6 log P_0 + constant.



Points are experimental, curve is theoretical.

The concentrations of ionic phosphates at various times in a solution of thionate alone are given for a typical run in Table 6.

TABLE 4. *Maximum yield of anticholinesterase from thionate alone at* 21·8°.

Thionate (mg./ml.)	0·360	0·276	0·181	0·090
Anticholinesterase	625	343	119	21
$P_0^{2·42}$ × constant	638	336	119	22

TABLE 5.

Thiolate (mg./ml.)	0·348	0·174	0·087
Anticholinesterase	1215	676	333
Anticholinesterase/[thiolate]	3490	3890	3830

Thionate concentration = 0·211 mg./ml. throughout.

TABLE 6. *The percentage of total phosphorus as ions at various times in a solution of thionate initially of* 0·411 *mg./ml. at* 21·8°.

Time (min.)	1·1	6·0	12·0	20·5	40·0	62·0	95·0
%, found	0·7	7·5	14·1	23·4	36·7	47·3	59·0
%, calc.	1·6	8·3	15·1	23·3	37·1	47·4	57·0

The anticholinesterase contributed a negligible amount to these concentrations. Curves drawn through these and other results indicate a short induction period of about 0·7 min., after which they rise smoothly. I have assumed that this period is required to complete the solution or solvation of the thionate, and ignore it in the following discussion.

DISCUSSION

In trying to find a sequence of reactions which fits the results, several were tried which failed. As these are new, they are described briefly in the experimental section. A satisfactory sequence was found by considering the suggestion [4] that the isomerisation of the diethyl homologue of the thionate may go *via* an ionic intermediate:

$$(EtO)_2PS{\cdot}O{\cdot}CH_2{\cdot}CH_2{\cdot}SEt \longrightarrow (EtO)_2PO{\cdot}S^-{\cdot}\overset{\ddagger}{C}H_2{\cdot}CH_2{\cdot}\overset{+}{S}Et$$

This seems to have been vizualised as an ion pair, which does not fit my results. If,

[4] Fukuto and Metcalf, *J. Amer. Chem. Soc.*, 1954, **76**, 5103.

however, it is assumed that the phosphorothioate and dimethylene-ethylsulphonium ions separate, and that the latter is unstable, the following reactions may be written:

$$(MeO)_2PS{\cdot}O{\cdot}CH_2{\cdot}CH_2{\cdot}SEt \xrightarrow{k_1} (MeO)_2PO{\cdot}S^- + \overset{\frown}{CH_2{\cdot}CH_2{\cdot}\overset{+}{S}Et} \quad . \quad . \quad . \quad . \quad (1)$$

$$(MeO)_2POS^- + \overset{\frown}{CH_2{\cdot}CH_2{\cdot}\overset{+}{S}Et} \xrightarrow{k_2} (MeO)_2PO{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}SEt \quad . \quad . \quad . \quad . \quad . \quad (2)$$

$$\overset{\frown}{CH_2{\cdot}CH_2{\cdot}\overset{+}{S}Et} \xrightarrow{k_3} \text{products} \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (3)$$

$$\overset{\frown}{CH_2{\cdot}CH_2{\cdot}\overset{+}{S}Et} + (MeO)_2PO{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}SEt \xrightarrow{k_5} \text{unstable anticholinesterase} \quad . \quad . \quad . \quad (4)$$

$$\text{unstable anticholinesterase} \xrightarrow{k_4} \text{non-toxic products} \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (5)$$

Dimethyl phosphorothioate ions are produced by reaction (1). If k_2 and k_3 are much greater than k_1, the ionic phosphorus compounds found consist of that phosphorothioate which is not used in reaction (2). As the sulphonium and phosphorothioate ions are produced in equivalent amounts, the phosphorothioate remaining is equivalent to the sulphonium ion decomposed, so that equation (3) also represents the rate of accumulation of ions containing phosphorus. With the constants given in Table 8, the correct dependence of the maximum concentration of thiolate on the initial concentration of thionate is found (see Figure), and the calculated concentrations of ionic phosphates, and of unstable anticholinesterase from solutions of thionate, both with and without added thiolate, agree with those found, as shown in Tables 6, 2, and 3. In Table 6 the agreement is poor at the beginning, where the induction period affects the results. In Tables 2 and 3 the theoretical results are roughly corrected for hydrolysis of the anticholinesterase, and are correct to within 2%.

It follows from the proposed scheme that adding *OO*-dimethyl phosphorothioate to a solution of the thionate at the start of a run should increase the yield of thiolate on thionate by favouring reaction (2) over reaction (3). In such an experiment the yield was increased from 24·5% to 84·6%. The yield calculated from the rate equations is 82·2%, agreeing within experimental error.

The thiolate alone in water also gave the unstable anticholinesterase. As the thiolate is much more stable than the anticholinesterase, the latter reached a concentration after a few hours at 37° which changed only slowly. In Table 7 this concentration is shown to be proportional to the square of the thiolate concentration. The formation of the anticholinesterase can be explained if reaction (2) is reversible:

$$(MeO)_2PO{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}SEt \xrightarrow{k_6} (MeO)_2PO{\cdot}S^- + \overset{\frown}{CH_2{\cdot}CH_2{\cdot}\overset{+}{S}Et} \quad . \quad . \quad . \quad . \quad . \quad . \quad (6)$$

Near the beginning of the reaction the concentration of the thiophosphate ion is small, so the reverse of reaction (6) can be ignored. Also, k_6 is very much smaller than the other constants. Then $[A] = k_5k_6[PO]^2/k_3k_4$, in accordance with the observed proportionality.

TABLE 7. *Unstable anticholinesterase from thiolate alone at* 37°.

Thiolate (mg./ml.)	7·52	4·51	3·01
Anticholinesterase, found	14,250	4,840	2,080
Anticholinesterase/[thiolate]^2	252	238	230

The constants for thionate–thiolate mixtures at 37° were also determined, and are shown in Table 8. From these, and the results in Table 7, k_6 at 37° is found to be $3{\cdot}05 \times 10^{-5}$ min.$^{-1}$, which represents the rate of decomposition in water of the thiolate by the above mechanism. The overall rate of decomposition at infinite dilution, where the reactions giving anticholinesterases do not contribute, is represented by the constant

$4{\cdot}18 \times 10^{-5}$ min.$^{-1}$, so that apparently some decomposition, represented by $k = 1{\cdot}13 \times 10^{-5}$ min.$^{-1}$, takes place by some other mechanism.

Now the sulphoxide and sulphone of the thiolate, $(MeO)_2PO{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}SO{\cdot}Et$ and $(MeO)_2PO{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}SO_2{\cdot}Et$, cannot give the cyclic sulphonium intermediate, yet they hydrolyse in dilute acids. It seems likely that the hydrolysis rates of the thiolate, its sulphoxide, and its sulphone should form a continuous series, as successive oxidation of the side-chain should increase inductive effects. The first-order rate constants for the sulphone and sulphoxide are $2{\cdot}60 \pm 0{\cdot}01 \times 10^{-5}$ min.$^{-1}$ and $2{\cdot}18 \pm 0{\cdot}01 \times 10^{-5}$ min.$^{-1}$, which do form such a series with $1{\cdot}13 \times 10^{-5}$ min.$^{-1}$ for the thiolate. Thus the value of this constant suggests that the thiolate is directly attacked by water to some extent:

$$(MeO)_2PO{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}SEt + H_2O \xrightarrow{k_7} \text{ionic products} \quad . \quad . \quad . \quad . \quad . \quad . \quad (7)$$

Water does not take a direct part in the decomposition of the thionate and thiolate *via* the cyclic intermediate. Thus a stored dry sample of the thionate contains thiolate, ionic products, and anticholinesterases.[2] Some rough experiments on the dry thiolate show that it is less stable than the sulphoxide, which is already known to be much more stable than the sulphone, as it is reduced to the thiolate when heated.[2]

The scheme explains why the rate constant for the decomposition of thiolate in water decreased with time, especially at higher concentrations.[2] As *OO*-dimethyl hydrogen phosphorothioate is relatively stable, its concentration increases as the reaction proceeds, until the reverse of reaction (6) becomes important.

From the rate constants at different concentrations of the thiolate alone the I_{50} of the unstable anticholinesterase is calculated to be $1{\cdot}4 \times 10^{-9}$M. The value found directly is $6{\cdot}9 \times 10^{-9}$M, but is likely to be high.

The account of the reactions of the thiolate and thionate in water is completed by adding those for the formation and decomposition of the stable inhibitor:

$$2(MeO)_2PO{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}SEt \xrightarrow{k_8} (MeO)_2PO{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}\overset{+}{S}EtMe + MeO{\cdot}PO_2^-{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}SEt \quad (8)$$

$$(MeO)_2PO{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}\overset{+}{S}EtMe \xrightarrow{k_9} \text{non-toxic products} \quad . \quad . \quad . \quad . \quad . \quad . \quad (9)$$

These have been discussed elsewhere.[2,3]

The constants for all nine reactions are given in Table 8. The ratio k_3/k_5 is given in terms of the I_{50} value of the unstable anticholinesterase. The results are very insensitive to k_3/k_2, changes of 10% barely affecting them.

TABLE 8. *Rate constants.*

	10^2k_1	$10^3k_3/k_2$	$10^8I_{50}k_3/k_5$	10^5k_6	10^5k_7	10^4k_8	10^3k_4	10^5k_9
37·0°	7·5	1·93	7·89	3·05	1·13	6·5	7·0 *	3·7 *
21·8°	1·5	1·52	4·61	—	—	—	1·3	—

All constants are in min. mole l.$^{-1}$ units. * Taken from Heath and Vandekar.[2]

EXPERIMENTAL

Determinations of Anticholinesterase Activity.—Such determinations were carried out as described by Heath and Vandekar.[2]

Phosphorus Analyses.—Phosphorus in samples was determined after combustion to phosphate [5] except that a little bromine water was added before ashing to avoid evaporation losses.[6]

O-2-*Ethylthioethyl* OO-*Dimethyl Phosphorothionate, and* S-2-*Ethylthioethyl*, S-2-*Ethylsulphinylethyl, and* S-2-*Ethylsulphonylethyl* OO-*Dimethyl Phosphorothiolates.*—For the sources and careful purification of these compounds, see Heath and Vandekar.[2]

Potassium OO-*Dimethyl Phosphorothioate.*—*OO*-Dimethyl *p*-nitrophenyl phosphorothionate was hydrolysed with ethanolic potassium hydroxide, acidified to pH 4 with hydrochloric acid, the product diluted with water, and the solution extracted six times with chloroform. The

[5] Fiske and Subbarow, *J. Biol. Chem.*, 1925, **66**, 375.
[6] Gardner and Heath, *Analyt. Chem.*, 1953, **25**, 1849.

aqueous layer was made slightly alkaline and concentrated to dryness *in vacuo*. The crude product, containing potassium chloride, gave no precipitate with barium chloride in the cold, and a copious one when heated with concentrated nitric acid for a few minutes. Analysis [7] gave thiophosphate equivalent to 63% of the phosphorus present.

2-Ethylthioethylsulphonium Derivative of the Thiolate, $(MeO)_2PO{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}\overset{+}{S}Et{\cdot}CH_2{\cdot}CH_2{\cdot}SEt$. —Thiolate (0·5 g.), 2-bromoethyl ethyl sulphide [8] (0·5 g.; b. p. 69·5—70·5°/10 mm.), and ethanol (2·5 ml.) were kept at 37° for 16 hr. 1 ml. of product was concentrated at <20°/0·5 mm., water (1 ml.) added, and the solution extracted with chloroform (2 × 4 ml.). The aqueous layer (0·37 ml.) was subjected to electrophoresis [2] on a strip (20 × 36 cm.) of Whatman 3 MM paper. The strip between 4 and 16 cm. from the starting line towards the cathode, *i.e.* the section containing positive ions, was soaked for 30 min. in water (30 ml.), the paper removed, and the solution stored at −30°.

Samples were also prepared from thionate and thiolate, alone and mixed, in 0·01N-hydrochloric acid. As in all experiments with thionate, ethanol was added to the dilute acid at the rate of 5 ml. of ethanol/100 ml. of acid. Thionate (about 0·4 mg./ml.) and thionate (about 0·2 mg./ml.) + thiolate (about 0·4 mg./ml.) were kept at 21·8° for 300 min., and thiolate (5 mg./ml. and 10 mg./ml.) at 37° for 200 min. The products were extracted thrice with chloroform, and the aqueous layers stored in the cold. Compounds prepared similarly had been shown to be positively charged.[2]

Hydrolysis Rates of the Unstable Anticholinesterases.—Samples prepared as above were stored at 21·8° or 37°, portions extracted from time to time, and their anticholinesterase activities determined. After 4—5 half-lives a portion was kept at 37° for 1 day, which decomposed the unstable anticholinesterase completely, but only about 5% of the more stable methylsulphonium inhibitor. In preparations from the thiolate alone or from the synthetical method, 5—10% of the initial activity was due to this compound. Other samples contained less than 1%. This residual activity was subtracted from the previous results, which then agreed with those expected for first-order decomposition, with the constants given in Table 1. As one constant depended on two points only no standard error is given for it.

Hydrolyses.—Runs were carried out at 21·80° ± 0·05° or 36·95° ± 0·05°.

For runs with thionate alone, the ester in ethanol was added to the acid to give 5 ml. of ethanol in 100 ml. of acid, and shaken briskly. The thionate concentrations were found by phosphorus analysis. The ionic products were determined at set times by removing samples, extracting them twice with an equal volume of chloroform, estimating phosphorus in the aqueous layer, and multiplying the results by 1·029 to correct for the increase in volume of that layer due to extraction of ethanol from the chloroform, which contained it as a preservative. This factor was obtained by extracting potassium phosphate solutions similarly.

For runs containing thiolate, a solution of the freshly purified compound [2] was heated rapidly to the correct temperature, and a sample withdrawn for analysis. Thionate, when required, was then added in ethanolic solution of known concentration. Runs were then carried out as before.

The anticholinesterase formed was determined on samples after extraction of non-ionic compounds with chloroform. Times were taken from the first moment of shaking with chloroform. Dissolved chloroform was blown out by air, and the determinations carried out immediately, or the samples stored at −30°. They were never left at room temperature for more than 15 min. between extraction and analysis.

The hydrolysis rates of the thiolate at two concentrations in 0·005N-hydrochloric acid at 37° are given by Heath and Vandekar.[2] Those of its sulphoxide and sulphone were determined at the same acid concentration and temperature by the chloroform-extraction method,[9] volume changes being allowed for as described above.

Thiolate from Thionate Solutions.—The concentration of thiolate produced by the end of a run with thionate alone was determined either by the difference between the concentration of ionic products at the end of the run and the initial concentration of thionate, or by the determination of the phosphorus extracted by chloroform. The precision of both methods was only about 5%, in the first because the thiolate concentration was only about 20% of the initial thionate concentration, and in the second because it proved difficult to evaporate the chloroform without losing phosphorus.

[7] Foss, *Acta Chem. Scand.*, 1947, **1**, 8.
[8] Steinkopf, Herold, and Stöhr, *Ber.*, 1920, **53**, 1007.
[9] Heath, *J.*, 1956, 3796.

These methods assume that the only compound extractable by chloroform at the end of a run is thiolate. This was demonstrated in two ways. First, a chloroform extract from the end of a run was concentrated, and subjected to counter-current analysis in a 54-tube stainless steel, Craig [10] machine. One phosphorus band only was found, with partition coefficient between light petroleum (b. p. 100—120°) and water of 1·61, *i.e.*, that of the pure thiolate.[2] Secondly, an aqueous solution was prepared from another extract, kept at 37° for 5 hr., and the phosphorus content and anticholinesterase activity determined. On comparison with the results given in Table 7, the two determinations agreed to within 2% on the assumption that the only phosphorus compound present was the thiolate.

Decomposition of Dry Thiolate.—Thiolate was dried *in vacuo*, and kept at 76° in a stoppered capillary tube. Samples were withdrawn at set times, and the fraction of the phosphorus not extractable by chloroform was determined. The rate of decomposition increased with time, reaching 40% at 16 hr. In the same time 13% of sulphoxide and 1% of sulphone decomposed.

*Rate Equations and Calculations.**—Two reaction schemes which fail are considered first.

(i) $PS \xrightarrow{k_1} PO$, $PS \xrightarrow{k_2} I$, $PO + PS \xrightarrow{k_5} A$, $A \xrightarrow{k_4}$ non-toxic products, where PS = thionate, PO = thiolate, I = ionic phosphates, and A = unstable anticholinesterase.

For runs with thionate alone, this scheme gives $[PO]_\infty$ proportional to $[PS]_0$, not to $[PS]_0^{1\cdot6}$ as is found, and an incorrect dependence of A, the concentration of species A, on time.

(ii) $PS \underset{k_6}{\overset{k_1}{\rightleftharpoons}} X \xrightarrow{k_3} PO$, $PO + PS \xrightarrow{k_5} A$, $A \xrightarrow{k_4}$ non-toxic products, and $PS \xrightarrow{k_7} I$ or $X \xrightarrow{k_7} I$, where k_6 may be 0.

These schemes correspond to the assumption that the intermediate in the isomerisation is an ion pair. They all give $[PO]_\infty$ proportional to $[PS]_0$, which is strong evidence that this hypothesis is incorrect. Calculations also suggest that A is not formed by a direct reaction between PO and PS. Thus, for runs with thionate alone, constants can be chosen which give values of A in good agreement with experiment; but, for mixtures containing thiolate, these constants give values of A 30—50% too low. Thus the rate of formation of A can only depend on [PO][PS] if PS decomposes at a rate far from exponential, which is inconsistent with the rate of formation of ionic phosphates (Table 6), which is roughly exponential, and accounts for most of the decomposition.

The equations for [PS], [X], and [PO] can be obtained from the treatment given by Lowry and John [11] to similar schemes. That for A is:

$$\frac{A(b-a)}{k_5P_0} = \frac{K(k-a)\{\exp(-k_4t) - \exp(-at)\}}{a-k_4} - \frac{K(k-b)\{\exp(-k_4t) - \exp(-bt)\}}{b-k_4}$$
$$- \frac{k_1k_2P_0}{b-a}\left\{\frac{(k-a)\{\exp(-k_4t) - \exp(-2at)\}}{a(2a-k_4)} + \frac{(k-b)\{\exp(-k_4t) - \exp(-2bt)\}}{b(2b-k_4)}\right.$$
$$\left. - \frac{\{(k-a)/b + (k-b)/a\}\{\exp(-k_4t) - \exp(-at-bt)\}}{(a+b-k_4)}\right\}$$

where $P_0 = [PS]_{t=0}$, $Q = [PO]_{t=0}$, and $K = Q + k_1k_2P_0/ab$;

and, for $PS \xrightarrow{k_7} I$, $k = k_2 + k_6$, $k' = k_1 + k_7$, $2a = k + k' - \{(k+k')^2 - 4(kk' - k_1k_6)\}^{\frac{1}{2}}$ and $2b = k + k' + \{(k+k')^2 - 4(kk' - k_1k_6)\}^{\frac{1}{2}}$;

and for $X \xrightarrow{k_7} I$, $k = k_2 + k_6 + k_7$, $2a = k + k_1 - \{(k+k_1)^2 - 4k_1(k-k_6)\}^{\frac{1}{2}}$ and $2b = k + k_1 + \{(k+k_1)^2 - 4k_1(k-k_6)\}^{\frac{1}{2}}$.

(iii) This scheme accounts for the results adequately. In it B = cyclic sulphonium intermediate and C = *OO*-dimethyl phosphorothioate ion, then

$$PS \xrightarrow{k_1} B + C \xrightarrow{k_2} PO,\ B \xrightarrow{k_3} I,\ B + PO \xrightarrow{k_5} A,\ A \xrightarrow{k_4} \text{non-toxic products.}$$

To integrate the equations, it appears to be necessary to put $k_4 = 0$, which will introduce only

* In this section the use of brackets [ ] is restricted to the significance of concentration.

[10] Craig and Post, *Analyt. Chem.*, 1949, **21**, 500.
[11] Lowry and John, *J.*, 1910, 2635.

minor errors, as it is the smallest constant by a factor of about 10. Then, assuming that B is unstable, so that $d[B]/dt = 0$, we obtain:

$$[B] = k_1[PS]/(k_2[C] + k_3)$$

$$[C] = 2\{(1 + bP_0 - b[PS])^{\frac{1}{2}} - 1\}/b \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (1)$$

where $b = 2k_2/k_3$;

$$[PO] = P_0 - [PS] - [C] \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (2)$$

$$k_3bA/2k_5 = (1/b + Q)\{(a - bx)^{\frac{1}{2}} - 1\} - (P_0 - x) + \{(a - bx)^{3/2} - 1\}/3b \quad . \quad (3)$$

in which $x = [PS] = P_0 \exp(-k_1t)$, and $a = (1 + bP_0)$. The final equations were obtained by dividing equations of the type: dy/dt by $d[PS]/dt$. and integrating the resulting equations with respect to [PS].

The theoretical curve in the Figure was obtained by putting [PS] = 0 in eqn. (1) and plotting the calculated values over the experimental range.

The constants given in Table 8 for the thiolate–thionate reactions at 37° were calculated as follows from the results summarised here. $P_0 = 1{\cdot}07 \times 10^{-3}$M, $Q = 1{\cdot}48 \times 10^{-3}$M, and $C_\infty = 8{\cdot}7 \times 10^{-4}$M, whence $b = 1{\cdot}035 \times 10^3$ mole^{-1} from eqn. 1. At $t = 7{\cdot}0$, 8·5, and 9·5 min., respectively, $C = 3{\cdot}96 \times 10^{-4}$M, $4{\cdot}22 \times 10^{-4}$M, and $5{\cdot}00 \times 10^{-4}$M. The best value of k_1, 0·075 min.$^{-1}$, obtained by putting these values into eqn. 1, gives [C] = $3{\cdot}96 \times 10^{-4}$M, $4{\cdot}52 \times 10^{-4}$M, and $4{\cdot}83 \times 10^{-4}$M. At the same times, $A = 1780$, 1970, and 2030 units, whence, from eqn. 3, $k_5/k_3 = 1{\cdot}31 \times 10^7$, $1{\cdot}27 \times 10^7$, and $1{\cdot}22 \times 10^7$ (average, $1{\cdot}27 \times 10^7$) in terms of units of A, and moles of the other reagents.

If dimethyl phosphorothioate ions are added, there is an additional constant concentration, C_0, of C present throughout the reaction. Then, for the additional amount of C produced by decomposition of the thionate, $[C] = 2\{(d^2 + bP_0 - b[PS])^{\frac{1}{2}} - d\}/b$, where $d = 1 + bC_0/2$. At $t = \infty$, [PS] = 0, and $[PO]_\infty = P_0 - [C]$. In an experiment carried out at 21·8°, $C_0 = 6{\cdot}93 \times 10^{-3}$M, and $P_0 = 1{\cdot}32 \times 10^{-3}$M, whence, the constants in Table 8 being used, $[PO]_\infty = 0{\cdot}822P_0$.

For runs with thiolate alone, the only additional reaction to be considered is: PO $\xrightarrow{k_6}$ B + C, the reverse of one already given. Then: $d[B]/dt = k_6[PO] - k_5[B][PO] - k_3[B] = 0$. f $k_5[B][PO]$ is small compared to the other two terms, $[B] = k_6[PO]/k_3$. As the constants are all great compared with k_6 $dA/dt = k_5[B][PO] - k_4A = 0$, whence $k_6 = k_3k_4A/k_5[PO]^2$. From Table 7, $A/[PO]^2 = 240$; k_4 and k_3/k_5 are given in Table 8; whence $k_6 = 3{\cdot}05 \times 10^{-5}$ min.$^{-1}$.

The Activity of the Unstable Anticholinesterase.—A direct determination was carried out on the solution obtained by the electrophoresis of the synthetic sample. The solution, $1{\cdot}2 \times 10^{-4}$M in phosphorus, contained 12,000 units of anticholinesterase activity, of which 950 units were of the methylsulphonium derivative of the thiolate. This [2] has $I_{50} = 3{\cdot}9 \times 10^{-8}$M, whence its concentration was $0{\cdot}37 \times 10^{-4}$M. Hence the I_{50} of the substance A is given by $(1{\cdot}20 - 0{\cdot}37) \times 10^{-4}$M/(12,000 − 950), *i.e.* $6{\cdot}9 \times 10^{-9}$M.

This value may be very high. Only about 25% of the anticholinesterase activity placed on the strip was recovered although none was found elsewhere. Perhaps the anticholinesterase was not removed completely from the strip, but, as the strip became warm during electrophoresis, which lasted 2 hr., it is equally likely that the anticholinesterase was decomposed, probably after it had moved towards the cathode. The value is inconsistent with other results, as shown below.

The I_{50} value can be calculated from the rate constants for the production of ions of all kinds from the thiolate alone at two concentrations (already reported [2]). The constants are: $4{\cdot}50 \times 10^{-5}$ min.$^{-1}$ at 0·94 mg./ml., and $5{\cdot}77 \times 10^{-5}$ min.$^{-1}$ at 4·70 mg./ml. The difference being assumed to be due solely to second-order reactions producting anticholinesterases, at infinite dilution $k = 4{\cdot}18 \times 10^{-5}$ min.$^{-1}$, so that, at the higher concentration, the second-order reactions contribute $1{\cdot}59 \times 10^{-5}$ min.$^{-1}$. The concentrations of methylsulphonium compound found [2] require a constant of $1{\cdot}33 \times 10^{-5}$ min.$^{-1}$, leaving $0{\cdot}26 \times 10^{-5}$ min.$^{-1}$ for the rate of formation of the unstable anticholinesterase. This is equal to the rate of decomposition, *i.e.* to k_4A. When the concentration of PO is 4·70 mg./ml., A, in the usual units, is 5,300 (calculated from Table 7). Then $0{\cdot}26 \times 10^{-5} = k_4AI_{50}/[PO]$, whence $I_{50} = 1{\cdot}4 \times 10^{-9}$M.

By similar reasoning, it can be shown that, if the I_{50} is $6{\cdot}9 \times 10^{-9}$M, the difference between the rate constants at the two concentrations would be $2{\cdot}61 \times 10^{-5}$ and not $1{\cdot}59 \times 10^{-5}$ min.$^{-1}$,

as observed. This discrepancy appears too great to be accounted for by either experimental error or concentration effects.

If, in reactions of thiolate alone, k_5[B][PO] is not negligible, $k_6 = (k_4A[PO] + k_3/k_5)/[PO]^2$. For $I_{50} = 6{\cdot}9 \times 10^{-9}$M, $k_3/k_5 = 11{\cdot}4$ mg./ml., not much above the higher concentrations of the thiolate. Then A is not proportional to $[PO]^2$. For $I_{50} = 1{\cdot}4 \times 10^{-9}$M, $k_3/k_5 = 56{\cdot}2$ mg./ml., which is large compared with the concentration of PO, so that the original formula is a good approximation.

I thank Dr. G. Schrader and his colleagues for the phosphorus compounds, Dr. G. S. Hartley for the ethyl hydroxyethyl sulphide, Dr. H. A. Krebs and Dr. D. E. Hughes for the Craig counter-current separator, Dr. D. F. Elliott for a 50-tube separator of the type designed by Lathe and Ruthven [12] used in some trial experiments, and Mr. D. E. A. Groom for technical assistance.

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[*Received, October 3rd*, 1957.]

[12] Lathe and Ruthven, *Biochem. J.*, 1951, **49**, 540.