aldehyde moiety, which corresponds to positions 5 and 6 of the molecule. Further degradation indicated that most of the radioactivity of penicillin derived from [α - ^3H]cystine was located at position 6, as expected from intact incorporation of the labelled cystine.

4. In a fermentation experiment with [α - ^3H]cystine the molar radioactivity of penicillin was greater than that of the cysteine in the mycelial protein.

5. These results show that the α -hydrogen atom and one of the β -hydrogen atoms of cystine are retained during penicillin biosynthesis.

6. The significance of these findings is discussed with particular reference to possible mechanisms of β -lactam-ring formation.

We wish to thank Dr J. W. Cornforth, F.R.S., for most helpful discussions concerning the mechanism suggested in this paper for the biosynthesis of the β -lactam ring of penicillin.

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Some Spontaneous Reactions of *OO*-Dimethyl *S*-Ethylthioethyl Phosphorothiolate and Related Compounds in Water and on Storage, and their Effects on the Toxicological Properties of the Compounds

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Shortly after beginning to study the biochemistry of Metasytox (a mixture of *OO*-dimethyl *S*-ethylthioethyl phosphorothiolate and its thionate isomer) and some related compounds, we discovered that all our samples, tested biochemically, were impure, and that most of them increased in toxicity on storage. We therefore carried out a few preliminary experiments on the whole group of compounds:

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OO-dimethyl *S*-ethylthioethyl phosphorothiolate, Isosystox (the *OO*-diethyl homologue), the thionate isomer (dimethyl ethylthioethyl phosphorothionate) and the sulphoxide and sulphone of the thiolate (*S*-ethylsulphinylethyl- and *S*-ethylsulphonylethyl-*OO*-dimethyl phosphorothiolates). Contrary to expectation, much of the toxicity of all five compounds, as determined by intravenous injection into rats, remained in the water layers after chloroform extraction of their aqueous solutions.

Heating the four dimethyl compounds increased the toxicity of each considerably, again by the formation of compounds inextractable by chloroform. *OO*-Dimethyl *S*-ethylthioethyl phosphorothiolate, its thionate isomer and Isosystox also apparently underwent similar reactions in water at a much faster rate. In some experiments the toxicities were increased 100-fold.

Spontaneous reactions which can increase the toxicities of such widely used insecticides as Isosystox and Metasystox are obviously important. We also wished to provide a firm basis for biochemical work on some of the dimethyl compounds, which presented interesting features (Vandekar & Heath, 1957). We were therefore led to investigate the reactions of the five compounds in detail, with the results described here.

Work with each compound fell naturally into three parts: first, methods of purification were worked out, mainly using solvent extraction, and the purified compounds were tested as rigidly as possible for chemical purity. Secondly, the reactions of these purified compounds were studied both in water and alone, and the toxic derivatives isolated and identified. In the most complicated case, *OO*-dimethyl *S*-ethylthioethyl phosphorothiolate in water, a kinetic analysis of the reactions was undertaken to show that the reactions postulated accounted quantitatively for the products, thus providing evidence that no other reactions leading to toxic products took place. Thirdly, the basic toxic properties—oral and intravenous LD_{50} and I_{50} values—of the compounds and the main toxic derivatives were found, such precautions being taken as the previous findings indicated.

MATERIALS AND METHODS

Compounds. *OO*-Dimethyl *S*-ethylthioethyl phosphorothiolate $[(MeO)_2PO \cdot S \cdot CH_2 \cdot CH_2 \cdot SET]$, the corresponding sulphoxide and sulphone [*OO*-dimethyl *S*-ethylsulphinyethyl phosphorothiolate, $(MeO)_2PO \cdot S \cdot CH_2 \cdot CH_2 \cdot SO \cdot Et$, and *OO*-dimethyl *S*-ethylsulphonylethyl phosphorothiolate, $(MeO)_2PO \cdot S \cdot CH_2 \cdot CH_2 \cdot SO_2 \cdot Et$] and the thionate isomer, *OO*-dimethyl *O*-ethylthioethyl phosphorothionate

were kindly given to us by Dr G. Schrader, Farbenfabriken Bayer A.-G., Wuppertal-Elberfeld. We also prepared the sulphonyl derivative by the method of Heath & Lane (1952). *OO*-Diethyl *S*-ethylthioethyl phosphorothiolate, $[(EtO)_2PO \cdot S \cdot CH_2 \cdot CH_2 \cdot SET]$ was given to us by the courtesy of Dr G. S. Hartley, Fisons Pest Control Ltd., Little Chesterford, Essex.

Phosphorus analyses. These were carried out by the method of Fiske & Subbarow (1925). By preliminary oxidation with bromine water before ashing, evaporation losses were avoided (Gardner & Heath, 1953). Results on non-aqueous samples were generally a few per cent low.

Elementary analyses. These were carried out by Drs Weiler and Strauss, 164, Banbury Road, Oxford, and Miss J.

Lewis, National Institute for Medical Research, Mill Hill, London, N.W. 7.

Tests of chemical structure. The main test used was successive partitioning (Heath, Lane & Llewellyn, 1952; Heath, Park & Lane, 1955). A solution of the compound in solvent *A* was repeatedly extracted with an equal volume of solvent *B*. The first extract with *B* was then extracted several times with *A*. One solvent always contained water. Each aqueous fraction was analysed for phosphorus. The aim was to extract often enough to remove most of the compound from *A* and from the first extract in *B*. Extractions with one solvent were often followed by further extractions with others. The calculation of results is not affected by the number of solvents used, so that only the case of two solvents is considered in the next paragraph.

For a pure compound, the ratio of the concentrations in the phases *A* and *B* at equilibrium is a constant, the partition coefficient *K*. Thus the concentrations in solvent *A* before and after extraction with *B* are in the ratio $(K + 1) : K$. Deviations from this relationship indicate impurities. The apparent values of *K*, as determined by phosphorus analysis, may differ a little according as *A* is extracted with *B* or vice versa if volume changes occur during partitioning. With very volatile solvents such changes are difficult to avoid, so that the two sides, *A* extracted with *B*, and *B* with *A*, had occasionally to be treated as two independent series, with some loss of sensitivity. The test is most sensitive if *K* equals 1, so solvents were chosen normally so that *K* was about unity.

The test was used in three ways in this work, and the validity of the results depends in part on its reliability for each purpose. The partition coefficient of a compound between two solvents is a characteristic property. A change in the structure of a compound will nearly always change the partition coefficient by a factor of at least two (see Seidell, 1941; Collander, 1949), except where the change is isomerization without much change in the type of bonding throughout the molecule as, for example, in

and

which may have very similar coefficients. Thus the validity of the test for the three ways in which it was used may be summarized as follows:

(1) As a test of purity it was probably very sensitive except to *S*-methyl isomers, of which several per cent may be undetectable.

(2) As a check that a compound is unchanged by a given treatment, for example, heating or storage, the test was reliable unless isomerization to *S*-methyl isomers took place, or, very improbably, two or more successive reactions led to a product with the coefficient of the parent compound.

(3) As a method for identifying the products of reactions, the test indicated only the probable nature of the product. By suitable design the test separated products fairly completely from the parent compounds, and gave their partition coefficients. Agreement between these coefficients and those of known compounds likely to be found suggested that these products were identical with the known compounds. There may, however, be other compounds with the same coefficient which can be produced.

The rate at which cholinesterase recovered its activity, after being inhibited by a short period of contact with a

compound, was also used to test structure. The method is due to Aldridge (1953), who showed that the rate of recovery was dependent on the structure of that part of the inhibitor which remained on the enzyme. Thus in esters, $RR'PO \cdot X$, where R and R' are alkoxy or similar groups and X is a replaceable acidic radical, the rate of recovery depends upon R and R'.

The method of Aldridge (1953) was used, unchanged, except that the inhibited enzyme was washed three times to remove excess of inhibitor.

Electrophoretic separations. These were carried out at 4°, in the apparatus described by Flynn & Mayo (1951), on strips of Whatman 3MM filter paper, 36 cm. $\times$ 5.5 cm., at 550 v in an electrolyte of 0.35% sodium acetate trihydrate and 0.16% glacial acetic acid in water, pH 5.3. For analysis the strips were cut up and eluted by immersion in water for at least 30 min.

Anticholinesterase activities. These were determined with sheep-erythrocyte cholinesterase for 30 min. at 37°, followed by the determination of the cholinesterase activity remaining (Aldridge, 1950). The rates of CO_2 evolution were calculated by the method of Aldridge, Berry & Davies (1949). I_{50} values were determined by choosing at least four concentrations which produced 40–60% inhibition. For the dimethyl phosphate esters the graph of log (percentage activity) against concentration of inhibitor was not a straight line because the inhibition, as with all dimethyl phosphate esters, is reversible. It is, however, nearly straight over the short range 40–60%. The average gradient of the best straight line over this range does not pass through 2, i.e. log (100%) at zero concentration. From over a hundred curves the best line appeared to pass through 1.95. The line chosen was therefore that which passed through this point and gave the best fit to the experimental points, and the I_{50} was taken as the concentration on this line corresponding to 50% inhibition.

I_{50} values of purified compounds were determined by using solutions containing concentrations of inhibitor found by phosphorus assay. This enabled us to use freshly purified compounds with the minimum of delay.

Concentration of inhibitor in a sample. This was usually expressed as the number of times the sample had to be diluted to give an I_{50} concentration, and was called the I_{50} dilution.

Preparation of solutions for toxicity and anticholinesterase tests. Purified compounds were stored at –30°. Solutions in water were prepared immediately before testing. Solutions in non-aqueous solvents were concentrated *in vacuo*, water was added and the evacuation continued until the non-aqueous solvent had disappeared. Non-aqueous solvents dissolved in aqueous solutions were blown out with air. The aqueous solutions were either tested immediately, or stored at –30°.

Toxicity tests. These were carried out on male and female albino rats of the same stock. Intravenous injections were made by the tail vein. Surviving animals were observed until all symptoms disappeared and for at least 24 hr. Rough LD_{50} values were obtained on 4–6 animals. Those given in Table 11 were obtained on sixteen animals, estimated by the method of Thompson & Weil (1952), the tables for median-effective dose given by Weil (1952) being used. The concentrations of active compounds in the solutions used for test were determined by phosphorus assay.

Constitution and purification of samples

Our samples had been distilled, a process which can lead to complex breakdown of dimethyl phosphate esters (Macpherson & Johnson, 1956). Elementary analyses were carried out to show that gross impurities were absent. The samples were then subjected to successive partitioning, purified where necessary, and the rate of recovery of cholinesterase inhibited by them was determined.

OO-Dimethyl S-ethylthioethyl phosphorothiolate. Found: C, 31.4; H, 6.4; S, 29.7, 24.9; P, 13.1, 12.4, 13.1%. $C_6H_{15}O_3S_2P$ requires C, 31.3; H, 6.52; S, 27.8; P, 13.5%. As there is a general tendency for sulphur analysis to be somewhat inaccurate in compounds with P–S bonding and for phosphorus to be found a little low in fairly volatile compounds such as these, the results indicated that the sample consisted originally of the stated compound or its isomers.

Successive partitioning (Table 1) showed mainly one compound, of partition coefficient (light petroleum–water) 1.605, contaminated with 0.60% of compounds not extractable from water by light petroleum or $CHCl_3$. The compound was freed from water-soluble impurities by extracting a $CHCl_3$ solution with water.

Cholinesterase, inhibited *in vitro* with the purified compound, recovered its activity with a half-life of 1.3 hr., the characteristic half-life for cholinesterase inhibited with a dimethyl phosphate ester.

OO-Dimethyl O-ethylthioethyl phosphorothionate. Found: C, 31.0; H, 6.66; S, 29.3, 27.2; P, 12.0, 12.65, 13.3%. $C_6H_{15}O_3S_2P$ requires C, 31.3; H, 6.52; S, 27.8; P, 13.5%, in satisfactory agreement.

Successive partitioning revealed that 36.2% of the sample had the light petroleum–water partition coefficient of the thiolate isomer, presumably formed by isomerization

Table 1. Successive partitioning of OO-dimethyl S-ethylthioethyl phosphorothiolate

Numerals in parentheses refer to the serial numbers in the table. A solution of the compound in water (1) was extracted four times with light petroleum, b.p. 100–120°, and the water layers were assayed for phosphorus after each extraction (2–5). (5) was then extracted with $CHCl_3$, leaving (6). The first light-petroleum extract was extracted five times with water, and each water extract assayed (7–11). The calculated results were obtained assuming that (1) contained 324 μ g. of P/ml., that *K* (light petroleum–water) was 1.605, and that the fraction not extracted by $CHCl_3$ could not be extracted by light petroleum.

No.	Concn. of phosphorus in aqueous layers (μ g./ml.)	
	Found	Calc.
1	330, 325	324
2	125, 126	126
3	49.3, 49.9	49.4
4	19.9, 18.7	20.2
5	8.53, 8.71	8.96
6	1.96, 1.96	—
7	77.5, 76.9	76.2
8	46.0, 45.1	46.9
9	29.0, 29.0	29.0
10	17.5, 17.8	17.8
11	11.0, 11.0	11.0

Table 2. *Successive partitioning of purified OO-dimethyl O-ethylthioethyl phosphorothionate*

Light petroleum, b.p. 100–120°, and 20% (v/v) aqueous methanol were equilibrated at 4°. The thionate was added to aqueous methanol (1) and rapidly extracted six times with an equal volume of light petroleum, leaving six methanol layers for assay (2–7). The first light-petroleum extract was rapidly extracted four times with aqueous methanol (8–11). The whole process took 20 min. The results were calculated on the assumption that (1) contained 123.0 µg. of P/ml., of which 0.149 µg. of P/ml. could not be extracted, and that *K* (light petroleum–methanol) was 1.70 when methanol was extracted by light petroleum, but was 1.94 when light petroleum was extracted by methanol. The difference was due to carry-over in this rapid experiment, when to avoid decomposition of thionate time could not be spent in cleaning funnels between extractions.

No.	Concn. of phosphorus in methanol layers (µg./ml.)	
	Found	Calc.
1	127	123
2	45.0	45.3
3	17.0	16.9
4	6.33	6.35
5	2.46	2.44
6	0.92	1.01
7	0.50	0.46
8	28.4	29.8
9	20.3	19.7
10	13.0	13.0
11	8.86	8.45

on ageing (Henglein & Schrader, 1955). The thionate is more oil-soluble than the thiolate, and was therefore separated as follows: crude thionate (20 g.) was dissolved in light petroleum, b.p. 100–120° (400 ml.), and extracted rapidly three times with successive 400 ml. portions of 20% (v/v) aqueous methanol. The light-petroleum layer was evaporated rapidly *in vacuo* finally at 50° under 0.1 mm. Hg. The product is likely to contain some solvent. Phosphorus assay gave the thionate content as 95.7%. Successive partitioning of the product at 4° revealed no impurities (Table 2).

The compound reacted too rapidly with water to test the recovery of cholinesterase inhibited by it.

OO-Dimethyl S-ethylsulphinyethyl phosphorothiolate. A sample was prepared by the method of Heath & Lane (1952), and purified as follows: the product (5 g.) in water (50 ml.) was extracted with CHCl₃ (3 × 50 ml.) to separate it from H₂O₂. From the CHCl₃ extract a water solution was prepared. The impurities most likely to be present are the sulphide and sulphone. The aqueous solution was therefore extracted with three equal volumes of 1:2-dichloropropane, which removes all the sulphide and about 95% of the sulphone. The sulphoxide was then extracted with CHCl₃ and concentrated, finally at 60° under 0.1 mm. Hg. Phosphorus assay gave 93% of sulphoxide, the remainder being presumably CHCl₃.

Successive partitioning showed the compound to be substantially pure (Table 3). It contained no appreciable amount of material extractable by light petroleum, as shown by the low and equal concentrations of phosphorus found in the light petroleum fractions *A* and *B*. Samples

14–16, Table 3, show that some compound was present which had a partition coefficient near that of the sulphone. We have assumed that this was sulphone, because it was a likely impurity. The quantity estimated is very sensitive to the partition coefficient taken, so that it can be estimated only as 0.8 ± 2%. The remainder, except traces of in-extractable compounds (0.036%), was sulphoxide.

Table 3. *Successive partitioning of OO-dimethyl S-ethylsulphinyethyl phosphorothiolate*

A solution in water (1) was extracted with light petroleum, b.p. 100–120°, twice, yielding light-petroleum fractions *A* and *B*. The aqueous residue was then extracted with benzene, giving a water layer (2) and a benzene layer (*C*). The aqueous residue was then extracted twice with redistilled 1:2-dichloropropane, giving *D* and *E* and leaving an aqueous layer (3). This was extracted five times with methylene dichloride and twice with CHCl₃, leaving seven aqueous layers (4–10). The dichloropropane layers were bulked, extracted once with water (water layer 11) and concentrated 15.2-fold. The concentrate was then extracted four times with water (water layers 12–15), leaving a dichloropropane layer (16). The first methylene dichloride extract was extracted five times with water, giving water layers (17–21). The results were best fitted by assuming that the sample contained 99.16% of sulphoxide, 0.806% of sulphone and 0.036% of compounds inextractable by any solvent. Partition coefficients used in the calculation are given at the bottom of the table. The starred values were calculated from the results, the remainder determined independently.

No.	Phosphorus (µg./ml.)	
	Found	Calc.
1	357, 356	358
2	345, 342	351
3	286, 285	291
4	108, 110	108
5	40.8, 40.8	40.3
6	14.0, 15.3	15.0
7	5.72, 5.68	5.67
8	2.20, 2.13	2.19
9	0.51, 0.62	0.51
10	0.18, 0.19	0.20
11	26.8, 27.2	26.9
12	36.6, 36.6	38.3
13	5.03, 4.96	4.99
14	1.54, 1.47	1.47
15	0.79, 0.78	0.78
16	1.16	1.29
17	66.2, 64.8	68.0
18	42.2, 42.1	42.7
19	27.0, 26.4	26.8
20	16.9, 16.9	16.9
21	10.9, 10.6	10.6
<i>A</i>	0.021, 0.012	0.014
<i>B</i>	0.021, 0.012	0.014
<i>C</i>	7.58, 7.35	7.47
<i>D + E</i>	27.5, 29.2	30.0

	Partition coefficients			
	Light petroleum–water	Benzene–water	Dichloro–propane–water	Methylene dichloride–water
Sulphoxide	0.00001*	0.0189*	0.0955*	1.692*
Sulphone	0.001	0.36	1.70	∞

Cholinesterase, inhibited with the sample, recovered its activity with a half-life of 1.3 hr.

OO-Dimethyl S-ethylsulphonylethyl phosphorothiolate. The crystalline sample given us contained about 2% of compounds not extractable by CHCl_3 . To remove the *S*-ethylthioethyl compound, an aqueous solution, 5 g. in 100 ml., was extracted four times with light petroleum. The sulphone was then separated from the sulphoxide by extracting the aqueous solution twice with 100 ml. of 1:2-dichloropropane. The bulked extracts were washed with 40 ml. of water and

concentrated *in vacuo*, finally at 50° under 0.1 mm. Hg. Phosphorus assay gave 93.8% of sulphone.

Successive partitioning showed only traces of any impurity (Table 4). The discrepancies between the found and calculated phosphorus concentration in samples 9 and 10, Table 4, together with the low CHCl_3 -water partition coefficient, which, determined independently, is about 30, may indicate about 0.2% of sulphoxide.

Cholinesterase inhibited with the compound recovered its activity with a half-life of 1.3 hr.

OO-Diethyl S-ethylthioethyl phosphorothiolate. The sample was originally part of the highly purified compound used by Gardner & Heath (1953). Four years later, 17% was inextractable by CHCl_3 from water. To purify it, it was dissolved in light petroleum, b.p. < 40°, washed twice with water and concentrated *in vacuo*, finally at 50° and 0.1 mm. Hg. The purity was tested by using in turn reversed-phase partition chromatography and a simple form of successive partitioning. The chromatographic methods used were communicated privately by Dr I. K. H. Otter, Fisons Pest Control Ltd., Chesterford Park Research Station, Little Chesterford, Essex.

Table 4. *Successive partitioning of OO-dimethyl S-ethylsulphonylethyl phosphorothiolate*

An aqueous solution (1) was extracted three times with light petroleum, b.p. 100–120°, giving three light-petroleum extracts (2–4) and leaving an aqueous extract (5). This was extracted five times with 1:2-dichloropropane and twice with CHCl_3 , leaving aqueous extracts 6–12. The first dichloropropane extract was extracted five times with water, yielding 5 aqueous extracts, 13–17. Theoretical values are calculated on 99.976% of a compound with K (light petroleum–water) = 0.0010, K (1:2-dichloropropane–water) = 1.70, K (CHCl_3 –water) = 15.7, and 0.024% of compounds inextractable by any solvent.

No.	Phosphorus ($\mu\text{g./ml.}$)	
	Found	Calc.
1	234	233
2	0.26	0.23
3	0.24	0.23
4	0.24	0.23
5	231, 234	232
6	86.0, 87.0	86.1
7	31.3, 33.3	31.9
8	12.2, 11.7	11.9
9	4.27, 4.26	4.42
10	1.64, 1.62	1.67
11	0.159	0.159
12	0.056	0.056
13	54.3, 56.7	54.2
14	33.2, 34.4	34.1
15	20.8, 21.5	21.5
16	13.3, 13.5	13.5
17	8.0, 8.2	8.5

Spots of the sample in cyclohexane were placed on strips of Whatman no. 1 filter paper impregnated with 5% of silicone MS 550 (Hopkin and Williams Ltd.), dried, and equilibrated with moving phase at 25°. In one run the moving phase was 100 ml. of acetone + 15 ml. of water, in the other 100 ml. of ethanol + 15 ml. of water. Both were run by the descending-phase method and developed by Otter's (1955) method. Both gave only one spot, R_f 0.93 and 0.90 respectively. This showed that the sample contained no compounds more oil-soluble than the stated compound.

The compound was dissolved in light petroleum, b.p. < 40°, and extracted with water twice, giving two partition coefficients, 8.0₄ and 8.0₄. The first aqueous extract was back-extracted with light petroleum, giving K 8.30. This showed that any impurity present to more than 1% must have a partition coefficient very near that of the pure compound.

Cholinesterase, inhibited with the compound, recovered its activity very slowly, as expected when a diethyl phosphate inhibitor is used.

Partition coefficients. These are summarized for convenience in Table 5. All five compounds can be extracted from water practically completely by three successive extractions with CHCl_3 .

Table 5. *Partition coefficients of the compounds*

Partition coefficients were determined at room temperature (18–25°) except for those asterisked, which were determined at 4°.

Solvent system	(EtO) ₂ PO ¹	PO ²	PS ³	SO ⁴	SO ₂ ⁵
Light petroleum (b.p. 100–120°)–20% aq. methanol	~0.8	~0.07*	1.70*	—	—
Light petroleum (b.p. 100–120°)–water	~15	1.61	> 20	~0.00001	~0.0010
		1.52*			
Benzene–water	V. large	~20	V. large	0.019	0.36
1:2-Dichloropropane–water	V. large	V. large	V. large	0.096	1.70
Methylene dichloride–water	V. large	V. large	V. large	1.69	18.7
CHCl_3 –water	V. large	V. large	V. large	4.4	~30

¹ (EtO)₂PO = *OO*-diethyl *S*-ethylthioethyl phosphorothiolate.

² PO = *OO*-dimethyl *S*-ethylthioethyl phosphorothiolate.

³ PS = *OO*-dimethyl *O*-ethylthioethyl phosphorothionate.

⁴ SO = *OO*-dimethyl *S*-ethylsulphonylethyl phosphorothiolate.

⁵ SO₂ = *OO*-dimethyl *S*-ethylsulphonylethyl phosphorothiolate.

RESULTS

Reactions of the purified compounds

It was found that, when purified as already described, all but the sulphone gave toxic derivatives on heating, storage or in water. The compounds are now dealt with in turn.

OO-Dimethyl and OO-diethyl S-ethylthioethyl phosphorothiolates

As these compounds produced toxic derivatives most rapidly, and conveniently, in water, most attention was paid to aqueous solutions. The toxicity of a freshly prepared 1% solution of the dimethyl compound increased greatly, the intravenous LD_{50} decreasing from 60 to 2 mg./kg. in one day at 35°. All this increase in toxicity was due to the formation of compounds not extractable by chloroform. About 7% of the compound had decomposed in this time.

To prove that the toxic derivative was not produced by an impurity, e.g. the *S*-methyl isomer, we proved that throughout the decomposition of the compound in water the derivative was produced at the same rate from the same concentration. Table 6 shows that the LD_{50} of the aqueous layer obtained by storing an approximately 1% solution one day is substantially constant up to 80% decomposition. The slight upward drift in LD_{50} as the experiment

proceeded is explained below. An impurity could produce such results only if it possessed both the same solubility properties and the same rate of reaction with water. Qualitatively similar results were obtained with the diethyl compound, though the reaction was about ten times as slow.

We then showed that the rate of formation of toxic derivatives depends on the square of the concentration of the dimethyl compound, using anticholinesterase activity of samples of solution, freed from parent compound by extraction with chloroform, as a measure of their concentration. Table 7 shows that on diluting the parent compound tenfold the rate of production of inhibitor was decreased 100-fold. The phosphorus analyses showed that at any given time the percentage of the total phosphorus in the water layer was similar at both concentrations. Thus most of the phosphorus in the water layer was in non-toxic compounds and the toxic product was formed by a relatively minor side reaction.

The bimolecularity of the reaction explained the upward drift in LD_{50} in Table 6, as the concentrations of the parent compound drifted downwards during the experiment.

Separation and identification of the toxic derivative of the dimethyl compound. As the inhibitor could not be extracted from water by immiscible solvents we concluded that it was probably ionic, and therefore

Table 6. *Production of toxic derivatives from OO-dimethyl S-ethylthioethyl phosphorothiolate in water*

A 1% solution of dimethyl thiolate was stored for one day at 35°. The dimethyl compound was extracted with $CHCl_3$ and the LD_{50} of the aqueous layer determined. A 1% aqueous solution was prepared from the $CHCl_3$ extract and its LD_{50} found, and the solution stored for several days at 35°. It was then re-extracted, the percentage decomposed determined by phosphorus assay of the aqueous layer, and a 1% aqueous solution prepared from the $CHCl_3$ layer. This was again stored for one day and re-extracted, and the LD_{50} of the aqueous layer found.

Days from start of decomposition ...	1	8	19	30
Concn. of compound at start of day (mg./ml.)	9.7	6.7	8.9	6.0
LD_{50} of aqueous layer (intravenous) (mg./kg.)*	0.15-0.22	0.18-0.24	0.27-0.34	0.29-0.46
LD_{50} of $CHCl_3$ layer (intravenous) (mg./kg.)	48	—	35	—
Total decomposition	7.8	43	71	81

* Estimated as the parent compound.

Table 7. *Production of anticholinesterase from OO-dimethyl S-ethylthioethyl phosphorothiolate in 0.0001N-HCl at 37°*

To remove any toxic derivatives produced on storage, a benzene solution was extracted twice with water and a solution of about 5 mg./ml. in 10^{-4} N-HCl prepared rapidly from it. Some was diluted tenfold with acid of the same concentration and both solutions held at 37° in a thermostat. Samples were taken at set times and extracted three times with $CHCl_3$, and the I_{50} dilutions and phosphorus content of the aqueous layers found. The values quoted are I_{50} dilutions; the errors are about $\pm 10\%$.

Initial concn. (mg./ml.)	Concn. of anticholinesterase									
	Time (hr.)									
	2	4.5	6	8	25	48	72	146	312	648
5.08	4600	6600	7600	8500	11 300	13 200	16 800	20 100	19 000	8800
0.508	28	75	66	80	93	120	196	227	227	—

tried to isolate it by electrophoresis. The results are shown in Fig. 1.

The inhibitor appeared in a position indicating that it was positively charged; the displacement of the peak towards the cathode was more than two-thirds of the displacement of phosphate ion at pH 5.3 towards the anode, and the profile of the inhibitor and a phosphorus band were very close. Other phosphorus bands appeared in the neutral position and, as expected for hydrolysis products, towards the anode. The spread of the inhibitor band was due to overloading. Experiments with smaller quantities of material gave a well-defined inhibitor band only about 3 cm. wide, but the phosphorus content of such bands was too small for accurate analysis.

Cholinesterase inhibited by the compound recovered its activity with a half-life of 1.3 hr., showing that the compound was a dimethyl phosphate.

These results indicated that the compound was *OO*-dimethyl *S*-ethylsulphonioethylmethyl phosphorothiolate, formed by the reaction:

To confirm this the apparently identical compound was synthesized. Electrophoresis yielded a positively charged compound in 9% yield with the same I_{50} and hydrolysis rate (Table 8), and moving the same distance in the electrophoretic field.

The isomeric structure,

would also be consistent with most of the results, but is unlikely both for chemical and biochemical reasons. The sulphur atom attached to the phosphorus appears to be generally unreactive; oxidation of the *OO*-diethyl sulphide with hydrogen peroxide can give nearly quantitative yields of sulfoxide in which only the other sulphur is attacked (Heath *et al.* 1955). Biochemically the compound is a much more powerful inhibitor than the parent compound (see Table 11), which could reasonably be explained by the similarity between the groups

and

in acetylcholine. Dr H. S. Hopf (private communication) has shown also that conversion of compounds containing the group $\text{PO}\cdot\text{S}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{NR}_2$ into those containing the group $\text{PO}\cdot\text{S}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{NR}_3^+$ in-

Fig. 1. Electrophoresis of the ionic products from the action of water on *OO*-dimethyl *S*-ethylthioethyl phosphorothiolate at room temperature for 14 days. A 1% solution of the thiolate in 0.005 *N*-HCl was left for 14 days at room temperature (about 18°) and then extracted with CHCl_3 . Three electrophoresis papers (Whatman 3MM, 5.5 cm. $\times$ 36 cm.) were each treated with 0.25 ml. of the aqueous residue at the starting lines, and electrophoresis was carried out with acetate buffer, pH 5.3, at 500 v for 135 min. The papers were cut up into strips 1.5 cm. wide, starting at the starting lines. Equivalent strips from all three papers were bulked, left in 10 ml. of water for 1 hr. and then removed. The aqueous fractions were analysed, for phosphorus and anticholinesterase activity: phosphorus, —; I_{50} dilutions, - - -. A fourth paper strip, treated on the starting line with a few micrograms of schradan and KH_2PO_4 , was run simultaneously, and developed by the methods already described. The centres of the bands are marked by vertical lines. At pH 5.3 phosphate ion exists almost completely as H_2PO_4^- .

creases considerably the activity against the cholinesterase of the locust central nervous system.

The toxic derivative from stored samples. About 4% of a similar compound with very similar toxicological and charge properties was separated from a stored sample of the dimethyl compound. The intravenous LD₅₀ of this sample was about 1 mg./kg.

The toxic derivative from the diethyl compound. Electrophoresis of a stored sample gave a positively charged inhibitor. The ethylsulphonium derivative was synthesized from ethyl iodide as before, and showed the exceedingly toxic properties given in Table 11.

The methylsulphonium derivative of Isosystox was also synthesized and separated. This has already been examined by Fukuto, Metcalf, March & Maxon (1955*b*), but they found an intraperitoneal toxicity to mice of only 1–5 mg./kg., whereas we found the intraperitoneal toxicity to mice to be 0.10 mg./kg. This suggests that their product was probably very impure. Their method of separation would not isolate the compound from by-products, which are formed in quantity.

OO-Dimethyl O-ethylthioethyl phosphorothionate

Action of water. Henglein & Schrader (1955) showed that this compound isomerized to the S-ethylthioethyl isomer in water. The lowest concen-

tration they used was 1%. We wished to study true solutions (<0.2%). We therefore checked that the same reaction took place at such dilutions.

A solution, 0.77 mg./ml. in 9% (v/v) aqueous ethanol, was stored for 24 hr. at 37° and extracted with chloroform. Successive partitioning showed that the only chloroform-extractable compound had a partition coefficient [light petroleum (b.p. 100–120°)–water] of 1.6 ± 0.1 in agreement with that of the thiolate. The yield was, however, only 42%. Thus isomerization takes place, but with lower yields than at higher concentrations.

The intravenous toxicity of a solution of concentration 2 mg./ml. increased in 1 hr. at 37° from 220 to 2 mg./kg. This could not be explained either by the formation of thiolate (LD₅₀ 64 mg./kg., Table 11) or by the subsequent action of thiolate with itself to form the methylsulphonium derivative, which was a relatively slow reaction at this dilution. Thus the increase in toxicity must have arisen from reactions involving the thionate. An experiment designed to provide more evidence for this is described in Table 9.

Table 9 shows that a solution of thiolate alone produced very little inhibitor, as we expected at this low concentration. However, solutions containing thionate produced inhibitor rapidly, and this inhibitor was nearly completely decomposed by

Table 8. *Properties of OO-dimethyl S-ethylsulphonioethylmethyl phosphorothiolate prepared in two ways*

The compound was obtained by the action of water on OO-dimethyl S-ethylthioethyl phosphorothiolate, as described (see Fig. 1) and by the action of methyl iodide (0.1 ml.) on the same compound (0.1 ml.) in ethanol (1 ml.) for 39 hr. at 37°. Hydrolysis rates were calculated from I_{50} dilutions determined on samples taken at various intervals over several weeks from stock solutions. Errors are standard errors.

I_{50} $10^5 k_w$ (min. ⁻¹)	Compound prepared by the action on thiolate of	
	Water	Methyl iodide
	$3.75 \times 10^{-8} M$ 4.05 ± 0.44 in 0.01 N-HCl	$3.95 \times 10^{-8} M$ 3.66 ± 0.11 in 0.01 N-HCl 3.78 ± 0.27 in 0.001 N-HCl

Table 9. *Reactions of OO-dimethyl O-ethylthioethyl phosphorothionate in 0.0001 N-HCl with and without admixture of the thiolate isomer, compared with the reactions of thiolate at 37°*

Three solutions were prepared, containing respectively 0.215 mg. of thiolate/ml., 0.397 mg. of thionate/ml., and 0.215 mg. of thiolate + 0.397 mg. of thionate/ml., all in 9% (v/v) aqueous ethanol, and stored at 37°. Samples taken at various times were extracted with CHCl₃, and the aqueous layers assayed for phosphorus and anticholinesterase activity. The aqueous layers were then stored a further day at 37°, and the I_{50} dilutions redetermined.

Time (min.)	Thionate only (0.397 mg./ml.)			Thionate + thiolate (0.397 + 0.215 mg./ml.)			Thiolate only (0.215 mg./ml.)		
	P in aq. phase (%)	I_{50} dilutions		P in aq. phase (%)*	I_{50} dilutions		P in aq. phase (%)	I_{50} dilutions	
		0 days	1 day		0 days	1 day		0 days	1 day
0	0	1	—	0	1	—	0	1	—
10	42	136	5	41	2370	3	—	5	—
100	67	399	5	68	2500	5	—	—	—
1300	71	9	4	70	61	22	7	17	—

* The percentages in this column are estimated on the thionate alone.

storing for 1 day at 37°. Thus the inhibitor produced from the thionate was not the methylsulphonium derivative of the thiolate, which was considerably more stable than this (see Table 8). Its formation was greatly accelerated by thiolate, indicating that it was formed by a reaction between the thiolate and thionate. This hypothesis accounts also for the finding that, when thiolate was added, most of the inhibitor was formed in the first 10 min., whereas, when thiolate was not added, only one-third of the inhibitor was produced in this time. The rate of formation of inhibitor was probably proportional to the product of the concentrations of thiolate and thionate. Consequently, with thiolate added, the maximum rate would be attained immediately, but in the absence of additional thiolate would only be attained when the product of the concentrations reached a maximum, i.e. when the decomposition of thionate was about half complete. The maximum rate was thus only attained after about 10 min.; for at this time ionic products accounted for 41 % of the thionate phosphorus in both experiments, which, as isomerization proceeded simultaneously, indicated that about half of the thionate had been decomposed by both reactions.

In a further experiment with thionate alone it was shown that the final concentration of inhibitor was roughly proportional to the square of the thionate concentration, which agrees with the hypothesis given above.

Electrophoresis of a sample of the inhibitor from thionate alone showed that it was positively charged. The recovery was 72 %. No phosphorus could be detected in the band, from which it could be shown that the I_{50} was less than 10^{-6} M. The first-order constant for the decomposition of the inhibitor in the presence of other products of the reactions was $7 \pm 1 \times 10^{-3} \text{ min.}^{-1}$ at 37°.

The structure of the unstable inhibitor is considered in the Discussion. Whatever the reaction yielding it, it was a trivial side reaction, for the quantity of chloroform-inextractable phosphorus produced was the same whether thiolate was added or not (Table 9), although eight times as much inhibitor was formed in the first instance as in the second. This observation, and the figures in Table 9, show that the I_{50} of the unstable inhibitor must be less than 10^{-8} M, i.e. the inhibitor was very powerful.

Kinetics of the decomposition of OO-dimethyl S-ethylthioethyl phosphorothiolate in water

It has been shown that OO-dimethyl S-ethylthioethyl phosphorothiolate underwent simultaneous hydrolysis and self-methylation in water. The experiments here showed that another reaction sequence also took place and also affected the toxicity of aqueous solutions. All reactions were carried out in weakly acid solutions, as dialkyl

phosphate esters are less sensitive to acids than to bases.

A solution of thiolate in 0.005 N-HCl (5 mg./ml.) was held at 37° for 2 hr. and extracted with chloroform, and the inhibitor in the aqueous layer determined. The I_{50} dilution was 3800. The inhibitor solution was left for 17 hr. at 37°, and the concentration redetermined. The I_{50} dilution was only 370, i.e. 90 % had decomposed in 17 hr.

Now the only inhibitor found in a thiolate solution left for 14 days was the sulphoniothiolate of half-life 13.2 days (see Table 8). Therefore a less stable inhibitor was produced alongside the sulphoniothiolate in the early stages of the reaction. To eliminate the possibility that an unstable impurity produced this inhibitor the experiment was repeated with thiolate that had been pretreated with water for one day at 37° and then purified by extraction with chloroform. The results were the same; thus the unstable inhibitor was produced from the thiolate.

These observations could be explained if the thiolate was in dynamic equilibrium with thionate, with which, according to the results of the preceding section, it reacted to form the unstable inhibitor. As the two inhibitors differed considerably in their stability in water, mixtures could be analysed by determining the activity immediately, and after one day's storage. The first gave the total inhibitor, the second the stable inhibitor less the small, calculable proportion decomposed in one day. This was the basis of the following experiments, which were designed to check whether the reactions already postulated were adequate to account for the results observed when allowance was made for the formation of unstable inhibitor.

If the simplest theory is applicable, the rate of decomposition of thiolate is given by

$$-dA/dt = k_1 A + k_2 A^2, \quad (2)$$

where A is the concentration of thiolate. The first term is for the first-order hydrolysis, the second for the second-order production of inhibitors. First, this equation was checked experimentally, by using thiolate pretreated for 1 day at 37° with dilute acid.

In very dilute solution $k_2 A^2$ can be ignored. The rate of hydrolysis was determined by Heath's (1956) method, which is based on the assumption that ionic products cannot be extracted from water by chloroform. In 0.094 % solution in 0.005 N-HCl the rate was found to be first-order from 1 to 10 days. Slight deviations were found in the first day, indicating that some constituent of the sample (about 0.4 %) hydrolysed more rapidly; and the apparent rate became a little slower after 10 days. This was shown to be due to the formation of chloroform-soluble products by secondary reactions from the primary products of hydrolysis. However, the first

term of the equation was a very good approximation to the truth in very dilute solution, and, applied to the results, gave $k_1 = 4.50 \pm 0.03 \times 10^{-5} \text{ min.}^{-1}$.

The experiment was repeated at a higher concentration (0.47 %). The rate was initially 28 % greater; it decreased to that of the reaction in more dilute solution as the reaction proceeded, and $k_2 A^2$ became less. Thus the equation fitted the observed results in the form

$$-dA/dt = 4.50 A(1 + 0.2824 A/A_0) \times 10^{-5}. \quad (3)$$

Now the rate of formation of the stable inhibitor is given by:

$$dI/dt = k_3 A^2 - k_4 I, \quad (4)$$

where k_3 is the constant for the rate of formation of the stable inhibitor (and is less than k_2 , which is the constant for the formation of both inhibitors) and k_4 is the constant for hydrolysis of the inhibitor. In pure solution $k_4 = 3.66 \times 10^{-5} \text{ min.}^{-1}$ (Table 8). By a similar experiment it was shown that when 64.4 % of a 0.47 % solution of thiolate had decomposed, $k_4 = 7.26 \times 10^{-5} \text{ min.}^{-1}$. Thus k_4 varied with A . It was assumed that the relationship was linear.

Thus from (3) A could be found by computation at various times. Hence, by computation, and introducing the values for k_4 , I could be found from (4). Now k_3 could not be determined independently, but the only effect of arbitrary changes in k_3 is to change the units in which I is calculated, so that k_3 was taken arbitrarily to be unity. Then $I_{\text{calc.}}$ was a

constant factor times the true value of I . This factor was chosen to give the best fit in the following experiment.

The concentrations of both inhibitors in a solution of thiolate in 0.005N-HCl (0.47 %) were found at various times, and the concentration of the stable inhibitor is compared with those calculated in Table 10.

The agreement between the experimental and calculated concentrations was satisfactory except that the experimental values were somewhat high at the beginning. This indicates that the reactions postulated were the only major reactions leading to the stable inhibitor, but that some other minor reactions might have contributed.

The stable inhibitor accounts for nearly the whole difference between the rates at the two thiolate concentrations. From the residual difference the I_{50} of the unstable inhibitor is found to be $< 10^{-9} \text{ M}$. The higher concentration is near saturation, which may affect the rate. Even so, the I_{50} is unlikely to be greater than $5 \times 10^{-9} \text{ M}$.

OO-Dimethyl S-ethylsulphinylethyl phosphorothiolate

On storing the purified compound for several months at room temperature its toxicity increased, the LD_{50} by intravenous injection decreasing from 48 to 5 mg./kg. Greater increases were found after heating. The increases were due entirely to compounds not extractable from water by chloroform.

Table 10. Concentrations of OO-dimethyl S-ethylsulphonio-methylethyl phosphorothiolate produced by the action of 0.005N-HCl on the sulphide at 4.70 mg./ml. concentration at 37°

A nearly saturated solution of OO-dimethyl S-ethylthioethyl phosphorothiolate was left for one day at about 20° in 0.005N-HCl to remove any unstable impurities. The compound was purified by extraction into benzene, and a solution (0.47 %) in 0.005N-HCl prepared rapidly from it. The solution was held at $36.98 \pm 0.03^\circ$ throughout the experiment. At set times samples were removed and extracted rapidly 3 times with CHCl_3 , and the I_{50} dilutions of the aqueous layers determined immediately. The aqueous layers were then stored and the I_{50} dilutions redetermined 1 and 2 days after sampling. From the concentrations found at 1 and 2 days the concentration of the stable inhibitor (sulphoniothiolate) at the times of sampling were calculated (penultimate column). The concentrations of the unstable inhibitor were calculated from the difference between the observed I_{50} dilutions at sampling and those calculated for the stable inhibitor. In the last column are given the theoretical concentrations of the stable inhibitor, computed as described in the text. Some I_{50} dilutions were obtained from I_{50} graphs showing marked scatter. These are less accurate, and are marked ~. Other results are believed to be reliable to better than $\pm 10\%$. The concentrations of transient inhibitor after 3094 min. were less than the experimental errors of the determinations, so no values are given.

Time (min.)	Concn. of inhibitor (arbitrary units)					
	Observed			Transient inhibitor	Sulphoniothiolate at sampling	Sulphonio- thiolate (theor.)
	Total at sampling	1 day later	2 days later			
0	175	7	—	170	7	0
237	5 400	870	850	4430	918, 974	830
1 414	8 750	4 800	3 840	3800	4 950, ~ 4 300	4 520
3 094	11 500	8 200	8 280	2100	~ 8 700, 9 420	8 670
8 654	15 100	13 600	11 750	—	14 700, 13 800	15 400
11 550	17 000	14 300	13 260	—	~ 15 600, 15 800	16 000
18 720	—	13 770	11 290	—	13 800, 14 100	13 800
21 530	13 400	11 040	—	—	12 100	12 300
31 690	7 370	—	—	—	~ 7 000	7 070

No detectable toxic derivatives were found on storing an aqueous solution for one day.

The sulfoxide cannot form a methylsulphonium derivative directly but, if reduction to the thiolate takes place, the thiolate can be methylated in the usual way. The oxygen acceptor could be either the sulfoxide itself:

or degradation products.

We therefore looked for thiolate in aged and heated samples, using successive partitioning. The procedure was exactly as described under Table 3, except that the light petroleum layers were subjected to successive partitioning with water. Both from the aged sample and from one heated for 16 hr. at 76° about 5% of the total phosphorus was extractable by light petroleum, with a light petroleum-water partition coefficient of 1.60 ± 0.05 , in excellent agreement with that of the thiolate.

The proportion of sulphone was unchanged. This could imply either that sulphone was not formed from sulfoxide by the above reaction or that it was formed but was unstable, so that the final concentration of sulphone was similar to the initial concentration. It was shown by adding sulphone to the sulfoxide, heating for 16 hr. at 76° and analysing the product by successive partitioning, that the sulphone was not decomposed appreciably. Thus

the oxygen acceptor was not the sulfoxide sulphur. In all experiments more phosphorus was found as compounds inextractable by chloroform than was found as thiolate; so that there were no inconsistencies in the hypothesis that degradation products were the oxygen acceptors.

OO-Dimethyl S-ethylsulphonylethyl phosphorothiolate

Heating the purified compound for 16 hr. at 76° left it unchanged, except for the production of 1% of compounds inextractable by chloroform. The aqueous fraction after chloroform extraction was not toxic.

Toxic compounds were formed from the original sample on heating. They were therefore produced from impurities, probably the sulfoxide. The purified compound did not form toxic derivatives.

Toxic properties of the compounds

The I_{50} values, and the oral and intravenous toxicities to rats of the purified compounds, are given in Table 11, those precautions being taken in their determination that are indicated by the results of previous sections.

OO-Dimethyl *S*-ethylthioethyl phosphorothiolate and its thionate isomer reacted too rapidly with water to be applied as aqueous solutions or suspensions, and were therefore injected, undiluted, by micro-syringe. The remaining compounds were made up as aqueous solutions immediately before use. A possible impurity, in addition to those

Table 11. *Toxic properties of the compounds*

95% fiducial limits are given below values obtained on sixteen rats. In those instances marked (-) all four rats died at twice the dose at which all four survived, so that no limits can be calculated.

Compound	LD ₅₀ values to white rats (mg./kg.)		I_{50} (M)
	Intravenous	Oral	
<i>OO</i> -Dimethyl <i>S</i> -ethylthioethyl phosphorothiolate	64.6, ♀ (54.4-76.8)	62.5, ♀ (45.5-85.9)	6.5×10^{-5}
<i>OO</i> -Dimethyl <i>O</i> -ethylthioethyl phosphorothionate	216, ♀ (-)	676, ♀ (-)	—
<i>OO</i> -Dimethyl <i>S</i> -ethylsulphinylethyl phosphorothiolate	47.2, ♂ (-)	65, ♀ (54.4-76.8)	4.1×10^{-5}
<i>OO</i> -Dimethyl <i>S</i> -ethylsulphonylethyl phosphorothiolate	21.7, ♀ (17.5-27.0) 23.7, ♂ (-)	32.4, ♀ (27.3-38.5)	2.3×10^{-5}
<i>OO</i> -Diethyl <i>S</i> -ethylthioethyl phosphorothiolate	—	—	5.4×10^{-6}
<i>OO</i> -Dimethyl <i>S</i> -ethylsulphonioethylmethyl phosphorothiolate	0.0615, ♀ (0.0523-0.0724)	9.8, * ♀	3.9×10^{-8}
<i>OO</i> -Diethyl <i>S</i> -ethylsulphonioethylmethyl phosphorothiolate	0.0160, ♀ (0.0143-0.0180)	20, † ♀	4.7×10^{-9}
<i>OO</i> -Diethyl <i>S</i> -ethylsulphoniodiethyl phosphorothiolate	0.010, * ♀	—	2.6×10^{-9}

* Approximate values obtained on 6-8 rats.

† This dose killed in two days, higher doses considerably more rapidly. Doses of 5 mg./kg. showed no effect.

already discussed, in all the dimethyl compounds before purification was tetramethyl pyrophosphate. Except for the thionate, every compound was left long enough in water during purification to degrade this practically completely. The very low toxicity of the thionate and the peculiar symptomatology it produced, described below, showed that tetramethyl pyrophosphate was absent from this compound also.

The I_{50} of the thionate could not be determined, because it reacted so rapidly with water. That of the thiolate isomer was determined by making up an aqueous solution of about $2 \times 10^{-4}M$ very rapidly in water, and using it immediately. At this dilution the formation of methylsulphonium derivatives is too slow to influence the results appreciably.

Intravenous injection of all compounds except the thionate produced typical symptoms of anticholinesterase poisoning in rats at doses close to the LD_{50} . The thionate, however, produced immediate inco-ordination, followed by accelerated respiration and weakness lasting several hours. Lethal amounts produced deep anaesthesia (with occasional jerking) which lasted about 30 min. Respiration then became more and more rapid until the animal died within 1–2 hr. At no stage were typical anticholinesterase symptoms observed. A similar, but weaker, anaesthetic stage was observed in rats injected intravenously with the thiolate isomer, but this was followed by the typical symptoms of anticholinesterase poisoning, from which the animals died. In neither case did injection of about 40 times the organic solvent in the samples induce any noticeable symptoms. Oral administration produced typical anticholinesterase symptoms with all compounds including the thionate. The duration of symptoms was markedly longer than after intravenous injection. The methylsulphonium compounds of both dimethyl and diethyl phosphorothiolate were very much less toxic orally than intravenously, although no accurate figure was obtained for the diethyl homologue. The symptoms from this compound developed exceptionally slowly, leading with fatal doses only to death after 24 hr. Bracketing the LD_{50} was therefore very difficult, especially as we had only a few milligrams of the compound.

A detailed study of the toxic action of some of these compounds is given by Vandekar & Heath (1957).

DISCUSSION

Although the tests of purity in the first section of the results were considerably more sensitive than the combination of elementary analysis and infrared spectroscopy used by most of those working with organophosphorus compounds, they were necessarily incomplete. The impurities which may not have been revealed were the *S*-methyl isomers. It is possible that the rates of recovery of cholin-

esterase inhibited by the various compounds would have indicated the presence of these compounds, as the rate of recovery is dependent upon the less easily replaced groups, i.e. R and R' in $RR'PO \cdot X$. The sensitivity of this test would depend on the relative I_{50} values of the isomers, about which nothing is known. However, the study of the reactions undergone by the compounds constituted a further series of tests of purity, as we were not forced to postulate impurities in order to account for the numerous products revealed. We therefore feel reasonably confident in treating the compounds as pure in the discussion that follows.

The reactions undergone by samples of four of the compounds in water and on storage profoundly affected their toxicological and biochemical properties, and presented some interesting chemical features.

In dilute solution in water *OO*-dimethyl *O*-ethylthioethyl phosphorothionate reacted very rapidly (half-life, about 10 min. at 37°). The main products were ions, with no detectable toxic properties, and the thiolate isomer in 30% yield. A very powerful, positively charged anticholinesterase was also formed, which was unstable in water (half-life, 100 min. at 37°). Much more of this was formed by adding thiolate to the thionate (Table 9); so, whatever its structure, it appears certain that it was produced by a reaction between thionate and thiolate.

Both *OO*-dimethyl and *OO*-diethyl *S*-ethylthioethyl phosphorothiolate underwent reactions in water and on storage. The main products were non-toxic ions, but, in addition, self-alkylation led to alkylsulphonium derivatives. Various types of evidence established that the main toxic product from the dimethyl homologue was its methylsulphonium derivative, and that in water it was produced by second-order self-alkylation [see equation (1) above]. This compound was very toxic, administered by intravenous injection (LD_{50} , 0.062 mg./kg.), a powerful inhibitor of cholinesterase (Table 11) and fairly stable in water (Table 8). The diethyl homologue underwent similar reactions at about one-tenth the rate. Simultaneously, however, the dimethyl compound produced an unstable inhibitor very similar to that produced by mixtures of thionate and thiolate. This (see Table 10) contributed considerably more to the increase in the anticholinesterase activity of solutions of the dimethyl compound in the first few hours than the stable inhibitor formed simultaneously, but contributed relatively little after a few days storage. These results can be explained if the thiolate was in dynamic equilibrium with the thionate, with which it reacted to form the unstable inhibitor. The equilibrium would be heavily on the side of the thiolate.

Because of the complexity of these reactions it was essential to show that the reactions postulated could account quantitatively for the formation of the stable inhibitor. Only in this way could we feel any confidence that no reactions of any importance producing toxic derivatives had been obscured. Table 10 shows that the experimental results were in good agreement with theory, but indicates that some minor reaction may also have contributed during the first day or two.

The thiolates and thionate underwent similar reactions on storage. The main products were non-toxic ions and, from the thionate, its thiolate isomer.

the complex then reacting according to the equation

Some alkylsulphonium derivatives were, however, formed, and in sufficient yield to increase the intravenous toxicities of old samples of the dimethyl thiolate and thionate 100-fold. *OO*-Dimethyl *S*-ethylsulphinyethyl phosphorothiolate on storage at room temperature and on heating was partially converted into the thioethyl compound, which then reacted to form alkylsulphonium compounds. The intravenous toxicity was thereby increased about 40-fold. The main products were non-toxic ions. Storage in 1% aqueous solution for one day did not, however, affect the toxicity.

These reactions are in some ways surprising. Thus both of the thiolates, the thionate and the sulphoxide produced ionic non-toxic compounds in the absence of water. Water accelerated the reactions in the case of all but the sulphoxide, but was plainly not necessary. Also the dimethyl thiolate reacted much more slowly with water than the dimethyl thionate did, which is directly contrary to theoretical expectation and to what is observed with similar isomers (Heath, 1956). Work is proceeding at present on these problems, which appear to be related to the fact that we obtained smaller yields of thiolate from thionate dissolved in water than Henglein & Schrader (1955) obtained from thionate suspended in water.

The simplest theory to account for the formation of the unstable inhibitor is that the thiolate methylated the thionate to give the methylsulphonium derivative of the thionate:

Against the structure proposed for the compound were the facts that it decomposed more rapidly in water than its thiolate isomer ($k = 7 \times 10^{-3} \text{ min.}^{-1}$ at 37° against $3.7 \times 10^{-5} \text{ min.}^{-1}$ at 37°) and that it was a more powerful anticholinesterase ($I_{50} = 5 \times 10^{-9} \text{ M}$ against $3.8 \times 10^{-8} \text{ M}$). Another possibility which may avoid these difficulties is that the unstable inhibitor was formed by the reaction of the thiolate with the transition complex in the thiolate-thionate equilibrium. Thus, adapting the mechanism of isomerization suggested by Fukuto & Metcalf (1954) to equilibrium between thionate and thiolate, their equation becomes

More detailed study was impossible because, owing to its instability, we could not separate enough to carry out analyses. There is therefore some doubt about its structure, and we shall refer to it below as the unstable inhibitor.

The reactions yielding toxic compounds are probably not confined to compounds of the types described in this paper. These reactions are self-alkylations, which implies that dialkyl phosphate esters can act as alkylating agents (Heath, 1957), though many may be very weak. Any dialkyl phosphate ester containing an acceptor atom, such as binary sulphur or ternary nitrogen, will therefore alkylate itself. It is probable that the acceptor atoms have to be in certain positions in the molecule relative to the phosphorus atom if the products are to be more toxic than the parent compounds, but, whether the products are more or less toxic than the parent compounds, the effect is a change in biochemical and toxicological properties on storage which may lead to inconsistent results. It is advisable, therefore, to check the purity frequently of any compound which might undergo such reactions during biochemical work with it.

We now consider the effects on biochemical and toxicological properties of the reactions already discussed, and some of their implications.

The intravenous toxicities of dimethyl thiolate and thionate increased rapidly on storage at room temperature. Although no exact experiments have been carried out, it appears likely that the original toxicity of the pure compound was at least doubled in a week. The conversion into alkylsulphonium derivatives far outweighed in its effect conversion into inert ionic products, although this was more rapid. For example, a 2-year-old sample of the

thiolate contained 56 % of inert ionic products, and 4 % of sulphoniothiolate and was about 100 times as toxic as the pure compound. In water the thionate increased in toxicity very rapidly. The increase was dependent on the concentration, and in a 0.2 % solution was over 100-fold. The increase in toxicity of thiolate solutions at 37° was slower, and was in the first day mainly to be accounted for by the formation of the unstable inhibitor. After this the stable inhibitor became the more important. In nearly saturated solutions (1 %) the intravenous toxicity was increased by a factor of a hundred. The toxicity of the diethyl thiolate increased relatively little on storage and in water, because the decomposition of the compound to inert compounds nearly kept pace with the formation of alkylsulphonium derivatives. The symptomatology was, however, changed somewhat.

In biochemical work with organophosphorus compounds the high toxicities of samples contaminated with minute traces of sulphonium derivatives underline the necessity for proving that no physical or physicochemical methods of fractionation can change the properties of samples under test. Aldridge & Davison (1952) had already shown that many results initially attributed to various diethyl phosphate esters in fact arose from contamination with tetraethyl pyrophosphate. Both in their work and ours no purely chemical or physical method of analysis existed that was sensitive enough to detect impurities in quantities able to control completely any biochemical observation made. Probably the group of compounds we have studied are extreme in their behaviour, but the need for caution is obvious in interpreting results obtained even with compounds subjected to stringent tests of purity.

The reactions of the dimethyl thiolate and thionate are expected to lead to difficulties in obtaining reproducible results with the insecticide Metasystox, which is a mixture of the isomers. The history of a sample of Metasystox from the factory to its degradation in the crop reveals a very large number of factors which are essentially uncontrollable and which may not influence the action of other systemic insecticides to the same extent. The time for which and the temperature at which the sample is stored will affect its constitution, as isomerization of thionate to thiolate, breakdown to non-toxic ionic products, and self-alkylation to toxic products take place on storage. Similar factors will influence the same reactions in the spray tank when the spray is made up. During and after spraying the aqueous spray is concentrated, leading to a considerable acceleration in the formation of toxic derivatives. How far these reactions proceed will depend on the rates at which the various constituents are absorbed by the leaves, or dissolved in or adsorbed on non-polar constituents on the leaf

surface, factors in turn dependent upon such variables as humidity, temperature and wind-speed. What is found inside the leaf will depend on the relative rates of absorption of the compounds, and their relative stabilities in the plant in question. Possibly also the plant itself may methylate these compounds to sulphonium derivatives. We conclude that some variations in toxicity to insects may be observed under different conditions. Dr Harlow and Dr Hopf (I.C.I. Ltd., Jealott's Hill Research Station, Bracknell, Berks) have informed us privately that our sample of the sulphoniothiolate showed only weak contact action against some insect species. Fukuto, Metcalf, March & Maxon (1955b) found, however, that the methylsulphonium derivative of Isosystox exerted systemic action, and Heath *et al.* (1955) found, in crops treated with Isosystox, a non-chloroform-extractable anticholinesterase, which it is now reasonable to suppose was its ethylsulphonium derivative.

The presence of alkylsulphonium derivatives in crops at the time of harvesting may lead to an overestimate of the toxic content of the crop when analyses are carried out by determinations of the anticholinesterase activity of plant extracts. These derivatives are very powerful anticholinesterases, but relatively non-toxic when taken by mouth (Table 11). In this context the margin of safety is increased.

These considerations are probably relatively unimportant with the sulphoxide, which produced toxic derivatives relatively slowly on storage and in water, and probably do not apply to the sulphone, which appeared to be a stable compound.

On the assumption that our compounds were pure we can discuss their toxic properties as summarized in Table 11.

The intravenous toxicity increased up the dimethyl phosphate series thionate, thiolate, sulphoxide and sulphone, as up the diethyl phosphate series of homologues (Fukuto, Metcalf, March & Maxon, 1955a). The compounds were, however, markedly less toxic than their homologues, and weaker inhibitors of cholinesterase. The oral and intravenous LD₅₀ values are very similar, in spite of the slower rate of absorption after oral administration. This suggests either that the compounds persisted in the body (Vandekar & Heath, 1957) or that they were converted into more toxic compounds.

The sulphonium derivatives of the thiolates were all highly toxic by intravenous injection, and were powerful inhibitors of cholinesterase. There is marked similarity between the $\text{OP} \cdot \text{S} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{SR}_2^+$ grouping in these compounds and

in acetylcholine, which probably results in strong adsorption on the anionic site of cholinesterase.

Orally, they were relatively much less toxic, perhaps because their ionic charge interfered with absorption. It was impossible to determine the LD_{50} values of the unstable inhibitor, as not enough was separated. It must, however, have been of comparable toxicity to the other methylsulphonium compounds, as the increase in water in the toxicity of aged samples of thionate and thiolate was considerable (20–22 mg./kg.), although in similar experiments with pure compounds the phosphorus content of the compound was undetectable (Table 9). As already stated, the I_{50} of the unstable inhibitor was at most 5×10^{-9} M.

The oral LD_{50} values of stored thiolate samples containing a few per cent of the sulphonium derivatives were similar to that of the pure thiolate. The effect of the impurities was obvious only when samples were injected intravenously or tested against cholinesterase *in vitro*.

The thionate did not produce cholinergic symptoms when given intravenously, but produced an anaesthetic effect. This was also observed with the thiolate, though in this instance cholinergic symptoms developed later. The observations led to the work described by Vandekar (1957), who showed that several phosphate esters of low toxicity produce anaesthetic effects. Given orally, both compounds produced cholinergic symptoms only. The metabolic reactions that these compounds undergo *in vivo* are being investigated.

It should be plain from the preceding discussion that the characteristic and marked differences in the properties of the compounds, both chemical and biochemical, appeared only because of the care taken in purifying them.

SUMMARY

1. *OO*-Dimethyl *S*-ethylthioethyl phosphorothiolate and its diethyl homologue, thionate isomer, sulphoxide and sulphone have been purified by solvent-extraction procedures, and their purities tested. Their oral and intravenous LD_{50} values to rats and I_{50} values against cholinesterase have been determined.

2. On storage or in water the intravenous toxicities of all but the sulphone increased 40- to 100-fold, owing to the formation of alkylsulphonium derivatives. Thus the thioethyl thiolates alkylated themselves to alkylsulphonium thiolates; the dimethyl thioethyl thiolate appeared to be in dynamic equilibrium with its thionate isomer, with which it reacted to give a sulphonium derivative; and the sulphoxide gave some thioethyl thiolate, which was also alkylated to sulphonium compounds. The sulphone was stable.

3. The alkylsulphonium compounds were very powerful anticholinesterases, very toxic by intra-

venous injection (LD_{50} values of 0.01–0.06 mg./kg.), but much less toxic orally.

4. Sulphonium compounds present in amounts below the limits of physical and chemical detection can interfere with biochemical and toxicological observations. The importance of using biochemical tests of purity is stressed.

5. The effects of these facile reactions on the study of biochemical and toxicological effects are pointed out with reference to insecticidal action, analysis of residues in crops and the practical precautions which have to be taken to obtain reproducible LD_{50} values by intravenous injection.

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