Further Studies on S-Substituted Phosphorothioic Acids

III *. Rates of Hydrolysis and Dissociation Constants

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1. An investigation of the rate of hydrolysis in the pH-range 1—7 of 10 compounds containing the $-SPO_3^{2-}$ group has shown the monoanion $RSPO_3H^-$ to be the most easily hydrolyzable ionic species.

2. Dissociation constants of the monoanion $RSPO_3H^-$ have been determined for 11 compounds.

3. The mechanism of hydrolysis and the influence of substituents on the rate of hydrolysis is discussed.

Under certain conditions S-substituted phosphorothioic acids of the general formula $RSPO_3H_2$ are easily hydrolyzed to orthophosphate and a thiol (RSH). A half life of less than one minute was thus found for the investigated compounds in 1 M HCl at 100°. A study of the effect of pH on the rate of hydrolysis in the region pH 1—7 has shown a maximum rate of hydrolysis for all compounds in the pH region 2—4. At pH values above 7 the rate of hydrolysis of the S—P bond in S-substituted phosphorothioic acids is so slow that it is immeasurable with the technique employed at the lower pH values.

The rate maximum observed at pH 2—4 was suggested by Herr and Koshland [1], who studied S-butyl phosphorothioate, to be due to the monoanion $(RSPO_3H^-)$, which was implied to be the reactive ionic species in the hydrolysis. However, no measurements of dissociation constants were performed to substantiate the theory. Åkerfeldt [2] determined the apparent dissociation constants for cysteamine S-phosphate at 25°. The maximum rate of hydrolysis of this compound coincides well with the maximum concentration of the mono-anion. Ramsay [3] has later reinvestigated the rate of hydrolysis of S-butyl phosphorothioate and confirmed Herr and Koshland's suggestion that the monoanion is the most reactive ionic species. However, Ramsay also finds the undissociated acid $(RSPO_3H_2)$ to be quite easily hydrolyzed.

The availability of a number of S-substituted phosphorothioic acids (Part II of this series [4]) has now made possible a more general study of the S—P

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* Part II: *Acta Chem. Scand.* **16** (1962) 1897.

bond in this class of compounds. In the present paper a study of the hydrolysis in the pH-region 1—7 of 10 compounds of this category has been performed. The results are discussed with special emphasis on the monoanion possibly beeing the most reactive ionic species in the hydrolysis within the investigated pH-range. Furthermore, the influence on the rate of hydrolysis of certain substituents has been studied and led to propositions regarding the mechanisms of hydrolysis.

pH OPTIMUM OF THE HYDROLYSIS

If the monoanion ($RSPO_3H^-$) were the sole ionic species responsible for hydrolysis, the first order rate of hydrolysis constant (k_{obs}) should be proportional to [$RSPO_3H^-$] and the curve k_{obs} versus pH should be a bell shaped one. [$RSPO_3H^-$] is given by the expression:

$$[RSPO_3H^-] = C/\left(1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}\right) \tag{1}$$

where [] denotes concentration, K_1 and K_2 the first and second dissociation constants of $RSPO_3H_2$, $C = [RSPO_3H_2] + [RSPO_3H^-] + [RSPO_3^{2-}]$.
(1) gives a maximum of [$RSPO_3H^-$] when [H^+] $= \sqrt{K_1K_2}$, i.e. when pH $= 1/2\ (pK_1 + pK_2)$.

A perfect fit of the experimental curve k_{obs} versus pH to the one calculated from (1) would establish beyond doubt that the monoanion were the sole ionic species responsible for the observed hydrolysis. However, since pK_1'-values of the S-substituted phosphorothioic acids were found to lie in the approximate region $pK_1' = 1—2$, they are difficult to determine with any degree of accuracy. From the following it will be seen, however, that knowledge of pK_1' is not an absolute requirement for the discussion if it is limited to pH-values greater than that were maximum rate of hydrolysis is observed.

If the monoanion were the sole rate determining factor in the hydrolysis, half maximal rate of hydrolysis should occur when the concentration of the monoanion is 50 % of its maximal value. At these points pH should equal the respective pK' values of the acid. If the point for half maximal rate is chosen on the branch of the bell shaped curve which is closest to higher pH values, this point should equal pK_2'. For the compounds under study pK_2' was found to lie in the range 4.4—5.9 at 35° ($I = 0.35$) and was therefore easy to determine with accuracy. It is evident from Table 1 that a very good agreement is obtained between pH at 50 % maximal rate of hydrolysis and pK_2' for all investigated compounds. The dianion ($RSPO_3^{2-}$) has thus been shown to be inactive, or nearly so, as a rate determining factor in the hydrolysis of S-substituted phosphorothioic acids.

A possible influence of undissociated acid ($RSPO_3H_2$) on the rate of hydrolysis at pH values smaller than that of rate maximum is possible in view of the findings of Ramsay [3]. By plotting k_{obs} versus pH (from Table 1) curves are obtained which usually are very nearly symmetrical on each side of the maximum. In some cases (e.g. for compound 3, Fig. 1) the curve is noticeably assymmetrical in such a way as to indicate a more rapid hydrolysis of the

Table 1. Rate of hydrolysis constants and pK_2'-values. Measurements were performed at $(34.8 \pm 0.2)°C$; pK_2'-values were determined at an ionic strength of 0.35, which corresponded closely to the ionic strength of the buffers used in the hydrolysis experiments.

No.	Formula of acid	$k_{obs} \, 10^2 \, (min^{-1})$ at pH [a]							Conditions at maximum rate of hydrolysis		pK_2'	pH at half maximal rate of hydrolysis [b,f]
		0.60	1.16	2.47	3.34	4.19	5.19	6.23	pH (g)	$k_{max} 10^2 (min^{-1})$ (b)		
1	$C_2H_5SPO_3H_2$	1.82	3.35	4.95 ± 0.15	4.95	4.57	3.63	1.05	3.0	5.05	5.90 ± 0.03 [e]	5.8
2	$HOCH_2CH_2SPO_3H_2$	2.94	5.10	5.85 ± 0.28	5.91	5.70	3.61	0.72	3.0	6.00	5.45 ± 0.02	5.5
3	$HOCH_2CH_2CH_2SPO_3H_2$	—	3.50	4.42	4.76 ± 0.16	4.19	3.02	0.98	3.5	4.76	5.61 ± 0.02	5.6
4	$NH_2CH_2CH_2SPO_3H_2$	—	—	—	3.86	3.47	1.49	—	3.0	4.9 [c]	4.65 ± 0.05	4.5 [c]
5	$NH_2(CH_2)_3SPO_3H_2$	—	3.82	4.14 ± 0.23					2.5	4.15	5.12 ± 0.02	5.0
6	$CH_3NHCH_2CH_2SPO_3H_2$	2.06	2.69	2.69 ± 0.18	2.34	1.75	0.46	—	2.0	2.85	4.63 ± 0.01	4.5
7	$(CH_3)_2\overset{+}{N}CH_2CH_2SPO_3H_2$	1.38	1.65	1.77 ± 0.07	1.62	1.08	0.20	—	2.5	1.77	4.37 ± 0.01	4.4
8	$(CH_3)_3\overset{+}{N}CH_2CH_2SPO_3H_2$	1.27	2.11	2.11 ± 0.12	2.02	1.67	0.76	—	2.0	2.14	4.91 ± 0.02	4.9
9	$[(CH_3)_2CH]_2NCH_2CH_2SPO_3H_2$ $\overset{NH}{\diagdown}\,\overset{+}{N}H$	1.10	1.27	1.44 ± 0.06	1.18	0.94	0.30	—	2.5	1.44	4.65 ± 0.01	4.6
10	$\underset{\overset{+}{N}H_3}{\overset{NH}{C}}NHCH_2CH_2SPO_3H_2$	—	2.96	3.85 ± 0.12	3.25	2.78	1.24	—	2.5	3.85	5.02 ± 0.01	4.9
11	$\underset{\overset{+}{N}H_3}{\overset{NH}{C}}NHCH_2CH_2CH_2SPO_3H_2$	2.52	3.63	4.44 ± 0.32	4.14	3.82	2.46	0.64	2.5	4.44	5.35 ± 0.01	5.4
12	$HOOCCH_2CH_2SPO_3H_2$ [d]	—	—	—	—	—	—	—	4.0	6.4	—	—

[a] variations in pH before and after hydrolysis was less than ± 0.04. Errors listed in k_{obs} are standard deviations (see text).
[b] values extrapolated from k_{obs} *versus* pH curves.
[c] values calculated from data in Ref.[2]; pK_2'-value redetermined at ionic strength 0.35 and 34.8°.
[d] values calculated from data in Ref.[11]
[e] maximal deviations from the mean.
[f] uncertainty estimated to ca. ± 0.1 pH unit.
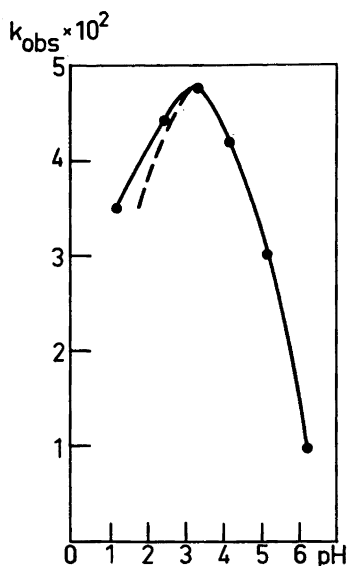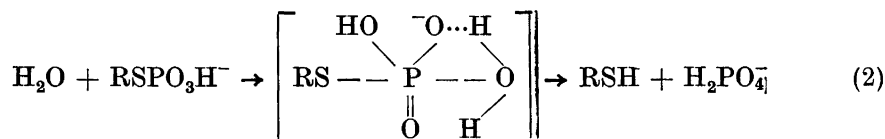[g] estimated to the nearest 1/2 pH unit.

Fig. 1. Rate of hydrolysis of S-(3-hydroxy-propyl) phosphorothioate (4 mM) as a function of pH. Complete symmetry around the maximum is indicated by the dotted line.

undissociated acid than of the dianion. However, a closer study of this question is outside the scope of the present investigation. The fact that a maximum rate of hydrolysis is obtained when the concentration of the monoanion is maximal, or very nearly so, indicates that the rate of hydrolysis of the undissociated acid is less than that of the monoanion. The monoanion has thus been found to be the most active ionic species in the hydrolysis of all studied S-substitued phosphorothioic acids.

According to the scheme suggested by Herr and Koshland [1] for the hydrolysis of the monoanion of S-butyl phosphorothioate the negatively charged oxygen atom of the phosphate residue should activate a water molecule and receive a proton from it. The proton of the undissociated HO group of the phosphate residue is simultaneously transferred to sulfur forming a thiol:

$$H_2O + RSPO_3H^- \rightarrow \left[\begin{array}{c} HO \quad {}^-O{\cdots}H \\ RS --- P --- O \\ \parallel \quad \diagdown \\ O \quad H \end{array} \right] \rightarrow RSH + H_2PO_4^- \qquad (2)$$

A similar mechanism of hydrolysis of certain mono orthophosphates has been discussed by Vernon [5]. For an intermediate or transition state containing water, *cf.* Haake and Westheimer [12]. A recent discussion of phosphate ester hydrolysis has also appeared in Ref.[14]

It is shown in the following paragraph of this paper that the most likely mechanism of hydrolysis is a direct phosphorylation of water according to (2).

Jaenicke and Lynen [6] write about the results of Herr and Koshland: "the fact that acid catalysis is not observed may also be interpreted, however,

by assuming that the approach of the proton to sulfur is hampered because of the high charge density at the sulfur, whereas completely ionized phosphate precludes the approach of OH^-". It would seem, however, that the experimental results presented in the present paper have proven a direct involvement of the monoanion as such in the hydrolysis of S-substituted phosphorothioic acids in the studied pH-range.
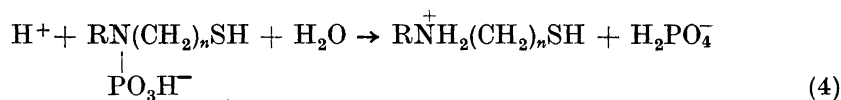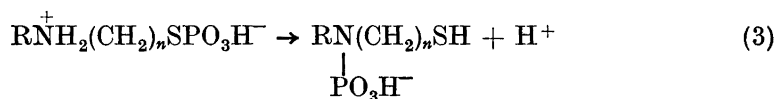
FATE OF THE PHOSPHORYL GROUP DURING HYDROLYSIS

Complete hydrolysis of S-substituted phosphorothioic acids in aqueous solution results in the quantitative formation of a thiol and of orthophosphate.

During the hydrolysis, which follows first order kinetics, the phosphoryl group in most of the investigated compounds could principally be split off in two ways: (a) by direct reaction with water, or (b) by the intermediary formation of O- or N-phosphorylated compounds, obtained by intramolecular transfer of the phosphoryl group to a substituent on the carbon chain (cf. eqn. (3)).

Alternative (b) is excluded as far as the hydroxyl group containing substances compounds (Nos. 2 and 3 in Table 1) are concerned since it was shown in an earlier paper [4] that phosphate group migration can not be demonstrated to occur in these compounds.

Alternative (b) is excluded also for S[2-(trimethylammonium)ethyl], for S[2-(dialkylammonium)ethyl] and for S-alkyl phosphorothioates since they lack accepting hydrogen atoms.

$$R\overset{+}{N}H_2(CH_2)_nSPO_3H^- \rightarrow \underset{\underset{PO_3H^-}{|}}{RN(CH_2)_nSH} + H^+ \qquad (3)$$

$$H^+ + \underset{\underset{PO_3H^-}{|}}{RN(CH_2)_nSH} + H_2O \rightarrow R\overset{+}{N}H_2(CH_2)_nSH + H_2PO_4^- \qquad (4)$$
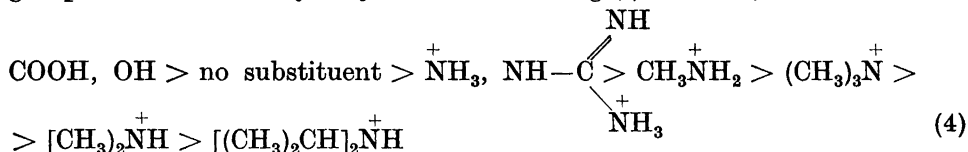
It is evident from (3) that the formation of an N-phosphate is accompanied by the release of one proton. Attempts to demonstrate proton formation at the pH of maximal rate of hydrolysis were, however, unsuccessful. The formation of stable or relatively long lived N-phosphorylated intermediates can therefore be excluded. A rapid hydrolysis of the N—P bond in such intermediates would not much change the pH as seen from (4). However, if this were the mechanism one would expect a greater difference in hydrolysis rates between compounds having and those lacking a phosphorylable N-atom, than those actually observed. Similarly, compounds containing a carboxyl group could give rise to acyl phosphates which can be analyzed for by the hydroxylamine method of Lipmann and Tuttle [7]. No traces of acyl phosphates could be demonstrated in such experiments.

In conclusion, the most likely mechanism for the formation of orthophosphate by hydrolysis of S-substituted phosphorothioic acids in aqueous medium is by a direct transfer of the phosphoryl group from sulfur to water.

EFFECT OF SUBSTITUENTS ON THE RATE OF HYDROLYSIS

Table 1 contains rate of hydrolysis constants for 12 compounds containing the $-SPO_3^{2-}$ group. The first order rate of hydrolysis constant for S-butyl phosphorothioate at pH 3 was given by Herr and Koshland[1] as about 0.055 min^{-1} ($I = 1.0$) at 35°. Ramsay[3] finds a slightly higher value. For the following discussion the rate constants at the pH where k_{obs} is maximal (k_{max}) will be utilized. It is evident from Table 1 that regardless of the nature of the substituent on the carbon chain the k_{max} values are similar in order of magnitude, the highest beeing only about four times as large as the smallest. It has been mentioned that the rate of hydrolysis of the undissociated acid $RSPO_3H_2$ in some cases probably contributes somewhat to k_{max}. For reasons already discussed this influence must be relatively small. From the difference between pK_2' and pH at 1/2 k_{max} (Table 1) it can be estimated * that the error in k_{max} due to hydrolysis of $RSPO_3H_2$ is maximally of the order of $\pm$ 10 %, but probably much less. The relative effect of substituents on substances with similar k_{max} values must, however, be interpreted with caution.

For substituted S-ethyl phosphorothioic acids the influence of the various groups on the rate of hydrolysis is the following (cf. Table 1) **

$$COOH, OH > no \ substituent > \overset{+}{N}H_3, \ NH-\overset{NH}{\underset{\overset{+}{N}H_3}{C}} > CH_3\overset{+}{N}H_2 > (CH_3)_3\overset{+}{N} >$$

$$> [CH_3]_2\overset{+}{N}H > [(CH_3)_2CH]_2\overset{+}{N}H \tag{4}$$

The data show that the presence of a hydroxyl or a carboxyl group increases whereas substituents carrying nitrogen decrease the rate of hydrolysis of S-substituted phosphorothioic acids as compared to the conditions for the unsubstituted compound. Three main effects of the substituents are believed to be responsible for the observed differences in k_{max}, namely: (a) intramolecular hydrogen bonding between the substituent and the phosphoryl group; (b) inductive effect of the substituent through the carbon chain; and (c) electrostatic interaction between the positive charge on the nitrogen and the negative charge on the phosphoryl group.

All factors would be expected to induce a small positive charge on the P-atom thus making it more reactive towards a nucleophilic attack by a water molecule according to (5a) or (5b) and (6).
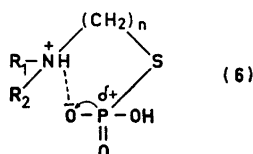

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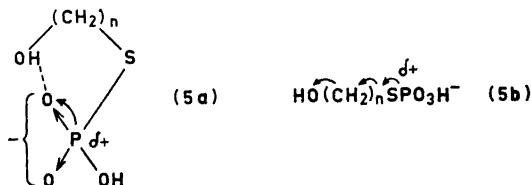
* The k_{obs} *versus* pH curves are used. By inserting pH = pK_2' values of k_{obs} are obtained which equal 1/2 k_{max} within $\pm$ 0 $-$ 8 %.

** The rate of hydrolysis constant of S-propyl phosphorothioic acid is lacking but the data for derivatives fit into (4).

An increase in k_{max} as compared to the unsubstituted compound would result. This is observed, *e.g.* for compounds 2 and 12 in Table 1, and explains why these compounds are more rapidly hydrolyzed than compound 1.

The effects of nitrogen containing substituents on the rate of hydrolysis of the SPO_3H^- group appear to be quite complicated. In the following the most likely ones will be discussed. However, since some effects should increase and others decrease the hydrolysis rate, it is sometimes difficult to predict which effect is the predominant one.

At the pH of maximal rate of hydrolysis all compounds having nitrogen containing substituents carry a positive charge. This will be expected to attract the negatively charged oxygen atom of the phosphate residue thus form a ring structure of a zwitterion type:

$$R_1 - \overset{+}{N}H \underset{R_2}{\diagdown} \overset{(CH_2)_n}{\diagup} \diagdown S \diagdown \overset{\delta+}{O-P-OH} \diagdown \overset{\parallel}{O} \qquad (6)$$

The negatively charged oxygen atom, which is vital for maximum rate of hydrolysis, is thereby screened more or less effectively depending on the bulkyness of R in (6). Approach of water molecules will be hindered and the rate of hydrolysis slower.

As seen from Table 1 bulky alkyl groups on the nitrogen atom markedly reduce the rate of hydrolysis. However, the trimethylammonium group affects the rate of hydrolysis less than does the dimethylammonium group. An explanation may be found in the fact that the nitrogen atom in this compound is so effectively screened by the methyl groups as to prevent a close contact between the negative oxygen of the phosphate group and the positive nitrogen atom. The negatively charged oxygen could thus be more available for water activation than in some other compounds containing N-groups. Such a view was supported by the study of atomic models.

It is highly probable that in addition to the electrostatic attraction effects discussed above intramolecular hydrogen bonding occurs between a hydrogen of the N-group (when present) and an oxygen of the phosphate residue (6). This is suggested by the fact that cysteamine S-phosphate is not hydrolyzed at a much slower rate than S-ethyl phosphorothioate in spite of the presence in the former compound of a positively charged nitrogen atom.

The inductive effect of the positively charged nitrogen atom through the carbon chain may also be of importance (*cf.* 5b).

Lengthening of the carbon chain decreases reaction rate for compounds 2, 3 and 4, 5 but increases the rate in 10, 11. Cyclization of compounds 2, 3 and 4, 5 by intramolecular hydrogen bonding would give rise to 7- and 8-membered rings, of which the 7-membered ones should be more stable and thus have the greater inductive influence on the phosphorus atom. Induction through the carbon chain could also explain the differences.

Compounds 10, 11 upon cyclization by intramolecular hydrogen bonding would form a 9- resp. 10-membered ring. Due to the large size of the ring the

masking effect of the guanidino group on the negatively charged oxygen atom of the phosphate residue would be expected to be less pronounced in compound 11 than in 10 thus explaining the slightly higher rate of hydrolysis observed for the former compound. This view was supported by the study of atomic models. Obviously induction through the carbon chain does not offer an explanation.

In Fig. 2 the first order rate of hydrolysis constants of 1,2-dimercapto-3-propanol S_1,S_2-diphosphate (I) and of 1,3-dimercapto-2-propanol S_1,S_3-diphosphate (II) are plotted *versus* pH; k_{obs} was calculated from the amount of totally liberated thiol and involves both the $-SPO_3^{2-}$ groups. The curve in Fig. 2 has the same general shape as those for compounds containing only one $-SPO_3^{2-}$ group. At the rate maximum close to pH 3 the k_{max}-value is 0.0545 min^{-1}. This value is considerably less than the one expected for two $-SPO_3^{2-}$ groups per molecule being independently hydrolyzed. Under such circumstances the expected k_{max}-value should be about two times k_{max} for compound 3 ($k_{max} = 0.0476$ min^{-1}). The most pronounced retarding effect on the rate of hydrolysis in compounds I and II is probably the mutual screening effect that the two $-SPO_3^{2-}$ groups impose on each other. This will be expected to hamper the approach of water molecules.

Little is known about the effect on the rate of hydrolysis of substituents in the carbon chain in esters of phosphoric acid. In works like that of Cherbuliez [10] (*cf.* table in Ref.[13]) a limited number of data are found, but few conclusions can be drawn from the material at hand.

A most striking difference between esters of phosphorothioic acid and those of phosphoric acid is the extreme lability of the S—P bond in the former (in mildly acid solution) and the great stability of the O—P bond in the latter.

EXPERIMENTAL

Materials. The S-substituted phosphorothioic acids used in the investigation were prepared as described previously [4]. The compounds that were isolated as barium salts in Ref.[4] were converted to sodium salts before use by trituration with a slight excess of sodium sulfate (molar ratio 1:1.25).
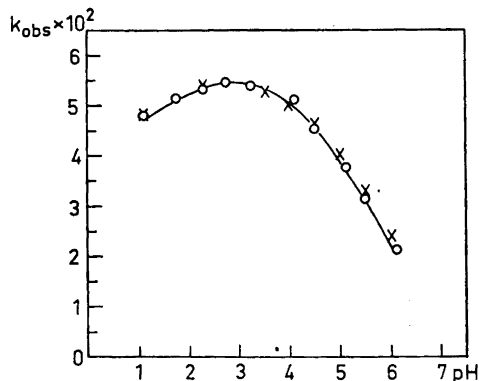

Fig. 2. Rate of hydrolysis constants (min^{-1}) of 1,2-dimercapto-3-propanol S_1,S_2-diphosphate (1.0 mM, crosses) and 1,3-dimercapto-2-propanol S_1,S_3-diphosphate (1.0 mM, circles) as a function of pH. Ionic strength 0.35, temperature $(34.8 \pm 0.2)°$. The hydrolysis was followed by colorimetric determination of the liberated thiols by the 2,6-dichlorophenolindophenol method [4].

Table 2. Comparison between the nitroprussiate and the 2,6-dichlorophenolindophenol method in determination of rate of hydrolysis constants. (Mean values are given).

Compound No.	pH	k_{obs} 10² (min⁻¹) as determined with	
		nitroprussiate	2,6-dichlorophenol indophenol
2	2.47	5.87	5.85
3	3.34	4.70	4.76
7	2.47	1.77	1.82
10	2.47	3.82	3.85

Determination of rate of hydrolysis constants. The rate of hydrolysis of the compounds (with the exception of S-ethyl phosphorothioate) was followed by measuring the liberated thiols colorimetrically by (*a*) the nitroprussiate method of Grunert and Phillips [8], and (*b*) the 2,6-dichlorophenolindophenol method of Åkerfeldt [4]. For these measurements a Beckman Model B spectrophotometer was used. As shown in Table 2 methods (*a*) and (*b*) were in close agreement. To ascertain the reliability of the results all compounds were investigated by both methods at least at one pH. The hydrolysis of 4 mM solution of the S-substituted phosphorothioic acids dissolved in buffers of an ionic strength close to 0.35 was followed. At a pH value close to that of optimal rate, the rate of hydrolysis was followed for 30 to 60 min by sampling every 4—5 min. This was done in order to ascertain for each compound] that the hydrolysis followed first order kinetics; k_{obs}-values were then calculated by Guggenheim's [9] method or directly from (7). The error in k_{obs}-values (standard deviations), as listed in Table 1, was calculated from these experiments. At other pH-values the rate of hydrolysis constants were calculated from multiple thiol determinations after the hydrolysis had been allowed to proceed for a fixed length of time, usually 15 min. The rate constants were then calculated from the expression

$$k_{obs} = \frac{2.303}{t} \log \frac{C_o}{C_t} \quad (C = \text{concentration, } t = \text{time}) \tag{7}$$

The rate of hydrolysis of S-ethyl phosphorothioate could not be followed by the above methods because of liberation of volatile ethanethiol during hydrolysis. Instead

Table 3. Demonstration of the constancy of pK'_2 during the course of the titration. Ion titrated: S-(3-guanidinopropyl) phosphorothioate. For details see text.

Added H⁺, μmoles	Observed pH	Calculated amount of $RSPO_3^{2-}$ present, μmoles	pK'_2
117.0	5.57	119.0	5.36
127.0	5.50	181.0	5.35
137.0	5.44	171.0	5.35
147.0	5.38	161.0	5.34
167.0	5.28	141.0	5.35
187.0	5.15	121.0	5.34
207.0	5.03	101.0	5.34
227.0	4.91	81.0	5.36
			5.35 ± 0.01

the following technique was used (*cf.* Ref.[3]): 1 mmole of the compound was dissolved in 50 ml of buffer of 34.8° (ionic strength 0.35) and incubated at 34.8° for the desired length of time, whereafter pH was immediately adjusted to 7.5 by the addition of a sufficient amount of a 1 M phosphate buffer of pH 9. Residual ethanethiol formed by hydrolysis was extracted with 50 ml of carbon tetrachloride. Remaining S-ethyl phosphorothioate was determined by adjusting a sample of the water phase to pH 1 — 2 (glass electrode) with 5 M HCl and adding an excess of iodine. Unreacted iodine was then titrated with sodium thiosulfate. The calculations were based on the fact that at pH 1 — 2 one equivalent of iodine reacts with one mole of S-ethyl phosphorothioate.

Determination of pK'$_2$-values. About 300 μmole of substance were dissolved in 50 ml of 0.35 M KCl. The solution was kept in a glass jacket equipped for magnetic stirring and equilibrated at $(34.8 \pm 0.2)°$ by running water from a thermostated waterbath. With the aid of a Gilmont micropipet 1 M HCl was added in quantities calculated to correspond to 30 — 70 % titration of the dianion $RSPO_3{}^{2-}$. Usually eight additions were made within 2 — 3 min. pH measurements were performed with a Beckman Expanded Scale pH-meter, standardized with biphthalate buffer of pH 4.02. From the equation

$$pK'_2 = pH - \log \frac{[RSPO_3{}^{2-}]}{[RSPO_3H^-]} \tag{8}$$

pK'$_2$ was calculated. The error in pK'$_2$ listed in Table 1 constitutes the maximum deviation of the mean. To avoid excessive hydolysis of the compounds during the titrations, they were speedily performed. Some hydrolysis of the compounds during the titrations was unavoidable. However, as is exemplified in Table 3, no drifting in pK'$_2$ values as the titration proceeded was observed, indicating a negligible influence of the liberated orthophosphate on the calculated value of pK'$_2$.

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