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Further Studies on S-Substituted Phosphorothioic Acids *

Mixed Lithiumsodium Salts of S-(1-Carboxyethyl) Phosphorothioic Acid and S-(2-Carboxyethyl) Phosphorothioic Acid

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1. The two title compounds were prepared and their rate of hydrolysis as a function of pH was studied at 37°. *Ortho*phosphate and the corresponding thiol were formed and a maximum rate of hydrolysis was observed at about pH 4 for both compounds.
2. The reaction between iodine and the two title compounds was investigated.
3. Methods for the colorimetric determination of S-substituted phosphorothioic acids are described and discussed.

The S—P bond in S-substituted phosphorothioic acids has proven to be a very easily hydrolyzable one. Indeed, the lability of this bond in hot acid solutions places S-substituted phosphorothioic acids among the most labile phosphate esters known.

Only a few compounds of this category have previously been synthesized by others [5–10]. Several of these compounds, however, were obtained in a highly impure state [9,10].

The rate of hydrolysis of the S—P bond in S-substituted phosphorothioic acids seems to be markedly effected by the pH of the medium. Thus, both S-(*n*-butyl) phosphorothioate [8] and cysteamine S-phosphate [4] are hydrolyzed at a maximum rate around pH 3 at 37°.

Investigations of the properties of the S—P bond in S-substituted phosphorothioic acids has now been extended to include two new compounds, namely the mixed lithiumsodium salts of S-(1-carboxyethyl) phosphorothioic acid and S-(2-carboxyethyl) phosphorothioic acid.

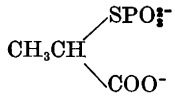
* Previous work by the author on this subject has appeared in Refs.[1-4]

RESULTS AND DISCUSSION

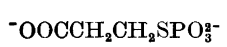
Synthetic methods. The S-substituted phosphorothioic acids synthesized earlier by others were without exception prepared from the corresponding thiol with the aid of a phosphorylating agent ($POCl_3$[5–9] or dimorpholinophosphinic chloride [10]). This method, however, has several drawbacks: a) thiols containing certain additional functional groups (hydroxyl-, carboxyl-, amino-groups *etc.*) are liable to yield polyphosphorylated products (*cf.* Ref.[6]); b) the thiol group often reacts poorly with the phosphorylating agent; c) the presence of large amounts of phosphates derived from unreacted phosphorylating agent in the reaction mixture introduces purification problems (*cf.* Refs.[5–10]); d) the final yield of S-substituted phosphorothioic acid is usually small depending on the factors mentioned in a), b) and c).

It was demonstrated earlier that trisodium phosphorothioate is a convenient starting material for the preparation of several S-substituted phosphorothioic acids [1,2]. With the use of this compound well defined reaction products were obtained in good yields.

S-(1-carboxyethyl) phosphorothioate (I) was prepared from 2-bromopropionic acid and trisodium phosphorothioate. The reaction proceeded rapidly at room temperature in the presence of excess 2-bromopropionic acid, and a quantitative reaction of the phosphorothioate ion was obtained.



I



II

S-(2-carboxyethyl) phosphorothioate (II) was prepared in two ways: a) by reaction of 3-bromopropionic acid with trisodium phosphorothioate; and b) by addition of trisodium phosphorothioate to methyl acrylate followed by hydrolysis of the methoxycarbonyl group with strong alkali. Method b) is based on the earlier observation [2] that methyl acrylate adds trisodium phosphorothioate to the double bond forming S-[2-(methoxycarbonyl)ethyl] phosphorothioate. This reaction proceeds rapidly at room temperature, and with an excess of methyl acrylate a quantitative reaction of the trisodium phosphorothioate was obtained. Treatment of the resulting compound with strong alkali at low temperature results in the rapid hydrolysis of the methoxycarbonyl group, whereas the S—P bond in the molecule is virtually unaffected by this treatment.

3,3′-Dithiodipropionic acid was previously isolated as the product obtained after acid hydrolysis and subsequent iodine oxidation of the reaction product between methyl acrylate and trisodium phosphorothioate. From this result the conclusion was drawn that the phosphorothioate residue was attached to carbon No. 3 of the propionic acid part of the molecule. A search for S-(1-carboxyethyl) phosphorothioate as a contaminant of the major reaction product has now been undertaken. It has been concluded from the following that the presence of this substance, even in trace amounts, is unlikely: a) S-(1-carboxyethyl) phosphorothioate and S-(2-carboxyethyl) phosphorothioate

can be separated from each other (and from *ortho*phosphate) by paper chromatography in the solvent systems (Whatman No. 4 papers, ascending method): I. *iso*-propanol: *tert.*-butanol: NH_3 (29 %) : H_2O, 40:20:1:39 (R_F's 0.56 and 0.77, respectively); II. *iso*-propanol:N,N-dimethylformamide: *n*-butanol: NH_3(29 %): H_2O, 20:20:20:1:39 (R_F's 0.40 and 0.62, respectively). *Ortho*phosphate has an R_F of 0.43 in I and 0.28 in II. The reaction product from trisodium phosphorothioate and methyl acrylate was analyzed in the above solvent systems after hydrolysis in strong alkali. Only a spot corresponding in position to that of S-(2-carboxyethyl) phosphorothioate was found in this way. b) Paper chromatography according to Ref.[11] of acid hydrolysate of the reaction product, coupled to 2-chloromercuri-6-nitrophenol in alkaline solution *, gave only one spot, identical in position to MNP-3-mercaptopropionic acid in all four solvent systems listed in Ref.[11] No trace of MNP-2-mercaptopropionic acid was detected on the chromatograms. (The R_F's of MNP- 2-mercaptopropionic acid in these solvent systems are 110—124 % of those of MNP-3-mercaptopropionic acid).

Both the S-substituted phosphorothioic acids described in this paper were isolated as their mixed lithiumsodium salts. This affords a simple means of removing small amounts of *ortho*phosphate formed as trilithium phosphate. The preparations obtained in this way were considerably more pure than products isolated as barium or sodium salts.

Both the prepared compounds were found to contain methanol of crystallization. Similarily, Wieland and Lambert [7] found that S-(*n*-butyl) phosphorothioate has a strong affinity for ethanol.

There exists the possibility that the bromopropionic acids used in the preparation of the S-substituted phosphorothioic acids mentioned here could also react with the oxygen atom of the phosphorothioate ion and thus form O-substituted phosphorothioic acids as contaminants. This could also be the case in the methyl acrylate-trisodium phosphorothioate reaction. It is evident from the following, however, that no detectable amounts of such contaminants are present: a) acid hydrolysis of the prepared compounds gave a quantitative yield of thiols, as determined by iodine titration; b) hydrogen sulfide or phosphorothioic acid were not formed in detectable amounts during acid hydrolysis of the compounds (silver ions give a black precipitate or a brown coloring with phosphorothioic acid, which is easily detectable when the phosphorothioate concentration is above *ca.* 100 μM, *i.e.* 20 μg of trisodium phosphorothioate per ml. This test is practically independent of the pH of the solution tested.)

The available evidence therefore indicates that the S-atom is the sole reactive site of the phosphorothioate ion in this type of reactions. Indeed, this was also found to be true when 2-bromoethylammonium bromide and bromoacetic acid react with trisodium phosphorothioate [1,2].

Effect of pH on the hydrolysis of the prepared compounds. Fig. 1 describes the rate of hydrolysis of S-(1-carboxyethyl) phosphorothioate and S-(2-carboxyethyl) phosphorothioate as a function of pH at 37°. The products of hydrolysis

* Preparing the MNP-thiols [11] in alkaline medium prevents the formation of "ghost" spot on the chromatograms.

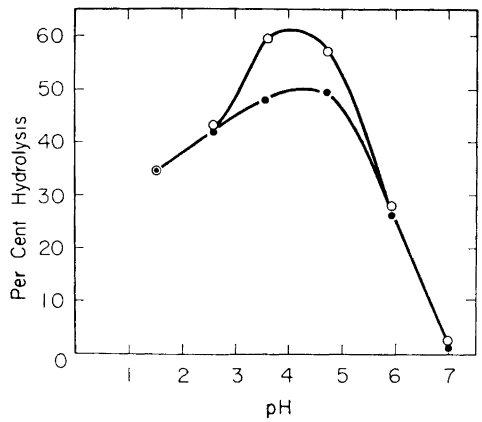


Fig. 1. Effect of pH on the hydrolysis of S-(1-carboxyethyl) phosphorothioate (2.2 mM; filled circles) and S-(2-carboxyethyl) phosphorothioate (2.1 mM; open circles) in 0.1 M citrate buffers. The samples were incubated for 15 min. at 37°C.

are *ortho*phosphate and the corresponding thiol. Both compounds show a maximum rate of hydrolysis at approximately pH 4. Only a very slow rate of hydrolysis is observed at pH 7. Similar hydrolysis curves were found earlier for S-(*n*-butyl) phosphorothioate [8] and for cysteamine S-phosphate [4]. In the case of S-(*n*-butyl) phosphorothioate a mechanism for the hydrolysis was proposed by Herr and Koshland to explain the observed effect of pH [8].

S-(1-carboxyethyl) phosphorothioate and S-(2-carboxyethyl) phosphorothioate are very rapidly hydrolyzed by heat in acid solution. Thus, 2 mM solutions of both compounds in 1 M HCl are completely hydrolyzed in about one minute at 100°, giving a quantitative yield of *ortho*phosphate and the corresponding thiol.

Reaction with iodine. One equivalent of iodine reacts instantaneously with one mole of the synthesized compounds at pH above *ca.* 2. During this process one mole of *ortho*phosphate is formed, and 2,2'-dithiodipropionic acid and 3,3'-dithiodipropionic acid, respectively, were isolated from the resulting solutions.

At pH's below *ca.* 1 the reaction between iodine and the two S-substituted phosphorothioic acids is very slow. Thus, 2 mmole of the compounds in 2 M HCl (50 ml) require more than 2.5 h to decolorize completely an equivalent amount of iodine at 25°C.

The effect of pH on the reaction between iodine and the two compounds described in this paper is similar to the one previously found in the reaction between cysteamine S-phosphate and iodine [4]. The mechanism suggested for the latter reaction [4] could apparently also be applied to explain the effect of pH on the reaction between iodine and the two S-substituted phosphorothioic acids described here.

Colorimetric determination of S-substituted phosphorothioic acids. The ease with which S-substituted phosphorothioic acids are hydrolyzed in hot acid

solutions offers a convenient means for their determination. Both *ortho*-phosphate and the liberated thiol can be determined colorimetrically in the hydrolysate (however, cysteine S-phosphate, as prepared by Binkley [6], seems to behave abnormally). Of these two methods, phosphate determination is the more accurate one (especially if a volatile thiol is formed).

Under certain circumstances it would be of value to have a method available for the liberation of *ortho*phosphate, which is more specific for S-substituted phosphorothioic acids than acid hydrolysis. It was previously shown [1] that cysteamine S-phosphate is rapidly hydrolyzed at room temperature by catalytic amounts of mercury(II) ions (or by 2-chloromercuri-6-nitrophenol), and this finding was utilized for the colorimetric determination of the phosphate residue in this substance. The hydrolysis of cysteamine S-phosphate by mercury(II) ions is not a unique property of this compound. Other phosphate esters, *e.g.* phosphoenolpyruvic acid [12], are known to be hydrolyzed by mercury(II) ions *. Coenzyme A S-phosphate is hydrolyzed by mercury(II) salts (in rather high concentrations [10]), and S-(*n*-butyl) phosphorothioate also appears to be hydrolyzed by this treatment [7].

The mercury method [1], when applied to the two S-substituted phosphorothioic acids described in this paper, gave satisfactory results with both compounds (it was found that S-(1-carboxyethyl) phosphorothioate is hydrolyzed quantitatively by the phosphate reagents alone). In the case of cysteamine S-phosphate and S-(2-carboxyethyl) phosphorothioate, copper(II) ions or silver ions can not substitute for the mercury(II) ions in similar concentrations. However, both copper(II) ions and silver ions (*e.g.* in concentrations of 0.5 mM) rapidly liberate *ortho*phosphate from the S-substituted phosphorothioic acids at pH 7.

Like iodine, bromine was found to liberate *ortho*phosphate rapidly and quantitatively from all S-substituted phosphorothioic acids studied by the author. The advantage of bromine over iodine in this respect is that bromine also seems to react rapidly with these compounds in strongly acid solution. Some results from experiments where *ortho*phosphate was determined after bromine treatment of S-substituted phosphorothioic acids are presented in

* However, with the small amounts of mercury(II) ions used in the authors method [1] phosphoenolpyruvate is only partially hydrolyzed.

Table 1. Recovery experiment for the determination of S-substituted phosphorothioic acids by the bromine method.

Substance	Added μmole	Found μmole	Error %
S-(2-Carboxyethyl) phosphorothioate	0.296	0.298	+ 0.7
	0.592	0.591	− 0.2
	0.888	0.885	− 0.3
Cysteamine S-phosphate	0.262	0.264	+ 0.8
	0.524	0.520	− 0.8
	0.786	0.787	+ 0.1

Table 1. Several other acid labile phosphate esters tested, *e.g.* adenosine-5'-triphosphate, do not liberate *ortho*phosphate by treatment with bromine water (*cf.* Ref.[14]). However, *e.g.* quinolphosphates [13] seem to be hydrolyzed by this treatment and phosphoenolpyruvate is hydrolyzed to some extent. Furthermore, Binkley [14] has reported that bromine liberates *ortho*phosphate quantitatively from the phosphorothioate ion.

EXPERIMENTAL

Materials. Trisodium phosphorothioate was prepared as described previously [4]. Methyl acrylate (stabilized with *p*-methoxyphenol) and the 2-resp. 3-bromopropionic acids used were purchased from Eastman Kodak Company.

General methods. Phosphate determinations were carried out using Gomori's [15] method, and thiols were determined according to Grunert and Phillips [16]. Standardization of the latter method was carried out with the use of acid-hydrolyzed solutions of the S-substituted phosphorothioic acids, the purity of which had been previously determined by iodine titrations and phosphate determinations. The hydrolysis rate experiments were performed by following the liberated thiols according to this method.

Methanol was determined using the method of Reid and Salmon [17] after passage of the sample through a double bed ion exchange column consisting of Dowex 50 W-X4 (H^+), top layer, and Dowex 21K (OH^-), bottom layer. Sodium and lithium were determined by flame spectrophotometry *. Lithium was also determined according to Thomason [18] after absorbtion on Dowex 50W-X4 (H^+) and subsequent eluation with 3 M HCl.

Determination of S-substituted phosphorothioic acids with the bromine method. To 0.10 ml of sample, containing 0—1 μmole of S-substituted phosphorothioic acid was added 0.20 ml of a saturated solution of bromine in water. The mixture was allowed to sit at room temperature for 10 min. 0.50 ml of "elon" [15], 1.00 ml of molybdate [15] and 3.20 ml of water were added. The color was developed for 1 h at room temperature and the absorbancy was read in a 1 cm cuvette at 660 mμ against a blank (containing water instead of sample). 1 μmole of S-substituted phosphorothioic acid in the sample gives an absorbancy of 0.712.

Lithiumsodium S-(1-carboxyethyl) phosphorothioate. 5.4 ml (60 mmole) of 2-bromopropionic acid in 30 ml of water were neutralized with 20 % lithium hydroxide under ice cooling. The cold bath was removed and 5.4 g (30 mmole) of trisodium phosphorothioate were added. The mixture was rapidly stirred at room temperature for 1 h, after which time all phosphorothioate had reacted (added silver ions give a clear yellow precipitate; a black or brownish precipitate indicates the presence of unreacted phosphorothioate).

7.0 g (170 mmole) of LiOH, H_2O were added and any precipitate (consisting of Li_3PO_4) was filtered off. 200 ml of N,N-dimethylformamide:methanol, 1:1, were then slowly added, the solution being cooled in an ice bath. The precipitate was filtered off, washed with methanol (100 ml), and redissolved in 30 ml of water. Any precipitate was filtered off and 200 ml of N,N-dimethylformamide:methanol, 1:1 were again added under ice cooling. The precipitate was filtered off and washed with methanol (100 ml). Yield **: 5.5 g (73 %; average of four prepns.). (Found: C 19.3; H 3.3; P 12.4. Calc. for $(PO_3SCH(CH_3)COO)Li_{2.1}Na_{0.9}$, CH_3OH (250): C 19.2; H 3.2; P 12.4. Found for ratios: Li/P = 2.10; Na/P = 0.91 and CH_3OH/P = 0.99.)

* These analyses were kindly performed by Miss Katherine Gallagher.

** All yields are calculated on the amount of Na_3SPO_3 used.

Synthesis of lithiumsodium S-(2-carboxyethyl) phosphorothioate

A. From methyl acrylate. 5.0 g (28 mmole) of trisodium phosphorothioate were dissolved in 50 ml of water and 5.0 ml (55 mmole) of methyl acrylate were added. The mixture was vigorously stirred for 5 min., after which time all the phosphorothioate had reacted (silver ion test; see above). The solution was extracted with 50 ml of benzene to remove excess methyl acrylate. 7.0 g (170 mmole) of $LiOH,H_2O$ were added and the solution was stirred in an ice bath for 30 min. The synthesized substance was precipitated with N,N-dimethylformamide:methanol, 1:1, and purified as described above. Yield: 5.1 g (80 %, average of four prepns.). The substance gave a completely negative hydroxylamine test for ester [20]. (Found: C 17.4; H 2.6; P 13.5. Calc. for $(PO_3SCH_2CH_2COO)$ $Li_{2.6}Na_{0.4}$ $(CH_3OH)_{0.3}(H_2O)_{0.5}$ (229): C: 17.3; H 2.8; P 13.5. Found for ratios: $Li/P = 2.63 \pm 0.04$; $Na/P = 0.37 \pm 0.02$ and $CH_3OH/P = 0.3 \pm 0.1$ (Average from two prepns. $\pm$ maximum deviation).

B. From 3-bromopropionic acid. 5.1 g (33 mmole) of 3-bromopropionic acid were dissolved in 45 ml of water. After neutralization under ice cooling with 20 % LiOH, 5.4 g (30 mmole) of trisodium phosphorothioate were added and the mixture was vigorously stirred for 1.5 h at room temperature, after which time all the phosphorothioate had reacted (silver ion test; see above). 7.0 g (170 mmole) of $LiOH,H_2O$ were added and after filtration the substance was isolated and purified as described under *A*. Yield: 5.7 g (84 %, average of four prepns.). (Found: C 17.3; H 2.6; P 13.6. Analysis for Li, Na and CH_3OH fell within the ranges given under *A*. For calculated values see under *A*.)

Acid hydrolysis and iodine titration of the compounds

1. Lithiumsodium S-(1-carboxyethyl) phosphorothioate. 1.98 mmole * of the substance were hydrolyzed in 30 ml of 5 % HCl at 90° for 5 min. H_2S or phosphorothioic acid were not detected as products of the hydrolysis. The hydrolysate consumed 1.99 matom of iodine as titrated with a standardized iodine solution. Extraction of iodine oxidized hydrolysate with ethylacetate (the water phase contained thiosulfate to reduce some oxidatively formed iodine) gave 2,2'-dithiodipropionic acid, m.p. 125—133° (unsharp, from benzene). Stoner and Dougherty [19] report 127—135° (from ethanol-water and then from benzene). Equiv. wt: found 106; calc. 105.

2. Lithiumsodium S-(2-carboxyethyl) phosphorothioate. The two substances from synthetic methods *A* and *B*, respectively, were hydrolyzed in 5 % HCl as described under *1*. H_2S or phosphorothioic acid were not detected as products of the hydrolysis. 2.18 mmole of substance from method *A* consumed 2.18 matom of iodine and 2.22 mmole of substance from method *B* consumed 2.21 matom of iodine. Extraction of iodine oxidized hydrolysates with ethylacetate (in the presence of thiosulfate) gave in both cases 3,3'-dithiodipropionic acid m.p. 154—155° (corr; from 4-methyl-2-pentanone), undepressed by admixture with an authentical sample.

Iodine treatment of the unhydrolyzed substances. 5.00 mmole each of the S-(1-carboxyethyl) phosphorothioate and the S-(2-carboxyethyl) phosphorothioate were dissolved in 30 ml of 20 % acetic acid. Titration with iodine showed a rapid reduction of 5 matom of iodine (endpoint not sharp; a slow further consumption of iodine was observed, probably indicating sulfoxide formation). Phosphate analysis of the resulting solutions showed the presence of 5.00 $\pm$ 0.04 mmole of *ortho*phosphate in both solutions, which were then acidified with conc. HCl, diluted with water and extracted with ethylacetate in the presence of thiosulfate. 2,2'-dithiodipropionic acid and 3,3'-dithiodipropionic acid were identified in the extracts as described above under *1* and *2*.

The author wishes to thank Miss Sarah Perry and Mrs. Mildred Gale for skillful assistance.

* The amounts taken are based on phosphate determinations.

Addendum. In his recent thesis [21], Ramsay has studied the effect of pH on the hydrolysis of S-(*n*-butyl) phosphorothioate and trisodium phosphorothioate. He finds a maximal rate of hydrolysis at pH 3.5 for the first compound and at pH 3.0 for the second. Jaenicke and Lynen [22] have recently elaborated on the chemistry of certain S-substituted phosphorothioic acids with special reference to coenzyme A S-phosphate.

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