

# Reducing Properties of Phosphorothioate

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Phosphorothioic acid ( $H_3PO_3S$ ) was shown to reduce ferricyanide in a mole to mole ratio, forming a phosphorothioate dimer. Electromotive force (e.m.f.) measurements have shown that the oxidation-reduction system  $2PS/PSSP + 2e$ , where PS and PSSP denote phosphorothioic acid monomer and dimer (or their corresponding salts), respectively, is a reversible one on a platinum electrode. The variation of the e.m.f. with the concentration of reductant, oxidant, and hydrogen ion was measured and found to obey the pertinent thermodynamic equations. The standard e.m.f. of the system at pH 7, referred to the standard hydrogen electrode, is approximately zero. The kinetics of the reduction of ferricyanide ion by phosphorothioate was found to be second order with respect to the reactants in the pH range 5.8 to 7.8 (ionic strength,  $\mu = 0.035$  to 0.06, 15 to 40°). The presence of free-radical intermediates in the reaction was demonstrated. The second-order rate constant of reduction of ferricyanide by PS showed a maximum at pH 6.2 ( $\mu = 0.06$  at 26°). The dependence of the reduction rate constant on ionic strength could be described by a linear Brønsted plot with a slope of 9 (pH 6.88, 26°). The rate constant of reduction (pH 7.24,  $\mu$  0.06) decreased on raising the temperature from 15 to 25°. Pertinent data on the ionization constants and absorption spectra of phosphorothioic acid and its dimer are presented.

## Introduction

Phosphorothioic acid is a derivative of orthophosphoric acid, in which one of the oxygen atoms has been replaced by a sulfur atom. The preparation and some of the properties of phosphorothioic acid and its salts have been described in the literature.<sup>1-3</sup> As far as we are aware, no data are available on the reductive properties of phosphorothioic acid, although its S-S dimer has been obtained on oxidation with iodine.<sup>4</sup> Our interest in the phosphorothioates as reducing agents was prompted by the observation that sodium phosphorothioate activates papain and ficin,<sup>5</sup> at equimolar amounts of reagent and enzyme. Furthermore, under the appropriate conditions, phosphorothioate has proved capable of splitting S-S bonds in proteins such as ribonuclease and lysozyme.<sup>6</sup>

In this communication we report on the reductive behavior of phosphorothioate toward ferricyanide. This system was chosen because of its simplicity and because only one electron transfer is involved in the reduction process. The kinetics of the oxidation of

phosphorothioate with ferricyanide as a function of pH, ionic strength, and temperature has been investigated. The oxidation-reduction potential of the system  $2PS \rightleftharpoons PSSP + 2e$  has also been studied,<sup>7</sup> and the pertinent data on the ionization constants of phosphorothioic acid and its dimer, and their absorption spectra, have been included.

## Experimental

**Materials.** Trisodium phosphorothioate,<sup>3</sup> dipotassium di(hydrophosphorothioate),<sup>4</sup> and triethyl thiophosphate<sup>8</sup> were synthesized according to the literature. Potassium ferricyanide (C.P.) was purchased from Agan Co., Tel Aviv, Israel. All other chemicals were of analytical grade.

Stock solutions of  $2 \times 10^{-2}$  M potassium ferricyanide,  $5 \times 10^{-2}$  M ceric sulfate in 0.1 N  $H_2SO_4$ , and 0.1 M iodine in 0.2 M KI were prepared using twice-distilled water and kept at 4°. Phosphate buffer solutions of ionic strength 0.1 were prepared according to Cohn,<sup>9</sup> using stock solutions of 4 M KOH and 1.5 M potassium dihydrophosphate. Since the apparent pK values of phosphoric acid are practically independent of concentration at buffer concentrations below 0.1 M, the ionic strength due to the phosphate buffer used is proportional to the total buffer concentration. The various buffer solutions were thus prepared by appropriate dilution of the stock phosphate buffer solutions of ionic strength 0.1, with conductivity water. The contribution of the reactants to the ionic strength (0.01) was added to that of the buffer.

**Absorption Spectra Measurements.** Spectrophotometric measurements were made with a Beckman Model DK1 automatic recording spectrophotometer, equipped with "thermospacers" for measurements at the desired temperature, or a Carl Zeiss Model PMQII spectrophotometer equipped with a temperature-controlled cell holder. Water from a thermostated bath, kept at a constant temperature ( $\pm 0.1^\circ$ ) in the range 15-40°, was circulated through the cell holders. Quartz cuvettes with a light path of 10 or 1 mm. were used.

**pH and Electromotive Force (E.m.f.) Measurements.** The pH measurements were carried out with a pH meter Model 22r, Radiometer, Copenhagen, using a glass electrode Type G-202B and a calomel electrode Type K4312. The electrodes were standardized with the aid of standard buffer solution.<sup>10</sup>

The measurements of the oxidation-reduction potentials were carried out with the above instrument, Radiometer 22r, using a shiny platinum wire as the

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(7) The notation PS will be used in the following for phosphorothioic acid or the phosphorothioate ion without specifying their state of ionization. The notation PSSP or phosphorothioate dimer will be used for  $(H_2PO_3S)_2$  without specifying its state of ionization.

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(9) E. J. Cohn, *ibid.*, **49**, 173 (1927).

(10) W. M. Clark, "Oxidation-Reduction Potentials of Organic Systems," Waverly Press, Inc., Baltimore, Md., 1960, p. 253.

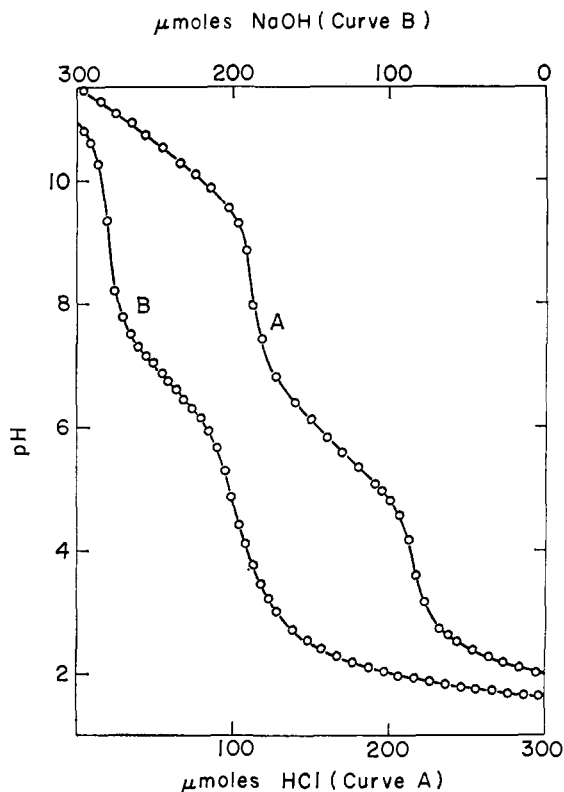


Figure 1. Potentiometric titration in water (5 ml.) of 100  $\mu$ moles of trisodium phosphorothioate with 1 *N* hydrochloric acid (curve A). Titration with 1 *N* sodium hydroxide of the oxidation products of 100  $\mu$ moles of PS obtained as described in the text (curve B). Both titrations were carried out at 23°.

indicator electrode and a calomel electrode Type K4312 as the reference electrode. The setup was such that a glass electrode was immersed permanently in the solution during the e.m.f. measurements, in addition to the calomel and platinum electrodes, so that the pH of the solution could be determined without dismantling the assembly.

The platinum electrodes were immersed in concentrated nitric acid between measurements and rinsed thoroughly with distilled water before use. It was noted that erratic results were often obtained when the platinum electrodes were not clean; different platinum electrodes were therefore used, as a rule, to check the reliability of the measurements. The pH measurements and the e.m.f. potentiometric titrations were carried out manually. Some of the experiments were conducted under nitrogen atmosphere.

**Kinetics of the Reduction of  $K_3Fe(CN)_6$  by Phosphorothioate.** The molar concentration of  $K_3Fe(CN)_6$ , at any given instant, was evaluated from optical density measurements at 420  $m\mu$ , using the molar extinction coefficient,  $\epsilon_{420m\mu}$   $1.00 \times 10^3$ .<sup>11</sup> Ferrocyanide ion, PS, and its oxidation product do not show any detectable absorption at this wave length.

In most of the kinetic experiments an excess of ferricyanide over phosphorothioate was used. Under these conditions practically all of the phosphorothioate is oxidized eventually (see Results). It is thus possible to determine the initial concentration of the phosphorothioate from the difference in the optical density at

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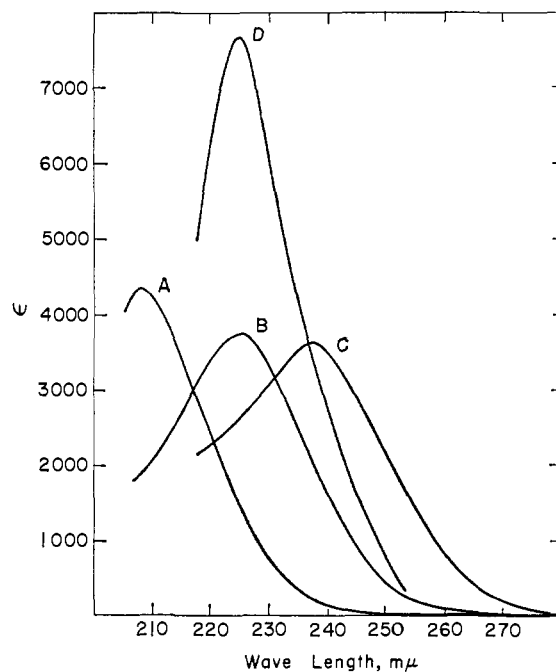


Figure 2. The absorption spectra of PS and PSSP in aqueous solution at different pH values. In the range of ionic strength measured (0.025–0.1), the spectra were independent of salt concentration.  $\epsilon$  is the molar extinction coefficient. Experiments were conducted at PS and PSSP concentrations of  $5 \times 10^{-4}$  mole/l.: curve A, PS, pH range 0.5 to 5.0; curve B, PS, pH range 5.5 to 10.0; curve C, PS, pH range 11.0 to 13.0; curve D, PSSP, pH range 1.0 to 10.0.

420  $m\mu$  of the reaction mixture at time  $t = 0$  and  $t = \infty$ . The concentration of phosphorothioate so determined corresponded in all cases to the weight of phosphorothioate initially introduced into the reaction mixture.

The kinetic experiments described were performed with a Carl Zeiss PMQII spectrophotometer. The temperature of the reaction mixture was checked at the end of each experiment.

## Results

**Potentiometric Titration of Phosphorothioic Acid and of Its Dimer.** The potentiometric titration in water of trisodium phosphorothioate with 1 *N* hydrochloric acid at 23° is given in Figure 1 (curve A). From the data obtained, three apparent dissociation constants could be evaluated:  $pK_1 < 2$ ;  $pK_2 = 5.75$ ;  $pK_3 = 10.4$ . (The first  $pK$  could not be determined because of the high uptake of protons by the solvent below pH 2.)

Figure 1, curve B, shows the titration curve of the dimer formed by the oxidation of phosphorothioic acid with iodine according to the equation  $H_3PO_3S + 0.5I_2 \rightarrow 0.5(H_2PO_3S)_2 + H^+ + I^-$ . The oxidation was performed in the titration vessel at the end of the titration of the monomer with hydrochloric acid. The titration of the reaction products was carried out from pH 1.6 to pH 11.5 with 1 *N* sodium hydroxide at 23°. One equivalent of the base added in the pH range 2.0 to 3.0 has probably been used to neutralize the hydroiodic acid liberated during the oxidation process of PS. Bearing this in mind, it follows from the course of the titration that 2 equiv. of base is consumed by the

oxidation product of PS up to pH 10.0, when calculated per half-dimer. The first equivalent of base has been consumed in the pH range of 2 to 3; the second equivalent at the pH range of 4.5 to 7.5. An apparent  $pK$  of about 6.5 could be derived from the course of titration in the neutral pH range.

The absorption spectra of PS at various pH values are shown in Figure 2. It should be noted that the absorption spectrum remains unaltered in the pH range of 0.5 to 5.0 (curve A). In this pH range a maximum in absorption at  $208\text{ m}\mu$  has been recorded. The absorption spectrum in the pH range of 5.5 to 10 possessing a maximum at  $225\text{ m}\mu$  (curve B) differs from that in the range of 0.5 to 5.0. A new spectrum with a maximum at  $238\text{ m}\mu$  (curve C) appears in the pH range of 11 to 13. Since marked changes in the spectrum appear at the pH values at which new ionized species appear, one may attribute spectrum A to un-ionized phosphorothioic acid ( $\text{H}_3\text{PO}_3\text{S}$ ) or mono-ionized phosphorothioate ion ( $\text{H}_2\text{PO}_3\text{S}$ )<sup>-</sup>, spectrum B to the diionized phosphorothioate ion ( $\text{HPO}_3\text{S}$ )<sup>-2</sup> and spectrum C to the triionized phosphorothioate ion ( $\text{PO}_3\text{S}$ )<sup>-3</sup>.

The spectrophotometric titration of the second and third ionization groups of phosphorothioic acid at  $240\text{ m}\mu$  is given in Figure 3. The inflection points occurring at pH 6.0 and 10.8 correspond to the apparent second and third dissociation constants, respectively, derived from the potentiometric titration (Figure 1). It is of interest that the absorption spectrum of O-triethyl phosphorothioate,  $(\text{C}_2\text{H}_5\text{O})_3\text{P}=\text{S}$ , in dioxane, ethanol, or cyclohexane, was found to be identical with that of phosphorothioic acid in the pH range 0.5 to 5 (see curve A, Figure 2).

**Reduction Properties of Phosphorothioate.** Phosphorothioate was found to reduce ferricyanide in a mole to mole ratio, at the pH range of 5 to 13. Excess of ferricyanide does not lead to any further oxidation of the reductant. In order to identify the oxidation product, phosphorothioate was oxidized with an equimolar amount of potassium ferricyanide at pH 11. The reaction mixture was acidified with hydrochloric acid to pH 3, and the ferrocyanide formed was quantitatively precipitated, as Prussian blue, with ferric chloride. The precipitate was removed by centrifugation, and the supernatant brought to dryness *in vacuo*. The residue left showed in aqueous solution an ultraviolet absorption spectrum identical with that of phosphorothioate dimer prepared according to the literature<sup>4</sup> (Figure 2, curve D). An independent proof for the formation of the dimer is given below.

E.m.f.-potentiometric titrations revealed that 1 mole of phosphorothioate reduces 8 equiv. of ceric sulfate in  $0.1\text{ M H}_2\text{SO}_4$ . Sulfate ion seems, therefore, to be the final oxidation product of the sulfur of phosphorothioate in this reaction.

According to the literature phosphorothioate dimer (PSSP) is formed when 1 equiv. of iodine is added per mole of phosphorothioate in  $5\text{ M HCl}$ .<sup>4</sup> It has been found (see next section) that the dimer is also formed at a similar iodine to PS ratio in more dilute acid solutions, such as  $0.1\text{ M HCl}$ . However, when the reaction was followed potentiometrically at  $0.1\text{ M HCl}$ , it was found that the oxidation continued beyond the dimer stage when more oxidant was added, up to a

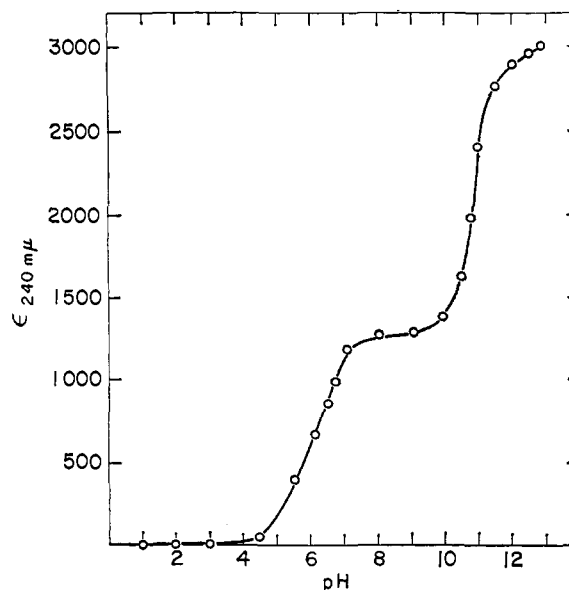


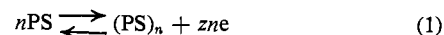
Figure 3. The spectrophotometric titration of PS at  $240\text{ m}\mu$ , at the PS concentration  $5 \times 10^{-4}$  mole/l.  $\epsilon$  is the molar extinction coefficient at  $240\text{ m}\mu$ .

ratio of 2 equiv. of oxidant to one of reductant. The final stages of the reaction are accompanied by liberation of elementary sulfur.

**Oxidation-Reduction Potential of the PS-PSSP System.** The potentiometric titration of phosphorothioate by ferricyanide is shown in Figure 4A. The e.m.f. values given are those obtained against the standard hydrogen electrode and were calculated by adding 0.246 v. to the voltages measured experimentally against the saturated calomel electrode.<sup>12</sup> Measurements were carried out in  $0.2\text{ M}$  phosphate buffer, pH 7.4.

After each addition of oxidant a sufficient amount of time (up to 15 min.) was allowed for the e.m.f. readings to reach a constant value, since it has been found that the reaction between PS and ferricyanide is time consuming.

In the following it will be shown that the e.m.f. measurements of the type recorded in Figure 5 give important information as to the nature of the oxidation products. At constant pH, the e.m.f. of the system



is given by

$$E = E_0' + \frac{RT}{znF} \ln \frac{[\text{O}]}{[\text{R}]^n} = E_0' + \frac{RT}{znF} \ln \frac{1}{n} + \frac{RT}{znF} \ln \frac{[\text{O}]'}{[\text{R}]^n} \quad (2)$$

where  $e$  is the electronic charge,  $E_0'$  is a constant at any given pH,  $z$  is the number of electrons donated during the reaction by one molecule of PS,  $n$  is the number of PS molecules combining to give the oxidation product,  $[\text{O}]$  and  $[\text{R}]$  are the molar concentrations of  $(\text{PS})_n$  and PS, respectively, and  $[\text{O}]'$  is the concentration of oxidant expressed in units of moles of monomer per liter ( $[\text{O}]' = n[\text{O}]$ ).

(12) The convention of Clark (ref. 10, pp. 95-97) regarding the signs of the standard potentials was used throughout.

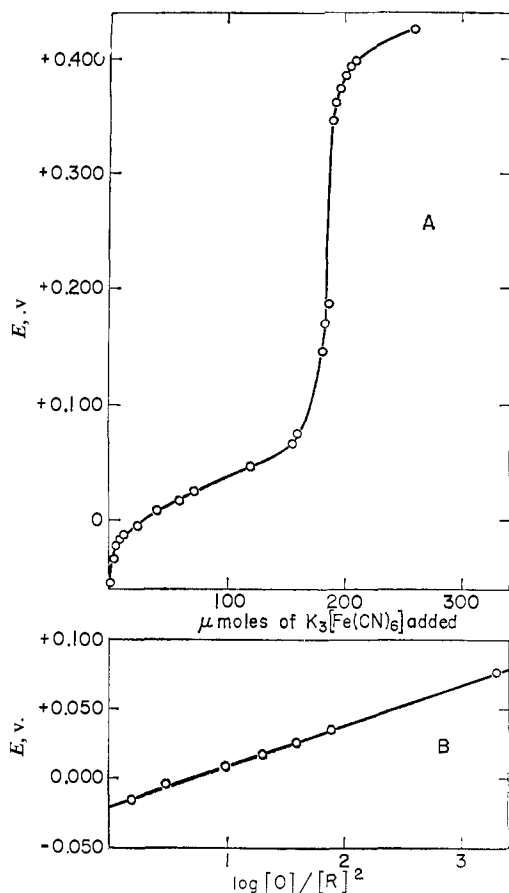


Figure 4. (A) The oxidation-reduction potentiometric titration of PS ( $1.8 \times 10^{-2} M$ ) in 10 ml. of 0.2 M phosphate buffer, pH 7.4, with ferricyanide (0.2 M). The potentials indicated are referred to the standard hydrogen electrode (22°). (B) A plot of the oxidation-reduction potentials obtained in the titration of the PS by ferricyanide (part A) vs.  $\log [O]/[R]^2$ .

At 23°

$$E = E_0' + \frac{0.06}{zn} \log \frac{1}{n} + \frac{0.06}{zn} \log [O]' - \frac{0.06}{z} \log [R] \quad (3)$$

A plot of  $E$  vs.  $\log [R]$  at constant  $[O]'$  should give a slope of  $-0.06/z$  v., while a plot of  $E$  vs.  $\log [O]'$  at constant  $[R]$  should give a slope of  $0.06/nz$  v.  $z$  and  $n$  can thus be evaluated from experiment.

Solutions containing 0.050 mole/l. of PS ( $[R] = 0.05$ ) and varying amounts of  $(PS)_n$  (prepared by oxidation of PS with ferricyanide or iodine as described in the section, "Reduction Properties of PS") in 0.2 M phosphate buffer, pH 7.3, were prepared and their e.m.f. determined. Similar measurements, in the same buffer, were performed on solutions containing  $(PS)_n$  at a concentration corresponding to  $[O]' = 0.05$  mole/l. and varying amounts of PS. Plots of  $E$  vs.  $\log [R]$  at constant  $[O]'$  and of  $E$  vs.  $\log [O]'$  at constant  $[R]$  are given in Figure 5. From the straight lines obtained, the values of  $z = 1$  and  $n = 2$  were calculated with the aid of eq. 3. The dimer constitution of the oxidation product of PS by ferricyanide has thus been demonstrated.

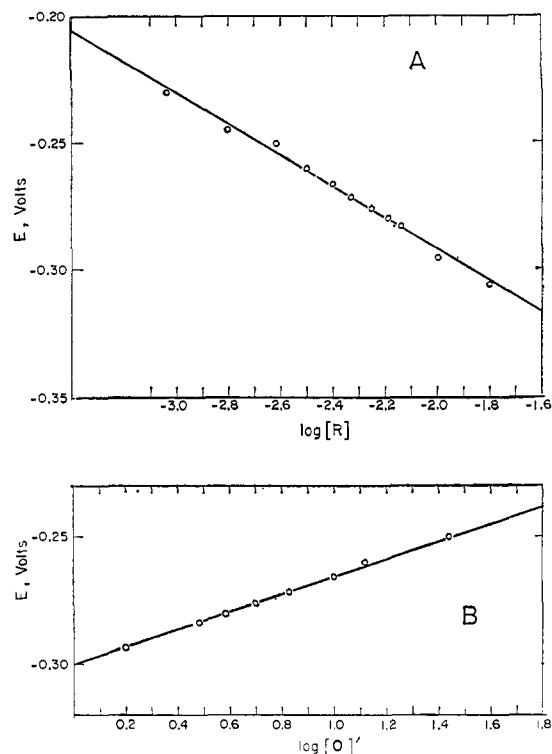


Figure 5. Oxidation-reduction potentials of mixtures of PS and  $(PS)_n$  in 0.2 M phosphate buffer, pH 7.3 (22°). Curve A: e.m.f. vs.  $\log [R]$ ;  $[O]' = 0.05$  mole/l. Curve B: e.m.f. vs.  $\log [O]'$ ;  $[R] = 0.050$  mole/l. (for definition of symbols, see text).

A plot of the values of  $E$  vs.  $\log [O]/[R]^2$ , taken from Figure 4A, yielded a straight line (Figure 4B) with the expected slope of  $\sim 0.030$  v.

The variation with pH of the e.m.f. of a system containing equimolar concentrations of PS ( $10^{-2} M$ ) and PSSP ( $10^{-2} M$ ) is given in Figure 6. The solutions investigated were prepared by adding the appropriate amount of ferricyanide (2 moles of ferricyanide per 3 moles of PS) to a solution  $3 \times 10^{-2} M$  in monomer. The oxidation of the phosphorothioate with ferricyanide was carried out in 0.1 M NaOH, and the pH of the solutions was subsequently varied by adding small amounts of HCl (4 M and 1 M) from a microburet, and the pH and e.m.f. readings were taken with at least two different platinum electrodes. After pH 1 was reached, small amounts of 1 or 4 M sodium hydroxide were added gradually and the pH and e.m.f. readings repeated. A similar procedure was followed when the partial oxidation of phosphorothioate was performed with iodine, except that the oxidation was carried out at pH 1 and the pH varied by adding 1 to 4 M sodium hydroxide up to pH 13. Measurements were repeated by adding 1 to 4 M HCl down to pH 1.0.

Oxygen was found to affect the e.m.f. measurements above pH 6 (see Figure 6). All e.m.f. measurements were, therefore, repeated under nitrogen atmosphere. The method of preparation of the phosphorothioate dimer had no effect on the variation of the e.m.f. with pH under nitrogen. From the data given in Figure 4B or 6, a standard e.m.f. of about zero at pH 7.3 was derived.

The relationship between the variation of the stand-

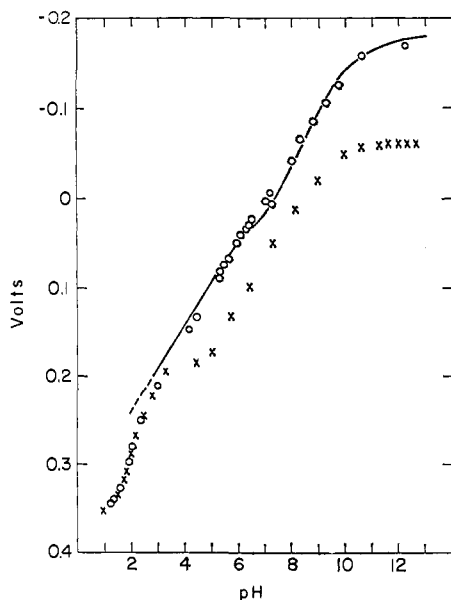


Figure 6. The variation of the oxidation-reduction potential of mixtures of PS and PSSP (each  $10^{-2} M$ ) with pH. The PSSP was prepared in the titration vessel from PS by oxidation with ferricyanide at pH 12.6; X, pH was lowered from 12.6 to 0.5 by adding acid (air not excluded); O, pH was lowered from 12.6 to 0.5 (under nitrogen atmosphere); —, calculated curve (see Discussion).

ard e.m.f. with pH and the ionization of monomer and dimer will be discussed later.

**Kinetics of the Reduction of Potassium Ferricyanide with Phosphorothioate.** The rate of reduction of ferricyanide by PS was found to depend on pH, ionic strength, and temperature. A thorough investigation of the order of the reaction, as well as the effect of the above factors on the rate of reduction, was therefore undertaken. Specific effects due to the ions of the buffer were also observed.

Each kinetic experiment to be reported below was analyzed according to the second-order equation

$$\log \frac{a-x}{b-x} = \frac{k}{2.303}(a-b)t + \log \frac{a}{b} \quad (4)$$

where  $a$  and  $b$  are the initial concentrations of ferricyanide and phosphorothioate, respectively, and  $x$  is the depletion in concentration of either of the reactants during the time  $t$  that has elapsed since the beginning of the reaction. It should be noted that  $(a-b)$  can be calculated directly from the optical density of the ferricyanide after it has reached the asymptotic value when the phosphorothioate has been essentially used up. Typical plots of  $\log (a-x)/(b-x)$  vs.  $t$  for the reduction of potassium ferricyanide by PS at four representative pH values and two ionic strengths are illustrated in Figure 7. The plots give straight lines throughout the reaction, showing that the reaction is second order with respect to the reactants in all the cases investigated. Similarly, kinetic experiments conducted with varying initial concentrations of ferricyanide or PS showed that the reaction is first order with respect to each of the reactants. The rate constants of the reaction were evaluated from the slopes of the plots of  $\log (a-x)/(b-x)$  vs.  $t$ . The exact timing of the beginning of the reaction is not possible because of experimental reasons; this is,

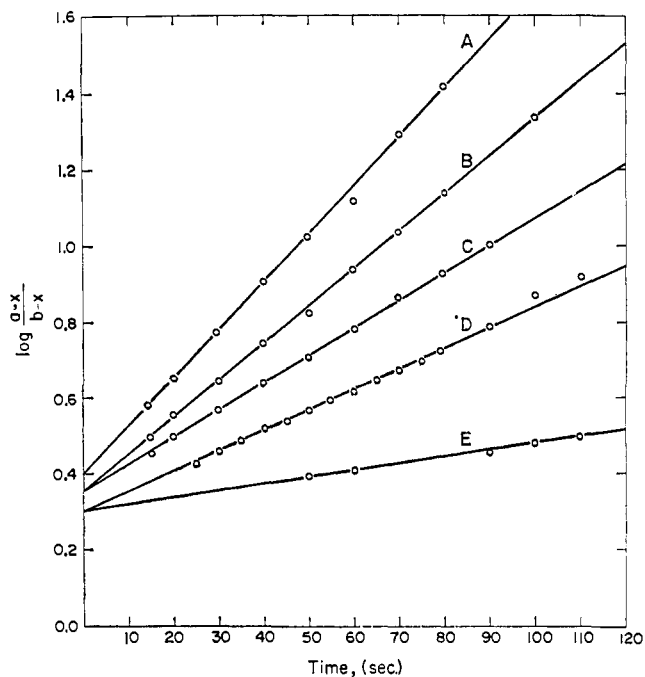


Figure 7. Typical plots of  $\log (a-x)/(b-x)$  vs. time for the reduction of potassium ferricyanide by PS ( $26^\circ$ ): (A) pH 6.2,  $\mu = 0.06$ ; (B) pH 6.0,  $\mu = 0.06$ ; (C) pH 6.85,  $\mu = 0.06$ ; (D) pH 6.88,  $\mu = 0.06$ ; (E) pH 6.88,  $\mu = 0.035$ .

however, not necessary for the calculation of the rate constants. The reproducibility of the results calculated from parallel experiments was within about  $\pm 7\%$ . Unless otherwise indicated, each result quoted below is an average of three to six parallel experiments done under similar conditions.

(a) **Effect of pH on the Rate of Reduction.** Qualitative observations revealed that reduction of ferricyanide by phosphorothioate occurs in the pH range of 5 to 13. No reaction could be detected below pH 4. Since the reduction between ferricyanide and phosphorothioate is very sensitive to ionic strength, the pH dependence of the rate of reduction was investigated at constant ionic strength. Preliminary studies have also shown that the nature of the anions and cations present may effect the rate constants of the oxidation-reduction reaction; for example, substitution of  $\text{Na}^+$  for  $\text{K}^+$  in the buffer caused a reduction of about 50% in the rate constants. It was thus impossible to investigate the rates of reduction over a wide pH range while keeping unaltered both ionic strength and nature of buffer. The investigation of the effect of pH on the reaction rate was limited to the pH range 5.8 to 7.8 and ionic strength 0.06, maintained by means of potassium phosphate buffer.

A typical set of experiments describing the pH dependence of the second-order rate constants of the reduction of ferricyanide by phosphorothioate, at  $26^\circ$ , in the above pH range and ionic strength, is given in Figure 8. It is noteworthy that the rate constant passes through a maximum at about pH 6.2.

(b) **Effect of Ionic Strength on the Rate of Reduction.** The reaction between ferricyanide and phosphorothioate was followed at pH 6.88 in solutions of ionic strengths 0.03 to 0.06. At ionic strengths higher than 0.06, the rate of reduction was too fast to follow with the

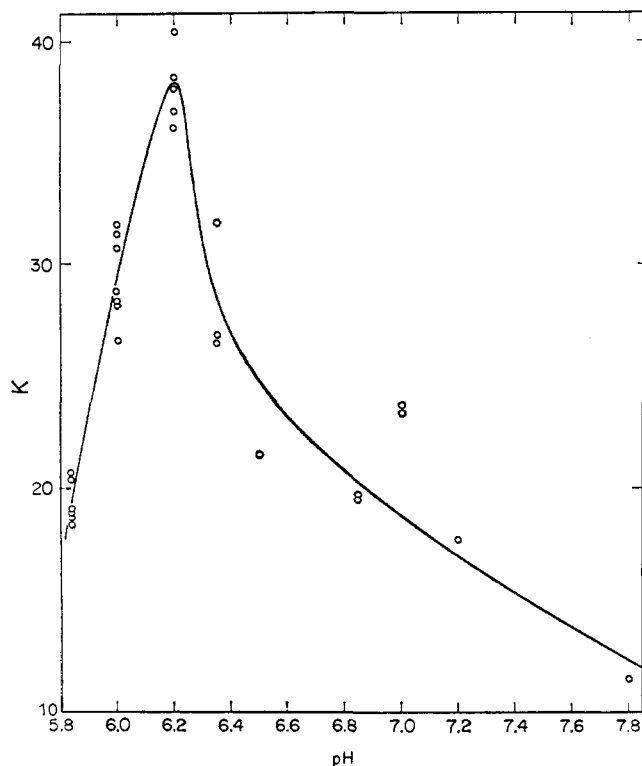


Figure 8. The dependence of the second-order rate constants of the reduction of potassium ferricyanide by potassium phosphorothioate on pH (26°, potassium phosphate buffers, ionic strength 0.06). Initial concentrations of ferricyanide and phosphorothioate  $1.33 \times 10^{-3}$  and  $0.66 \times 10^{-3}$  M, respectively. The rate constant  $k$  is given in  $\text{l. mole}^{-1} \text{sec.}^{-1}$ .

recording spectrophotometer. At ionic strengths lower than 0.03, the reactants and products contribute appreciably to the total ion concentration; changes in ionic strength as a result of the reaction cannot therefore be ignored.

A Brønsted plot of the logarithm of the oxidation-reduction rate constant  $k$  vs. the square root of the ionic strength  $\mu$ , in potassium phosphate buffer (pH 6.88), is given in Figure 9. The straight line obtained, characterized by a slope of 9, reflects the marked effect of ionic strength on the rate of the reaction.

(c) *Effect of Temperature.* The reduction of ferricyanide by PS was followed at several temperatures in potassium phosphate buffer, pH 7.24, ionic strength 0.06. The rate constants were 23, 20, 17, 15, 15, and 17  $\text{l. mole}^{-1} \text{sec.}^{-1}$  at the temperatures 15, 18, 21.5, 25, 33, and 39°, respectively. The anomalous result of a negative coefficient for the change of  $k$  with temperature between 15 and 25°, corresponding to a negative energy of activation of about  $-7$  kcal./mole, should be noted. Between 25 and 40°,  $k$  changes little with temperature.

## Discussion

The possible use of phosphorothioic acid as a reducing agent has been illustrated in the present work by its ability to reduce ceric sulfate, iodine, and ferricyanide under the appropriate conditions. The sulfur of phosphorothioic acid was converted to sulfate by treatment with ceric ion. Treatment of phosphorothioic acid with excess iodine resulted in the liberation

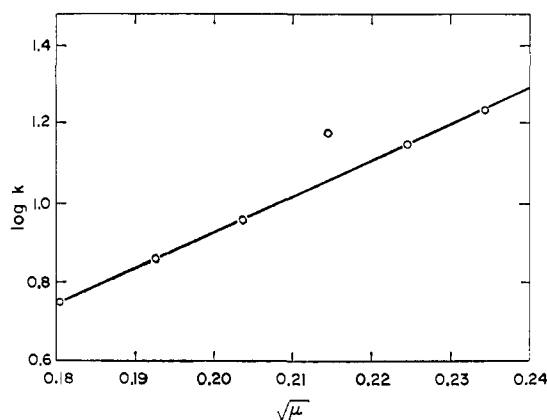


Figure 9. A Brønsted plot of the logarithm of the second-order rate constants of the reduction of potassium ferricyanide by potassium phosphorothioate vs. the square root of the ionic strength  $\mu$  of the solution mixture. Solutions were buffered with potassium phosphate buffers of different concentrations, pH 6.88 (26°). Initial concentrations of ferricyanide and phosphorothioate  $1.33 \times 10^{-3}$  and  $0.66 \times 10^{-3}$  M, respectively.

of elementary sulfur. Oxidation with an equivalent amount of iodine, on the other hand, led to the formation of the disulfide dimer (PSSP). The latter product was also obtained on oxidation of phosphorothioic acid with ferricyanide, the mildest of the oxidants used. The similarity of the oxidation product derived from phosphorothioic acid on treatment with ferricyanide to that obtained on treatment with an equivalent amount of iodine<sup>4</sup> was demonstrated by the identity of their ultraviolet absorption spectra. Independent evidence of the formation of PSSP as a result of oxidation of PS with either ferricyanide or an equivalent amount of iodine was obtained by the analysis given above of the variation of the oxidation-reduction potential of the system, phosphorothioate  $\rightleftharpoons$  oxidation products, with the concentration of both components.

Measurements of the oxidation-reduction potential  $E$  of the system  $2\text{PS} \rightleftharpoons \text{PSSP} + 2e$  revealed that  $E$  is determined by the concentrations of oxidant, reductant, and hydrogen ions, irrespective of the route by which concentrations were attained. It was thus suggested that the oxidation-reduction under investigation is reversible on the platinum electrode. In this connection it is pertinent to note that platinum black was found to catalyze the oxidation of phosphorothioate by ferricyanide.

A measure of the reducing power of phosphorothioate is given by its  $E_0'$  value,  $E_0'$  being the oxidation-reduction potential referred to the hydrogen standard when  $[\text{O}]/[\text{R}]^2 = 1$ . At neutral pH,  $E_0'$  is approximately zero. The dependence of  $E_0'$  on pH is given in Figure 6. Since the ionization constants of both oxidizing and reducing species have been determined, the pH dependence of  $E_0'$  could be calculated for the reaction  $2\text{H}_3\text{PSO}_3 \rightleftharpoons (\text{H}_2\text{PSO}_3)_2 + 2\text{H}^+ + 2e$ , with the aid of eq. 5 in which  $E_0$  denotes the standard oxidation-

$$E_0' = E_0 + \frac{RT}{nF} \ln \{ [\text{H}^+]^3 + K_{r1}[\text{H}^+]^2 + K_{r1}K_{r2}[\text{H}^+] + K_{r1}K_{r2}K_{r3} \}^{2/3} / \{ [\text{H}^+]^4 + K_{o1}[\text{H}^+]^3 + K_{o1}K_{o2}[\text{H}^+]^2 + K_{o1}K_{o2}K_{o3}[\text{H}^+] + K_{o1}K_{o2}K_{o3}K_{o4} \} \quad (5)$$

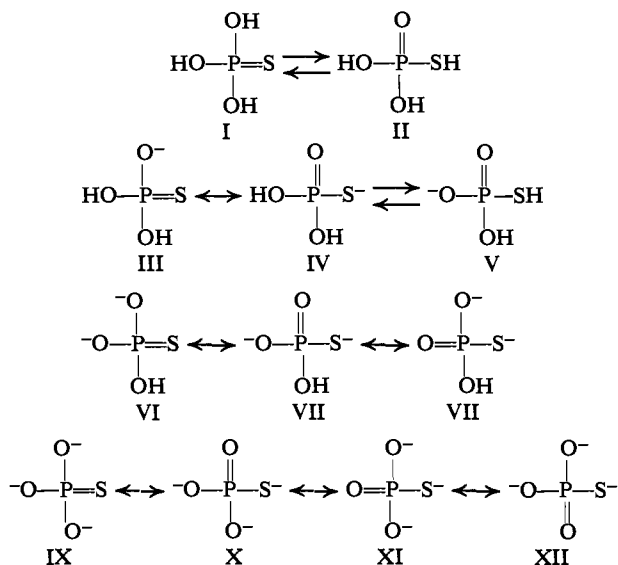
reduction potential in volts and  $K_{ri}$  ( $i = 1$  to 3) and

$K_{oi}$  ( $i = 1$  to 4) are the ionization constants of PS and PSSP, respectively.<sup>13</sup> For the system under investigation  $n = 2$ .  $K_{r1}$ ,  $K_{r2}$ , and  $K_{r3}$  were taken as  $10^{-2}$ ,  $1.78 \times 10^{-6}$ , and  $3.98 \times 10^{-11}$ , respectively;  $K_{o1}$ ,  $K_{o2}$ ,  $K_{o3}$ , and  $K_{o4}$  were taken as  $3.16 \times 10^{-3}$ ,  $3.16 \times 10^{-3}$ ,  $2.24 \times 10^{-7}$ , and  $2.24 \times 10^{-7}$ , respectively. The calculated values for  $E_0'$  below pH 2.5 are not reliable because of the uncertainty in the values of the first  $K$  values of reductant and oxidant.  $E_0 = 0.350$  v. at 22° was calculated with the aid of eq. 5 from the value of  $E_0' = 0.051$  v. measured at pH 6.0. The calculated  $E_0'$  values at the other pH values given in Figure 6 were derived from eq. 5 introducing  $E_0 = 0.350$  v. A close fit was found between the calculated and experimental data (Figure 6).

Since phosphorothioate can exist in solution in various forms, it is of interest to consider the forms which can participate in the reduction reaction. No reduction of ferricyanide occurred in the acid pH range of pH 1.0 to 4.0. It might, therefore, be concluded that the un-ionized forms (forms I and II) as well as the monoionized forms (forms III, IV, and V) cannot reduce ferricyanide at an appreciable rate.

An equilibrium between the two un-ionized forms (I and II) of PS might be expected. No information is available, however, as to the magnitude of the equilibrium constant. The monoionized forms III and IV are structures in resonance which can exist in equilibrium with PSH forms, such as form V. Reduction of the ferricyanide starts with the appearance of the diionized forms of PS on raising the pH above pH 5.

In the pH range 5 to 9, the resonance structures VI, VII, and VIII, as well as the corresponding resonance PSH forms, can exist. It is impossible at this stage to predict which of the forms predominates and which plays a major role in the reduction reaction. It should be noted, however, that in the hybrid made up of forms VI, VII, and VIII, an increase of negative charge density on the sulfur atom might be anticipated. The resonance structures of the triionized forms (IX, X, XI, and XII) should appear above pH 10. Reduction of ferricyanide in this pH range might also be correlated with the high negative charge density on the

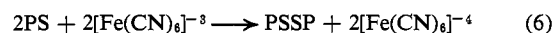


(13) Equation 5 was derived for the case under investigation by a procedure analogous to that adopted by Clark (ref. 10, pp. 118-130).

sulfur atom in the hybrid made up of structures IX, X, XI, and XII.

Parallel with the appearance of the various ionized species in solution on changing the pH from 0.5 to 12.6, there are marked changes in the absorption spectrum of phosphorothioate between 205 and 260 m $\mu$ . The most remarkable changes occur at about pH 6.0 and 11.0, indicating pronounced rearrangements in the electronic structure of the molecules on the formation of the di- and triionized species. Since orthophosphate does not absorb measurably in this spectral region, the high absorption of phosphorothioate is obviously due to the presence of sulfur in the molecule.

The second-order rate constants characterizing the reaction between phosphorothioate and ferricyanide were found to be strongly dependent on ionic strength and pH, but relatively mildly dependent on temperature. The stoichiometry of the reaction is given by eq. 6; however, since the order of the reaction is two,



one has to assume existence of intermediate stages in which a bimolecular reaction is the rate-limiting one. A possible route is as follows.



PS $\cdot$  is a free-radical species formed by the removal of an electron from PS.

Support for this scheme, in which the presence of free radicals is assumed, has been found in the observation that a solution of phosphorothioate and ferricyanide (pH 7.8) initiated the polymerization of acrylonitrile in water, a heavy white precipitate of polymer being formed within a few hours. Neither phosphorothioate nor ferricyanide by themselves were found to catalyze polymerization.

The rate constants of the above oxidation-reduction reaction at various ionic strengths (pH 6.88) were fitted to the Brønsted relationship

$$\log k = \log k_0 + 1.02z_A z_B \sqrt{\mu} \quad (9)$$

where  $k_0$  is a constant,  $z_A$  and  $z_B$  are the number of the electronic charges on the reacting molecules, and  $\mu$  is the ionic strength of the solution (see Figure 9). At pH 6.88 the phosphorothioate is predominantly doubly ionized, while the ferricyanide ion has three negative charges. The expected slope of the plot of  $\log k$  vs.  $\sqrt{\mu}$  is thus about 6. The slope found experimentally, 9, is, however, appreciably higher than this value. It should be noted that the Brønsted equation represents the effect of salt on the activity coefficients of the reactants and activated complex due to changes in the ionic atmosphere within the Debye approximation. The enhanced effect of salt concentration on the rate constants in our case is probably due to additional specific effects of the added salt on the reactants and activated complex. As a matter of fact, the nature of the cation of the added salt was found to affect the rates appreciably. Thus the reaction is faster in potassium salt than in sodium salt of the same concentration.

A plot of the rate constant describing the reduction of ferricyanide with PS as a function of pH has been given in Figure 8. In accord with the assumption made

above that reduction may be performed by the di-ionized form of PS, an S-shaped curve for the plot of  $k$  vs. pH might have been expected. The appearance of a maximum in the curve around pH 6.2 is therefore somewhat surprising. It should be remembered, however, that the experiments comparing the rates of oxidation-reduction at different pH values were conducted in phosphate buffers of constant ionic strength, and therefore necessarily of varying buffer concentration. Since there seems to be a specific ion effect superimposed on the effect of ionic strength on the rates, the pH profile of the rate constants reflects also the variation of buffer concentration on varying the pH.

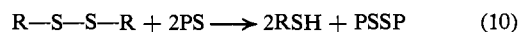
Between 18 and 25° the rate constant for the reduction of ferricyanide by PS (at pH 7.24) decreased as the temperature was raised. The formal energy of activation calculated from the measured decrease was about -7 kcal./mole. One possible explanation for this anomalous result might be that the reaction shown in eq. 8 is the rate-limiting step, while the reaction shown in eq. 7 is a fast pre-equilibrium reaction characterized by a negative enthalpy change. A similar proposal as to the nature of the rate-limiting step was made by Benesh and Benesh<sup>14</sup> in order to explain the observed maximum in the curve describing the pH dependence of the rate constants for the oxidation of various thiols. However, in the present case this explanation seems very unlikely in view of the fact that the reaction is strictly second-order; if reaction 8 were the rate-limiting step, the kinetics would be expected to be much more complex. On the other hand, while reaction 7 seems to be the rate-limiting one, it is conceivable that it is pre-

(14) R. E. Benesh, and R. Benesh, *J. Am. Chem. Soc.*, 77, 5877 (1955).

ceded by a fast equilibrium reaction of ion-pair formation between the reactants (or one of the reactants) and the counterions in the solution.<sup>15</sup> The decrease in the rate constant with temperature would then presumably result from decrease in ion-pair concentration with increasing temperature. This explanation is consistent with the conclusions reached above as to the role of the counterions in the reaction between ferricyanide and phosphorothioate.

The triionized form, as explained above, is also capable of reducing ferricyanide. No detailed studies on the reduction at constant ionic strengths, in the pH range at which the triionized form predominates, have been performed. The rate of the reaction in 0.035 *M* sodium phosphate adjusted to pH 12 was found comparable to the rate of the oxidation of the di-ionized species at pH 7.8 and ionic strength 0.05.

Phosphorothioate has been shown to react with organic S-S containing compounds such as cystine<sup>5</sup> as well as ribonuclease and lysozyme.<sup>6</sup> Because of the relatively low oxidation-reduction potential of PS ( $E_0' = 0$  at pH 7.0), in comparison with cysteine ( $E_0' = -0.32$  v. at pH = 7.0),<sup>16</sup> a simple reduction of S-S containing proteins by PS according to the formula



might be excluded. The cleavage of S-S bonds by PS must, therefore, involve a different reaction in which attachment of PS to the organic molecules occurs.

(15) Complexes of alkali metal ions with pyrophosphate and triphosphate ions were described by S. M. Lambert and J. I. Watters, *ibid.*, 79, 4262 (1957); J. I. Watters, S. M. Lambert, and E. D. Loughran, *ibid.*, 79, 3651 (1957).

(16) See ref. 10, p. 486.