789. *Interaction between Carbonyl Groups and Biologically Essential Substituents. Part III.*[1] *The Formation of a Thiazolidine Derivative in Aqueous Solution from Pyridoxal Phosphate and* L-*Cysteine.*

By F. Bergel and K. R. Harrap.

The absorption spectra of solutions containing L-cysteine and pyridoxal phosphate have been examined with a view to establishing the structure of the product. The inadequacy of this spectral evidence has been illustrated, and further evidence presented which establishes the nature of the product as the thiazolidine-4-carboxylic acid derivative, and its formation *via* the hemimercaptal.

In pursuing our study of the possible rôle of enzymes in carcinogenesis and cancer chemotherapy with particular reference to cysteine desulphydrase,[2,3] we discovered a model of its enzymic reaction which we have described in preliminary reports.[4,5] This model enzyme system consists of pyridoxal phosphate and a vanadium salt, and it became of interest to study its reaction with cysteine * in some detail. As a first step more information on the nature of the metal-free product was required.† The results of this work are described in the present paper; the mechanism of the reaction in presence of metal ions will be discussed in a later Part.

The function of pyridoxal phosphate as a coenzyme for the large number of enzyme-catalysed transformations of α-amino-acids has been reviewed by Snell [6] and by Braunstein.[7] Both these workers suggested schemes outlining the participation of pyridoxal phosphate in amino-acid metabolism, and its specific rôle as the coenzyme of cysteine desulphydrase has been demonstrated by Suda *et al.*[8] and by Braunstein and Asarkh.[9] On the other hand Snell and his collaborators [10] have studied the action of pyridoxal with amino-acids *in vitro*, laying particular emphasis on the function of metal ions in such model reactions.

The reversible equilibrium between mixtures of aldehydes or ketones and cysteine, representing an interaction between carbonyl and amino- or thiol-groups, has been studied repeatedly [11]; all these papers report the formation of thiazolidine derivatives. Heyl and his co-workers [12] described the preparation in aqueous ethanol of 2-(3-hydroxy-5-hydroxymethyl-2-methyl-4-pyridyl)thiazolidine-4-carboxylic acid, from pyridoxal and cysteine. However, the identity of the product formed from pyridoxal phosphate and cysteine derivatives in aqueous solution rests entirely on the evidence of ultraviolet absorption spectra, the aldehyde group of pyridoxals absorbing at 387·5 mμ and the condensation product at 330 mμ. For instance, du Vigneaud *et al.*[13] showed that the

* In this paper " cysteine " refers to the L-compound.

† Since this paper was submitted, two others have come to our notice: Buell and Hansen, *J. Amer. Chem. Soc.*, 1960, **82**, 6042; Naaken, *Acta Physiol. Scand.*, 1960, **50**, 109.

[1] Part II, preceding paper.

[2] Fromageot, Wookey, and Chaix, *Compt. rend.*, 1939, **209**, 1019.

[3] Smythe, " Methods in Enzymology," ed. Colowick and Kaplan, Academic Press, New York, 1955, Vol. II, p. 315.

[4] Bergel, Bray, and Harrap, *Nature*, 1958, **181**, 1654.

[5] Harrap, Bergel, and Bray, Internat. Congr. Biochem., Vienna, 1958, Abs., 4—97.

[6] Snell, " Vitamins and Hormones," ed. Harris, Marisan, and Thimann, Academic Press, New York, 1958, Vol. XVI, p. 77.

[7] Braunstein, " The Enzymes," ed. Boyer, Lardy, and Myrbäck, Academic Press, New York, 1960, Vol. II, p. 113.

[8] Suda, Risu, Saigo, and Ichihara, *Med. J. Osaka Univ.*, 1953, **3**, 469.

[9] Braunstein and Asarkh, *Compt. rend. Acad. Sci., U.R.S.S.*, 1950, **71**, 93.

[10] *E.g.*, Metzler, Ikawa, and Snell, *J. Amer. Chem. Soc.*, 1954, **67**, 648.

[11] Schubert, *J. Biol. Chem.*, 1936, **114**, 341; Ratner and Clarke, *J. Amer. Chem. Soc.*, 1937, **59**, 200; Woodward and Schroeder, *ibid.*, p. 1690; Sheehan and Yang, *ibid.*, 1958, **80**, 1154; King, Clark-Lewis, and Wade, *J.*, 1957, 881.

[12] Heyl, Harris, and Folkers, *J. Amer. Chem. Soc.*, 1948, **70**, 3429.

[13] du Vigneaud, Kuchinskas, and Horvath, *Arch. Biochem. Biophys.*, 1957, **69**, 130.

absorption spectrum of a mixture of pyridoxal phosphate and penicillamine was similar to that of the thiazolidine prepared by the method of Heyl *et al.*[12] from pyridoxal and penicillamine, and Matsuo [14] ascribed the spectrum of a mixture of pyridoxal phosphate and cysteine in water to the formation of the corresponding heterocyclic derivative. This cannot be considered as conclusive evidence because Metzler and Snell [15] have listed absorption data of 3-hydroxypyridine, pyridoxine, and their *N*-methyl derivatives, pyridoxamine, and pyridoxal in its hemiacetal form, showing absorption maxima in the range 313—330 mμ in neutral solution. In all these cases this absorption is attributable solely to the ionic form of the 3-hydroxypyridine nucleus. Consequently, the observation of similar absorption maxima of products from pyridoxal phosphate and cysteine does not represent definite proof of specific structural units.

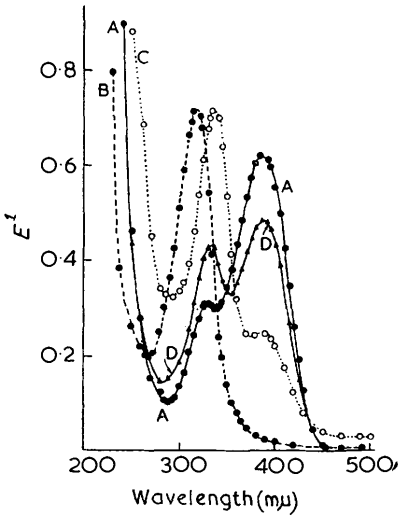


FIG. 1. Absorption spectra of $1{\cdot}2 \times 10^{-4}$M-pyridoxal phosphate alone (A) and in presence of $1{\cdot}2 \times 10^{-3}$M-KCN (B), $1{\cdot}2 \times 10^{-3}$M-mercaptoacetic acid (C), and $3{\cdot}0 \times 10^{-4}$M-Na_2SO_3, (D), in 0·1M-phosphate buffer of pH 6·0.

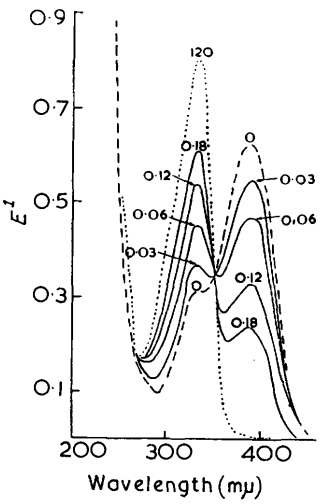


FIG. 2. Absorption spectrum of $1{\cdot}2 \times 10^{-4}$M-pyridoxal phosphate in the presence of varied concentrations of cysteine at pH 6·1. Millimolar concentrations of cysteine are shown on the peaks.

Fig. 1 shows the spectra obtained on separate addition of sodium sulphite, mercaptoacetic acid, and potassium cyanide to a solution of pyridoxal phosphate at pH 6·0. In each case the characteristic absorption of the carbonyl group of pyridoxal phosphate at 387·5 mμ is lowered and in the presence of a sufficient excess of reagent, is replaced by a single absorption band in the region of 330 mμ (although the cyanohydrin absorbs at somewhat lower wavelengths). A similar spectrum results from a mixture of pyridoxal phosphate and cysteine and Fig. 2 shows the spectra obtained when increasing quantities of cysteine were added to a solution of pyridoxal phosphate at pH 6·0. Isosbestic points are evident at 270 and 350 mμ (indicating that the spectral changes represent an equilibrium process between pyridoxal phosphate and the product of its reaction with cysteine [16]), and the aldehyde peak of pyridoxal phosphate has been replaced by a single absorption at 330 mμ. The similarity between the absorption spectra of mercaptoacetic acid–pyridoxal phosphate, and cysteine–pyridoxal phosphate mixtures demonstrates that it is not possible to distinguish between the hemimercaptal (I; X = H or NH_2), the dimercaptal (II; X = H or NH_2), and the thiazolidine (III) by spectrophotometric means alone.

[14] Matsuo, *J. Amer. Chem. Soc.*, 1957, **79**, 2011.
[15] Metzler and Snell, *J. Amer. Chem. Soc.*, 1955, **77**, 2431.
[16] Metzler, *J. Amer. Chem. Soc.*, 1957, **79**, 485.

The effect of pH on the extent of reaction between pyridoxal phosphate and cysteine is shown in Fig. 3, and it is evident that condensation has taken place over pH range 4—10 and maximally at about pH 8·0.

Equilibrium constants were measured for the reaction of pyridoxal phosphate with an equimolar amount of (*a*) cysteine and (*b*) mercaptoacetic acid at pH 6·0. For (*a*), values of $2{\cdot}5 \times 10^4$ l. mole^{-1} (standard deviation $3{\cdot}4 \times 10^3$), and for (*b*) $1{\cdot}36 \times 10^2$ l. mole^{-1} (standard deviation $1{\cdot}78 \times 10^1$) were obtained. These figures indicate that cysteine reacted far more extensively with pyridoxal phosphate than did mercaptoacetic acid, and they agree with Matsuo's observation [14] that the equilibrium constant for the cysteine–pyridoxal phosphate reaction (although not measured by him directly) was several powers of ten greater than that obtained for formation of Schiff's bases from pyridoxal phosphate and amino-acids. In our experiments we eliminated the possibility of transamination by subjecting mixed solutions of pyridoxal phosphate and cysteine after 24 hr. to paper electrophoresis.[17] No pyridoxamine phosphate was detected.

It has been implied by Metzler *et al.*[10] that desulphydration of cysteine by pyridoxal and metal salts [18] involved formation of a Schiff's base as intermediate. However, we

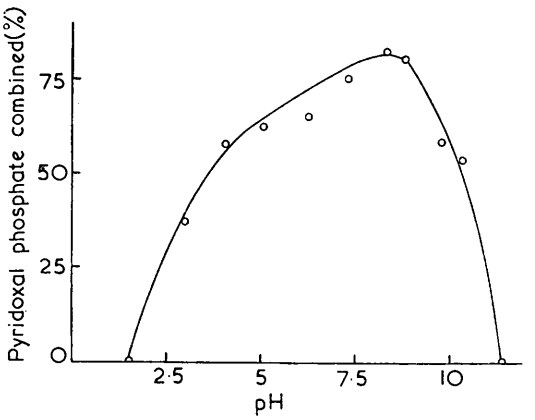


FIG. 3. Effect of pH on thiazolidine formation. Pyridoxal phosphate 3×10^{-4}M; cysteine 3×10^{-4}M.

have shown that the thiol as well as the amino-group of cysteine reacts with the aldehyde group of pyridoxal phosphate, by measuring the rate and extent of disappearance of the thiol group by means of 2,6-dichlorophenolindophenol.[19] Fig. 4 shows the effect of increasing concentrations of pyridoxal phosphate on the initial rate of reduction of a solution of the dye in the presence of cysteine.

The kinetics of the interaction of pyridoxal phosphate with mercaptoacetic acid and cysteine severally indicated that with the latter more than simple mercaptal formation took place. Under pseudo-first-order conditions pyridoxal phosphate reacted with an excess of the thiols in the manner shown in Fig. 5. The curve depicting the rate of decrease of $E^1_{387{\cdot}5}$ (the optical density at 387·5 mμ for 1 cm. light path) disclosed two distinct reactions. In the case of mercaptoacetic acid the two reactions were assumed to correspond to the formation of hemi- and di-mercaptal. With cysteine there could, in addition, be some thiazolidine formation during the second reaction. The initial reaction was too fast for analysis: the second gave a first-order rate coefficient of $3{\cdot}5 \times 10^{-3}$ sec.$^{-1}$ for cysteine and $0{\cdot}8 \times 10^{-3}$ sec.$^{-1}$ for mercaptoacetic acid at the concentrations employed (these figures illustrate only the difference in rate between the two reactions).

When equivalent amounts of thiol and pyridoxal phosphate were mixed, the rate curves indicated that the two thiols reacted differently with pyridoxal phosphate. As shown in Fig. 6, the mercaptoacetic acid reaction reached equilibrium within 10 seconds,

[17] N. Siliprandi, D. Siliprandi, and Lis, *Biochem. Biophys. Acta*, 1954, **14**, 212.
[18] Metzler and Snell, *J. Biol. Chem.*, 1952, **198**, 353.
[19] Basford and Heunnekens, *J. Amer. Chem. Soc.*, 1955, **77**, 3874.

whereas the rate curve for the cysteine reaction was of the two-step type obtained for an excess of thiol: after an initial rapid reaction, whose plot followed that of the mercaptoacetic acid curve, a slower secondary reaction took place. Under the conditions used,

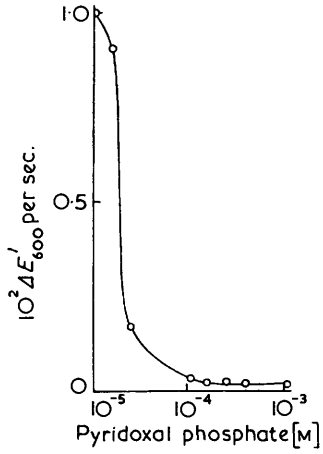


FIG. 4. Variation of initial rate of reduction of indophenol by cysteine in the presence of increasing concentrations of pyridoxal phosphate at pH 7·0. Indophenol 4·92 × 10^{-5}M; cysteine 4·0 × 10^{-5}M.

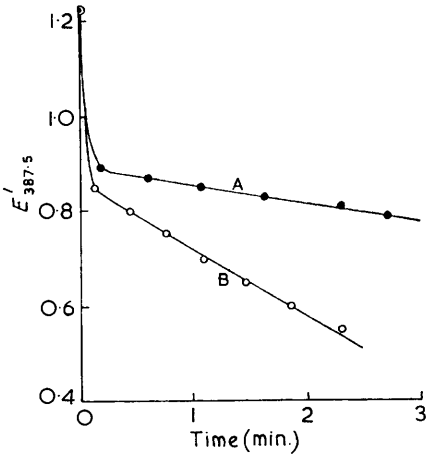


FIG. 5. Rate of change of $E^1_{387·5}$ for 2·4 × 10^{-4}M-pyridoxal phosphate in the presence of an excess (1·2 × 10^{-2}M) of mercaptoacetic acid (A) or cysteine (B) in 0·1M-phosphate of pH 6·0.

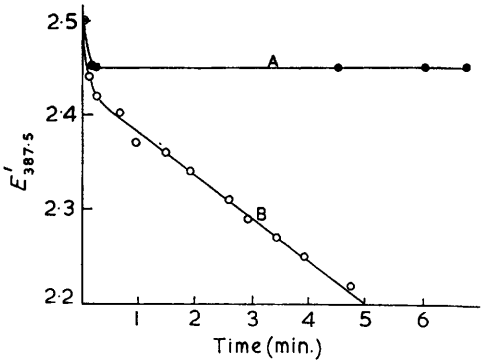
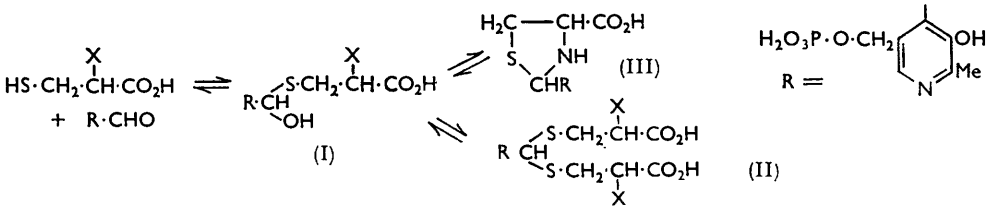


FIG. 6. Rate of change of $E^1_{387·5}$ for 4·8 × 10^{-4}M-pyridoxal phosphate in the presence of an equivalent concentration of 6·0 × 10^{-4}M-mercaptoacetic acid (A) or 4·8 × 10^{-4}M-cysteine (B) 0·1M-phosphate of pH 6·0.

no dimercaptal formation was possible, and the cysteine–pyridoxal phosphate reaction may be interpreted as, first, formation of hemimercaptal (by analogy with the mercaptoacetic acid reaction) and then ring closure to the thiazolidine. The kinetics of this reaction



(In the scheme the compounds are represented in their non-ionic forms.)

(calculations showed it to be of the second order) are complicated, as a more exact evaluation of the rate coefficients in the scheme shown above (I—III; X = NH_2) involves a

knowledge of the concentration of the hemimercaptal (I; X = NH_2) at any instant, and this cannot be obtained by the method adopted here. For precise calculation of the rate coefficients the concentration of this hemimercaptal cannot be ignored, particularly in the later stages of the reaction, when considerable amounts of thiazolidine (III) have been formed, the cysteine and pyridoxal phosphate concentration have fallen below their initial values, and the reverse reaction gained predominance. But when it was ignored and the necessary substitutions were made in the integrated rate equation for a reaction of the type A + B $\rightleftharpoons$ C, it was found that the apparent velocity coefficient decreased linearly with time. By extrapolating to zero time a value of 0·95 l. $mole^{-1}$ $sec.^{-1}$ was obtained which was probably determined largely by the constant for the ring closure (the rate-limiting step for the overall reaction), calculated under conditions where the concentration of reactants are high and that of thiazolidine very low.

Paper chromatography of a cysteine solution containing an excess of pyridoxal phosphate, resulted in the appearance of a new fluorescent spot (R_F 0·02). This developed a very faint yellowish-orange colour in the presence of ninhydrin, similar to that for, *e.g.*, 2-phenyl-, 2-*o*-hydroxyphenyl-, and unsubstituted thiazolidine-4-carboxylic acids whose colours ranged from yellow to orange when treated with ninhydrin on paper. Cysteine mercaptals would be expected to give the usual blue coloration of amino-acids in the presence of the reagent.

Attempts to isolate pure 2-(3-hydroxy-2-methyl-5-hydroxymethyl-4-pyridyl)thiazolidine-4-carboxylic acid 5′-phosphate gave colourless crystals with an absorption maximum at 330 mμ in water. These were very unstable and became yellow and moist in air; the analytical results were unsatisfactory. Nevertheless, all the data presented can be best explained by the formation of this phosphate acid (III) in solution.

As shown in the scheme (I—III), an excess of thiol with pyridoxal phosphate forms, first, the hemimercaptal (I; X = H or NH_2), and then the dimercaptal (II; X = H or NH_2). Equivalent amounts of pyridoxal phosphate and thiol give only the hemimercaptal. With cysteine, however, once the hemimercaptal (I; X = NH_2) has been formed, the amino-group is capable of further reaction, and ring closure takes place to the thiazolidine, demonstrated by the second phase in Fig. 6 and indirectly by the equilibrium constant and chromatographic results.

Experimental

Reagents and Methods.—Pyridoxal phosphate monohydrate was obtained from Roche Products Ltd. In solution it is photosensitive [20] and must be shielded from strong light. L-(−)-Cysteine hydrochloride, mercaptoacetic acid, and 2,6-dichlorophenol-indophenol were of B.D.H. Laboratory Reagent Grade. Buffer salts were of analytical or B.D.H. Laboratory Reagent Grade. Water was glass-distilled and then de-ionised before use. pH was measured on a Pye Universal pH meter. Absorption spectra were measured in a Unicam S.P. 500 spectrophotometer, which for kinetic work was fitted with a thermostatically controlled cell-housing set at 23—24°. A Carey recording spectrophotometer, model 11M—50, was used for the determination of the effect of pH on thiazolidine formation.

Absorption Spectra of Pyridoxal Phosphate Derivatives.—Pyridoxal phosphate (final concn. $1·2 \times 10^{-4}$M) was mixed with the following reagents in 0·1M-phosphate buffer (pH 6·0 [21]) and left in the absence of air for 1 hr., then the absorption spectra (Fig. 1) were measured: KCN $1·2 \times 10^{-3}$M; mercaptoacetic acid $1·2 \times 10^{-3}$M; Na_2SO_3 $3·0 \times 10^{-4}$M.

Pyridoxal Phosphate–Cysteine Spectra.—Pyridoxal phosphate in phosphate buffer (0·1M; pH 6·1) was mixed with varying concentrations of cysteine hydrochloride and diluted with the same buffer in 1 cm. stoppered cells to the final concentrations shown in Fig. 2. The cells were flushed with nitrogen for several minutes before closure and then left in the dark for equilibration.

Effect of pH *on Thiazolidine Formation.*—Cysteine hydrochloride and pyridoxal phosphate were mixed to give final concentrations of 3×10^{-4}M in the buffers listed below. A manifold was arranged so that nitrogen could be passed into the solns. for 15 min. Then the tubes were

[20] Morrison and Long, *J.*, 1958, 211.
[21] Green, *J. Amer. Chem. Soc.*, 1933, **55**, 2331.

sealed and left for equilibration overnight in the dark. The spectra, and finally the pH of the solutions, were measured. The extent of reaction was calculated on the basis of the $E^1_{387\cdot5}$ value.

Buffers: pH <3, 0·1N-HCl; pH 3—7, 0·1M-citric acid and 0·2M-sodium dihydrogen phosphate; [22] pH 8 and 9, 0·1M-disodium dihydrogen pyrophosphate and 0·1M-tetrasodium pyrophosphate; pH 10·0, 0·2M-sodium carbonate [22] and 0·2M-sodium hydrogen carbonate; pH >10, 0·1N-NaOH.

Equilibrium Constants.—Cysteine or mercaptoacetic acid (3×10^{-3}M; 0·1M with respect to EDTA, to obviate metallic contamination from the buffer) and phosphate buffer (pH 6·0) were mixed with pyridoxal phosphate (3×10^{-3}M) in 0·1M-phosphate buffer (pH 6·0) and diluted with the same buffer in duplicate tubes to the following concentrations: mercaptoacetic acid 1·8—$9\cdot6 \times 10^{-4}$M; cysteine 0·6—$12\cdot0 \times 10^{-4}$M. All operations were carried out in a glove box in an atmosphere of nitrogen (tested with alkaline pyrogallol to ensure absence of oxygen). The solutions were left overnight in the dark, then the optical density at 387·5 mμ was measured at 23—24°. Equilibrium constants were calculated on the basis of the amount of pyridoxal phosphate that reacted, from the following formulæ (P = pyridoxal phosphate):

$$\text{For cysteine (C):}\quad K = [(\text{I};\ X = NH_2) + (\text{III})]/[P][C]$$

$$\text{For mercaptoacetic acid (M):}\quad K = [(\text{I};\ X = H)]/[P][M]$$

Titration of Cysteine–Pyridoxal Phosphate Solutions with 2,6-Dichlorophenolindophenol.—Cysteine hydrochloride was kept with pyridoxal phosphate solutions of various concentrations at pH 6·0 and 23° for 1 hr. Portions (0·1 ml.) were then added to a 1 cm. stoppered cell containing 0·1M-phosphate (2·3 ml.) (pH 7·0 [21]) at 23—24°, followed by aqueous $1\cdot2 \times 10^{-3}$M-indophenol (0·1 ml.). The rate of change of optical density at 600 mμ was measured at 23—24°.

Kinetics of Pyridoxal Phosphate–Thiol Reactions.—(*a*) An excess of cysteine or mercaptoacetic acid (0·1 ml.; 0·3M) was added to a 1 cm. stoppered Unicam cell containing pyridoxal phosphate (0·15, 0·3, 0·6, 0·9, or 1·2 μmoles severally) in 0·1M-phosphate buffer (pH 6·0) at 23—24°. The rate of decrease of $E^1_{387\cdot5}$ was then measured (Fig. 5). The velocity constants were determined from the slope of the graph of log [pyridoxal phosphate] against time.

(*b*) Equivalent amounts of the reactants were treated as in (*a*) except that the final concentrations of aldehyde and thiols were 0·6, 1·2, 2·4, 3·6, or $4\cdot8 \times 10^{-4}$M severally (Fig. 6). For experiments lasting more than 10 min. solutions were stored under nitrogen.

Paper Chromatography.—A solution of pyridoxal phosphate (0·1M) and cysteine hydrochloride (0·01M) at pH 6·0 was left in the dark under nitrogen for 1 hr., then spotted on No. 4 Whatman paper and chromatographed in butanol–acetic acid–water (2 : 3 : 5).

Attempted Isolation of the Thiazolidine Derivative.—To pyridoxal phosphate (0·27 g.) in N-alcoholic potassium hydroxide (5 ml.) was added cysteine (1·21 g.) in the same solvent (40 ml.) under nitrogen. Concentrated hydrochloric acid (6 ml.) was added, the precipitate was removed and extracted with absolute alcohol (200 ml.) at 40°. The filtered solution was combined with the acid filtrate and evaporated. The resulting off-white solid was recrystallised from alcohol–ether and dried (P_2O_5–KOH) (yield 50 mg., 13%) (Found: C, 33·2; H, 5·35; O, 29·8; N, 6·7; S, 9·3; P, 7·8; Cl, 10·5. Calc. for $C_{11}H_{16}ClN_2O_7SP$: C, 34·1; H, 4·15; O, 29·0; N, 7·2; S, 8·8; P, 8·0; Cl, 9·15%). Infrared spectrophotometry, while showing the absence of thiol absorption at 2550 cm.$^{-1}$, was of no assistance in ascertaining the purity of the product.

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[22] Gomori, "Methods in Enzymology," ed. Colowick and Kaplan, Academic Press, New York, 1955, Vol. I, p. 138.