205. *Interaction between Carbonyl Groups and Biologically Essential Substituents. Part IV.*[1] *An Enzyme Model System for Cysteine Desulphydrase.*

By F. BERGEL, K. R. HARRAP, and (MISS) A. M. SCOTT.

Vanadium salts, in combination with pyridoxal phosphate, catalyse a highly specific elimination of hydrogen sulphide from cysteine. The experimental conditions controlling this enzymomimetic reaction have been investigated, and the principal products identified, among which are also ammonia, pyruvic acid, lanthionine, and cystine. The reaction mechanism is thought to involve vanadium-catalysed hydrolysis of the 1,2-bond of the thiazolidine intermediate,[1] followed by formation of a Schiff's base that decomposes immediately by an αβ-elimination similar to that proposed by Metzler *et al.*[2]

THE enzyme cysteine desulphydrase degrades cysteine;[3] because of the claims that leucocytes from certain leukæmic patients and animals showed an elevated or altered

[1] Part III, Bergel and Harrap, *J.*, 1961, 4051.
[2] Metzler, Ikawa, and Snell, *J. Amer. Chem. Soc.*, 1954, **76**, 648.
[3] Fromageot, Wookey, and Chaix, *Enzymologia*, 1940, **9**, 198; Smythe, *J. Biol. Chem.*, 1942, **142**, 387; Chatagner, Jollès-Bergeret, and Labouesse, *Compt. rend.*, 1960, **251**, 3097.

sulphur metabolism,[4] we undertook further purification of this enzyme which, we speculated, might control the levels of sulphur-containing amino-acids under biological conditions. These attempts failed with regard to the isolation of a homogeneous functional protein. Subsequently we studied the effect of pyridoxal phosphate (the coenzyme of cysteine desulphydrase [5]) on cysteine and related compounds in presence of metal ions, following the work of Metzler *et al.*[2] on enzyme models. In these studies, as briefly reported,[6] we discovered that a combination of pyridoxal phosphate and a vanadium salt degraded cysteine efficiently under anaerobic conditions and in physiological ranges of pH and temperature. Part III [1] of this series described the formation of a thiazolidine-4-carboxylic acid derivative from pyridoxal phosphate and L-cysteine: its behaviour in the presence of metal ions is discussed in the present paper.

While mixtures of pyridoxal and metal ions (*e.g.*, aluminium) at 100° produced a measurable degradation of cysteine (cf. Metzler and Snell [7]), a survey by us of several transition elements disclosed that a combination of vanadium salts with pyridoxal phosphate was a far more efficient catalyst. The results are set out in Table 1, where the metals are arranged in order of their desulphydrase-like activities.

TABLE 1.

Desulphydrase activities of various metals (0·38 mM) in combination with pyridoxal phosphate and cysteine (pyridoxal phosphate 0·38 mM, cysteine 1·2 mM; 37°; 0·1M-acetate; pH 5·8).

Salt	Activity (10^{-6} mole of H_2S/hr.)	Salt	Activity (10^{-6} mole of H_2S/hr.)
NH_4VO_3	0·390	$Ni(NO_3)_2,6H_2O$	0·012
$VOCl_2$	0·320	$Na_2WO_4,2H_2O$	0·012
$VOSO_4$	0·380	$AgNO_3$	0·012
$Na_3VO_4,14H_2O$	0·410	$HgCl_2$	0·011
$FeSO_4,(NH_4)_2SO_4,6H_2O$	0·049	$Zr(NO_3)_4$	0·011
$Ti_2(SO_4)_3$	0·043	$CuSO_4,5H_2O$	0·010
$Al_2(SO_4)_3,K_2SO_4,24H_2O$	0·042	$MnCl_2,4H_2O$	0·010
$KMnO_4$	0·038	$RuCl_3$	0·010
$CoCl_2,6H_2O$	0·038	$[(NH_4)_3Rh]Cl_6,1\frac{1}{2}H_2O$	0·010
$TlCl_2$	0·026	$PdCl_2$	0·010
$Fe_2(SO_4)_3,(NH_4)_2SO_4,24H_2O$	0·024	$K_2Nb_2O_6,7H_2O$	0·010
$MgSO_4,7H_2O$	0·020	$Ce(SO_4)_2,2(NH_4)_2SO_4,2H_2O$	0·009
$CrCl_3,6H_2O$	0·012	$CaCl_2,6H_2O$	0·006
K_2CrO_4	0·012	$K_2Cr_2O_7$	0·002
$Zn(OAc)_2,2H_2O$	0·012		

The ultraviolet absorption spectrum of the thiazolidine-4-carboxylic acid derivative was described in Part III,[1] and the addition of vanadyl ions to this compound results in an initial spectral change as shown in Fig. 1, namely, the maximum at 330 mμ moves to 310 mμ, and the adsorption at 275 mμ becomes more intense. The effect is not merely summation of the spectra of the constituents of the mixture, but suggests that vanadium has combined with the thiazolidine. By the method of continuous variations [8] it is possible to show that quadrivalent vanadium and the thiazolidine combine in a 1 : 1 ratio. If the original mixture is kept for 72 hr., the degradation involving loss of hydrogen sulphide results in further spectral changes shown in Fig. 1. Ferrous salts behave similarly, although their catalytic effects are less marked.

[4] Weisberger and Heinle, *J. Lab. Clin. Med.*, 1950, **36**, 872; Weisberger and Levine, *Blood*, 1954, **9**, 1082; White, Mider, and Heston, *J. Nat. Cancer Inst.*, 1943, **4**, 409; Contopoulos and Anderson, *J. Lab. Clin. Med.*, 1950, **36**, 929.
[5] Braunstein and Azarkh, *Compt. rend. Acad. Sci. U.R.S.S.*, 1950, **71**, 93; Suda, Kisu, Saigo, and Ichihara, *Med. J. Osaka Univ.*, 1953, **3**, 469.
[6] Bergel, Bray, and Harrap, *Nature*, 1958, **181**, 1654.
[7] Metzler and Snell, *J. Biol. Chem.*, 1952, **198**, 353.
[8] Martell and Calvin, " Chemistry of the Metal Chelate Compounds," Prentice-Hall Inc., New York, 1952, p. 29.

The questions next arose whether phosphate of pyridoxal was essential and whether other sulphur compounds than cysteine could serve as substrates. Substitution of pyridoxal for its phosphate resulted in a 25-fold decrease in the rate of evolution of hydrogen sulphide, but substitution of 5-deoxypyridoxal resulted in the same rate of evolution as was obtained in the presence of the phosphate, suggesting that the phosphate group was not involved in the reaction. The failure with pyridoxal was therefore attributed to its presence as hemiacetal.[9]

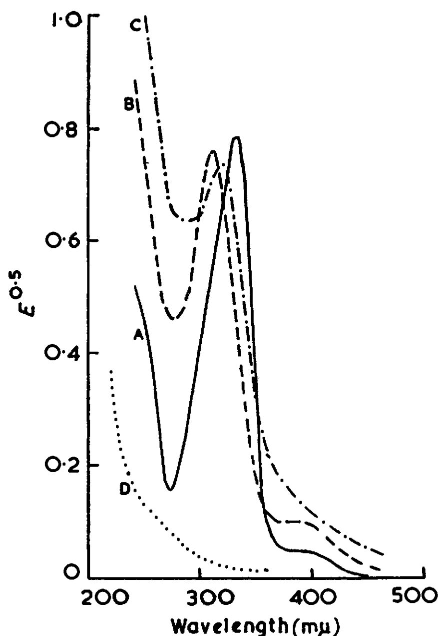


FIG. 1. Absorption spectra of mixtures of thiazolidine-4-carboxylic acid ($2 \cdot 4 \times 10^{-4}$M), cysteine ($1 \cdot 2 \times 10^{-3}$M), and vanadyl sulphate ($2 \cdot 4 \times 10^{-4}$M) in acetate buffer (0·1M; pH 6·0).

A, B, C, Thiazolidine-4-carboxylic acid (A) alone and (B, C) + VO^{2+} (B, immediate; C, after 72 hr.); D, cysteine + VO^{2+}.

A number of sulphur-containing compounds related to cysteine have been examined, with results assembled in Table 2 that show the great specificity of the model system for cysteine degradation. Alkylation of the α- or β-carbon atom of cysteine stabilises the molecule. However, the carboxyl group can be replaced by an ethoxycarbonyl group

TABLE 2.

Other substrates for model system: comparative rates of evolution of hydrogen sulphide.

Compound	Rate	Compound	Rate
L-Cysteine	100	L-Cysteine Et ester *	100
L-Cysteinylglycine	25	DL-Cysteinyl-D-valine *	10
L-Cystine	5		

The following were inert: *N*-acetyl- and *S*-ethyl-L-cysteine; α-methyl-DL-cysteine; DL-homocysteine; cysteamine; DL-penicillamine; DL-methionine; reduced glutathione; mercaptoacetic acid; urease.

* After reduction of the disulphide.

without affecting the rate of evolution of gas, although the influence of the carbonyl group cannot be negligible (presumably its mesomeric effect contributes to the forces governing the decomposition) since cysteamine is unaffected. Further, simple terminal dipeptides of cysteine (still containing the carbonyl group) are degraded more slowly than the uncombined amino-acid. The stability of homocysteine is probably due to the formation of a less-strained thiazan ring system. Alkylation of thiol or acylation of amino-groups protects the molecule against the action of the model system.

[9] Metzler and Snell, *J. Amer. Chem. Soc.*, 1955, **77**, 2431.

We have already indicated[6] that the pyridoxal phosphate–vanadium combination does not catalyse reactions of alanine and stimulated little those of serine. We wished to investigate whether our model system was effective in catalysing the synthesis of tryptophan from indole, described as an *in vitro* reaction by Metzler *et al.*,[2] who used serine,

TABLE 3.
Synthesis of tryptophan by model systems.

Amino-acid–metal combination	Yield (%) of tryptophan after heating		Yield (%) of tryptophan after storage	
Al = $Al_2(SO_4)_3,(NH_4)_2SO_4,6H_2O$ V = NH_4VO_3	Pyridoxal phosphate	Pyridoxal	Pyridoxal phosphate	Pyridoxal
Ser–Al	0·3	1·9	0·1	1·4
Ser–V	0	0·2	0	0·3
CySH–Al	,,	0·3	,,	0·2
CySH–V	,,	0	,,	0

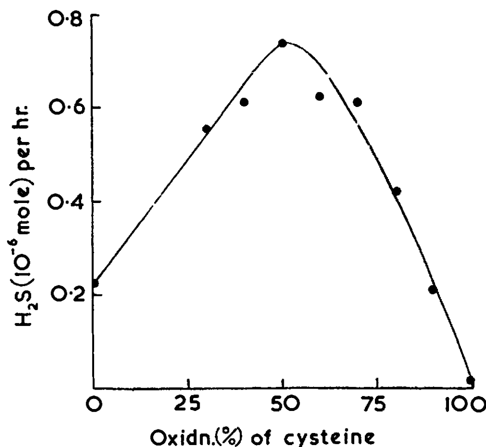


FIG. 2. Effect of redox potential on rate of evolution of H_2S. Total cysteine + cystine concn. (as cysteine), 7·7 × 10^{-3}M; pyridoxal phosphate and ammonium vanadate, each 3·8 × 10^{-4}M; in phosphate buffer (0·1M; pH 6·0).

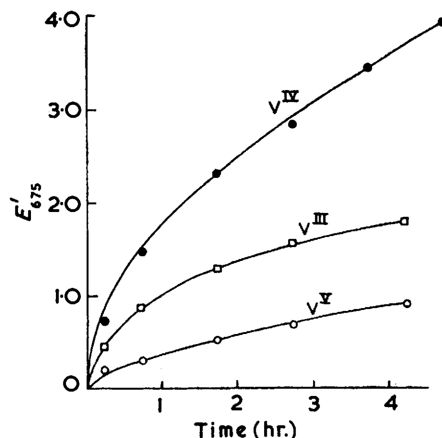


FIG. 3. Variation of rate of evolution of H_2S with valency of vanadium. Pyridoxal phosphate, 1·2 × 10^{-2}M; cysteine, 1·2 × 10^{-3}M; metal 1·2 × 10^{-3}M; buffer of pH 6·0.

pyridoxal, and aluminium. Repeating their experiment (heating at 100° for 30 min., or storage for 10 days at 23°), we compared it with others in which serine was replaced by cysteine, aluminium by vandium, and pyridoxal by pyridoxal phosphate (see Experimental and Table 3). It is apparent that cysteine gave lower yields than serine, and that vanadium was less efficient than aluminium in bringing about the synthesis of tryptophan. When pyridoxal was replaced by pyridoxal phosphate no tryptophan was found.

The rate of evolution of hydrogen sulphide from cysteine in presence of the enzyme model seems to depend on the oxidation–reduction potential of the system. In Fig. 2 are shown the results of an experiment with mixtures of varying proportions of cysteine and cystine, the concentrations of which were sufficient to maintain constant the redox potential of the system. Evolution of gas was fastest from the solution having 50% of its cysteine in the disulphide form. The relatively low rate of elimination from unpoised cysteine solutions cannot be attributed to the formation of a vanadium species of lower valency which is less active than that predominating at the favoured potential of the cysteine–cystine couple, because solutions of cysteine at pH 5·0 are not able to reduce vanadium below the quadrivalent state. A comparison of V^{III}, V^{IV}, V^{V} species indicated that the most active modification was quadrivalent vanadium (see Fig. 3).

The detection of hydrogen sulphide, pyruvic acid, ammonia, and alanine in incubated solutions of cysteine, pyridoxal phosphate, and vanadium salts was reported in our previous publications.[6,10] Lanthionine, cystine, and serine have also been detected by paper chromatography. Cystine and lanthionine were separated by applying hydrogen peroxide

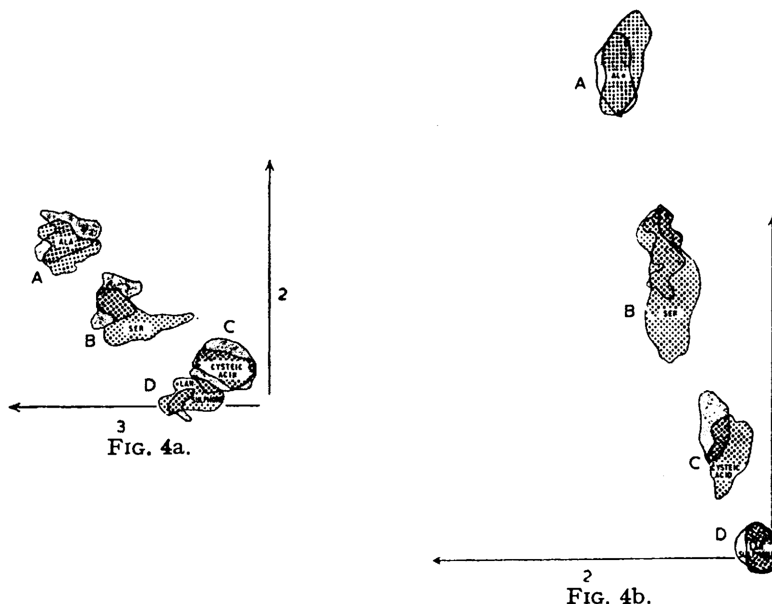


FIG. 4. Two-dimensional paper chromatography of model system after 24 hours' incubation (peroxide-treated) in solvents marked on the arrows. Solvent 1 run overnight, other solvents run to end of papers. Light stippling = standard amino-acids; dark stippling = amino-acids from degradation of cysteine.

A, Alanine; B, serine; C, cysteic acid; D, sulphone from lanthionine.

to the dried material on the paper before running the chromatogram, so that the spots in Fig. 4a and b corresponding to these amino-acids refer to cysteic acid and lanthionine sulphone; Dent [11] used this method for chromatographic separation of methionine as sulphone on paper. The quantitative results are recorded in Table 4. The material unaccounted for in the totals (averaging 15%) is represented by the contents of band A in

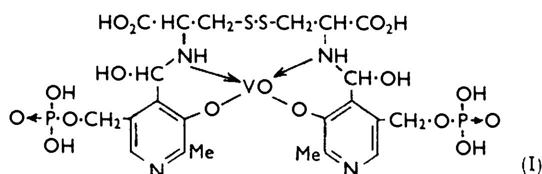


Fig. 5. Paper chromatography and paper electrophoresis have established the presence of vandium and pyridoxamine phosphate in this fraction, as well as a fluorescent ninhydrin-positive product which is associated with vanadium and is oxidised on paper chromatograms by hydrogen peroxide to cysteic acid. It is tentatively suggested that this unidentified component of band A is a disulphide such as (I). Traces of two other ninhydrin-positive spots in the chromatogram of band A have not been identified. Bands B and C represent lanthionine–serine and cystine–alanine as shown (Fig. 5). When

[10] Harrap, Bergel, and Bray, 4th Internat Congr. Biochem., Vienna, Supp. Int. Abs. Biol. Sci., 1958, p. 51.

[11] Dent, *Biochem. J.*, 1948, **43**, 169.

TABLE 4.

Analysis of model-system degradation products (duplicate experiments): $VOSO_4$ and pyridoxal phosphate 0·01M; cysteine 0·05M; acetate buffer 0·01M, pH 5·0; 24 hr. at 37°.

Component	Concn. of component (as % of initial cysteine concn.)	Groups in component (as % of total groups at time 0)	
		NH_2	S
Cysteine	3·4, 1·0	3·4, 1·0	3·4, 1·0
Cystine	4·5, 4·7	9·0, 9·4	9·0, 9·4
Lanthionine	23·4, 21·1	46·8, 42·2	23·4, 21·1
Alanine	0·9, 0·8	0·9, 0·8	
Serine	0·5, 0·8	0·5, 0·8	
Ammonia	20·0, 25·0	20·0, 25·0	
H_2S	40·0, 40·0		40·0, 40·0
Thiosulphate	5·0, 5·0		10·0, 10·0
Pyruvic acid *	20·0, 25·0		
Pyridoxamine phosphate †	8·0, 8·0	8·0, 8·0	
Total		88·7, 87·3	85·8, 81·5

* As pyruvic acid is lost to a large extent on incubation with vanadium salts at the above concns. and pH (see Experimental), the figures are calculated from the ammonia determinations.
† Measured during another run which produced the same amount of H_2S.

ammonium vanadate was substituted for vanadyl sulphate the yield of cystine increased, that of lanthionine decreasing accordingly.

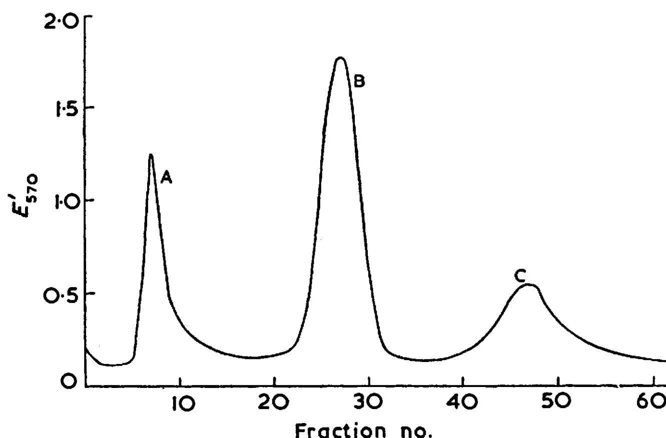


FIG. 5. Amino-acid elution pattern from column chromatography of products of cysteine degradation by the model system: Dowex 50 × 8 (initial composition of system: cysteine 0·05M, pyridoxal phosphate and $VOSO_4$ each 0·01M; acetate buffer (0·1M; pH 6·0).

A = unknown; B = lanthionine + serine; C = cystine + alanine.

Spectrophotometry and paper electrophoresis have failed to detect the presence of free pyridoxal phosphate in our reaction mixtures after 24 hours' incubation. However, addition of fresh cysteine to such mixtures resulted in the renewed formation of hydrogen sulphide, indicating the presence of a pyridoxal phosphate complex (cf. I). Heating aliquot parts of two reaction mixtures such as those of Table 4 with a thiophen reagent disclosed pyridoxal phosphate by formation of the coloured product described by Levine and Hansen,[12] concentrations being 15—19% of the total starting concentration of the coenzyme.

The reaction sequence was elucidated to a considerable extent by observing the rates

[12] Levine and Hansen, *Biochem. Biophys. Acta*, 1959, **31**, 248.

of reduction of dichlorophenolindophenol in presence of various thiazolidine derivatives, the formation of which from pyridoxal phosphate and cysteine has been described in Part III [1] and confirmed for the other amino-thiols by the use of similar techniques. The effect of ammonium vanadate is illustrated for the cysteine derivative in Fig. 6 and listed for cysteine and the other amino-thiols in Table 5. Thiol groups were liberated with three of the aldehydes used (Table 5), but, of these, only cysteine in combination with pyridoxal phosphate or *o*-hydroxybenzaldehyde evolved hydrogen sulphide.

TABLE 5.

Effect of ammonium vanadate on thiazolidine derivatives: amino-thiol or thiazolidine 4×10^{-5}M; pyridoxal phosphate (pyp) or aldehyde 5×10^{-4}M; NH_4VO_3 4×10^{-4}M; indophenol 4.8×10^{-5}M; pH 7·0.

Amino-thiol	Aldehyde	E^1_{600}/min.* With NH_4VO_3	E^1_{600}/min.* Without NH_4VO_3	Diff.	H_2S evolved
Cysteine	pyp	0·132	0·010	0·122	+
,,	*o*-HO·C_6H_4·CHO	0·046	0·022	0·024	+‡
,,	Ph·CHO	0·003	0·003	0	—
,,	Pyridine-4-aldehyde	0·006	0·006	0	—
α-Methylcysteine	pyp	0·015	0·004	0·011	—
Penicillamine	,,	0·004	0·004	0	—
Cysteamine	,,	0·050	0·050	0	—
Homocysteine †	,,	0·006	0·006	0	—

* Rate of reduction of indophenol at 600 mμ. † Forms a thiazan intermediate. ‡ At approx. ¼ of the rate obtained from cysteine and pyp under the same conditions.

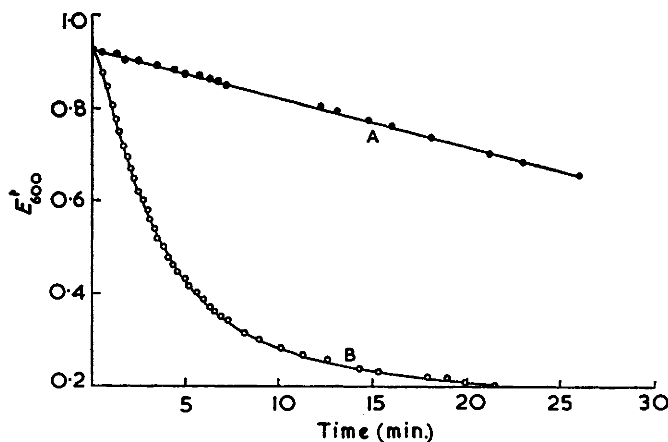


FIG. 6. Effect of ammonium vanadate on the rate of reduction of indophenol by thiazolidine: cysteine 4×10^{-5}M; pyridoxal phosphate 5×10^{-4}M; ammonium vanadate 4×10^{-4}M; indophenol 4.8×10^{-5}M; in phosphate buffer (0·1M; pH 7·0); 23—24°. A, thiazolidine alone; B, thiazolidine + NH_4VO_3.

From a quantitative point of view it is difficult to compare the rates of thiol and hydrogen sulphide formation in solutions containing cysteine, pyridoxal phosphate, and ammonium vanadate under the experimental conditions of Fig. 6; the yields of hydrogen sulphide are very low, and it is evident that considerable oxidation of the thiol group must take place. Thus, although ~70% (0.07×10^{-6} mole) of the available thiol has been revealed within 10 min. (see Fig. 6), it is almost impossible to detect any hydrogen sulphide within this time, while the total quantity released at completion of the reaction (after 4 hr.) was only one-tenth of the theoretical amount. This oxidation of thiol groups is illustrated further in Table 6. Comparison of mixtures 2 and 3 shows that by adjusting the vanadate concentration below that of cysteine it is possible to increase the rate of formation of hydrogen sulphide (even though the pyridoxal phosphate concentration is

also reduced). However, its formation in these mixtures is still slower than that of thiol groups in mixture 1, the reactant concentrations of which are lower than those in mixtures

TABLE 6.

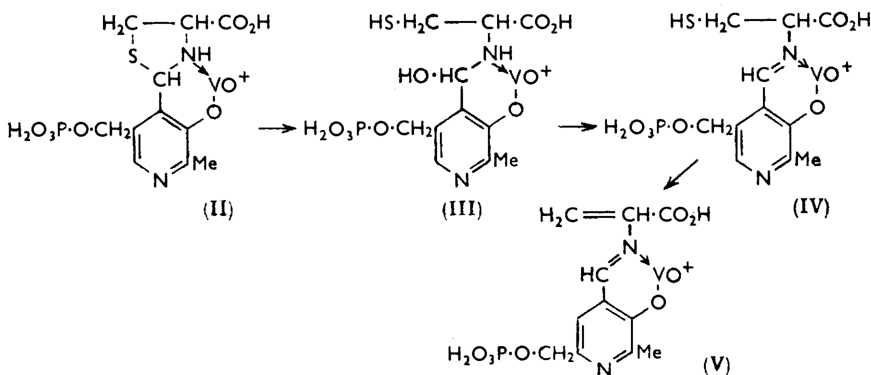
Effect of reactant concentration on rate of H_2S and –SH formation.

Mixture	Cysteine (10^{-4}M)	Pyridoxal phosphate (10^{-4}M)	NH_4VO_3 (10^{-4}M)	H_2S (10^{-6} mole/min.)	–SH (10^{-6} mole/min.)
1 (of Fig. 6)	0·4	5·0	4·0	2	16,000 '
2	12·0	120·0	12·0	2500	—
3	12·0	3·0	3·0	5000	—

2 and 3. It may be concluded, therefore, that two discrete reactions occur: (i) ring fission to provide thiol groups, and (ii) elimination of hydrogen sulphide. Neither of these occurred in presence of EDTA.

The formation of thiol groups on addition of ammonium vanadate to some thiazolidine solutions (cf. Table 5) cannot be attributed to the simple dissociation, thiazolidine + water $\rightleftharpoons$ cysteine + pyridoxal phosphate, uncombined cysteine in the mixture being oxidised by vanadate. Such behaviour would give free pyridoxal phosphate, which was not detected during the reaction. Quadrivalent vanadium ions reduce indophenol approximately half as fast as an equimolar quantity of cysteine, and in the experiment of Fig. 6 where the ammonium vanadate was in excess of the cysteine, it is likely that the thiol groups which were formed were oxidised by the V^{V}, the resultant V^{IV} actually reducing the indophenol. The net effect under these conditions is equivalent to direct reduction of indophenol by thiol groups.

The behaviour of cysteine analogues *vis-à-vis* the enzymomimetic system reflects again the specificity of this system for the degradation of cysteine. The only other thiazolidine derivative which on addition of vanadium salts showed an enhanced rate of reduction of the indophenol dye was α-methylcysteine. The absence of a hydrogen in the α-position explains its resistance to the elimination of hydrogen sulphide from an intermediate corresponding to (IV)



Chelation of vanadium must play an important part in the degradation of cysteine by the enzyme model since only those thiazolidines having a hydroxyl group in the *ortho*-position of the 2-substituent are labile (cf. II). The proposed reaction sequence (II—V) follows basically that put forward by Metzler *et al.*[2] The overall effects are consistent with a vanadium-catalysed hydrolysis of the $S-C_{(2)}$ bond of the thiazolidine (II), giving an amino-alcohol (III). Loss of water to form the Schiff's base (IV) is followed immediately by αβ-elimination of hydrogen sulphide, as shown by the formation of pyruvic acid and lanthionine, the latter produced by addition of cysteine to the aminoacrylic acid derivative

(V) and subsequent hydrolysis. Data for mixture 3 of Table 6 indicate that loss of hydrogen sulphide is a first-order process with a rate constant $k = 2{\cdot}2 \times 10^{-5}$ sec.$^{-1}$.

As pointed out above, ring fission (II ⟶ III) is faster than elimination of hydrogen sulphide (IV ⟶ V). However, since spectrophotometric observations failed to reveal the presence of the azomethine intermediate (IV) during the desulphydrase-like reaction, the intermediate must be degraded as fast as it is formed. This means that the amino-alcohol (III) must be considerably stabilised by chelation with vanadium; other amino-alcohols are normally less stable than the corresponding Schiff's bases.

Experimental

Materials.—Pyridoxal phosphate monohydrate (pyp) was obtained from Roche Products Ltd. It is photosensitive in solution [13] and such solutions were kept shielded from strong light. 5-Deoxypyridoxal was a gift from Dr. Karl Folkers (Merck, Rahway). We also acknowledge gifts of materials from Dr. H. R. V. Arnstein (DL-cysteinyl-D-valine), Dr. W. C. J. Ross (α-methylcysteine), Dr. R. Wade (L-cysteinylglycine and the 2-substituted thiazolidine-carboxylic acid derivatives [14]). 2-4′-Pyridylthiazolidine-4-carboxylic acid was prepared as follows by Dr. R. Wade: Cysteine (3·16 g.) and hydrated sodium acetate (2·72 g.) in water (40 ml.) were warmed on a steam bath for 10 min., then added to pyridine-4-aldehyde (2·02 ml.) in 95% ethanol (40 ml.) and shaken for 3 hr. The *acid* which had separated had m. p. 169—170° (from 50% aqueous ethanol) (4·3 g.) (Found: C, 50·9; H, 4·7; N, 13·1; S, 15·5. $C_9H_{10}N_2O_2S$ requires C, 51·4; H, 4·8; N, 13·3; S, 15·3%). L-(−)-Cysteine hydrochloride was of B.D.H. Laboratory Reagent grade; penicillamine was obtained from Aldrich Chemical Co. Inc., cysteamine hydrochloride from California Biochemicals, homocystine from Roche Products Ltd., and homocysteine from L. Light and Co. Other amino-acids, except lanthionine (Mann Biochemicals Inc.), were of B.D.H. Laboratory Reagent grade, as also was the 2,6-dichlorophenolindophenol. Metal salts and buffer salts were of "AnalaR" quality (where available), B.D.H. Laboratory Reagent grade, or Hopkin and Williams "Pure." Water was glass-distilled and de-ionised before use.

Absorption spectra were measured in a Unicam S.P. 500 spectrophotometer, which, for kinetic work, was fitted with a temperature-controlled cell-housing set at 23—24°.

Comparison of Desulphydrase Activities of Metals.—Bray's procedure [15] was used for the measurement of hydrogen sulphide: solutions of cysteine (finally $1{\cdot}2 \times 10^{-3}$M) were equilibrated for 20 min. before the addition of metal salts (finally $3{\cdot}8 \times 10^{-4}$M). The determinations were carried out in a constant-temperature box, at 37° ± 1°.

Spectra of Thiazolidine–Metal Mixtures (Fig. 1).—Cysteine (finally $1{\cdot}2 \times 10^{-3}$M) and pyridoxal phosphate (finally $2{\cdot}4 \times 10^{-4}$M) were allowed to equilibrate for 1 hr. under nitrogen in 0·1M-phosphate buffer (pH 6·0). Vanadyl sulphate, $VOSO_4$, was added to a final concentration of $2{\cdot}4 \times 10^{-4}$M, and the spectrum was measured immediately. The cuvette was flushed with argon, then stoppered, and the spectrum was measured after 72 hr. The procedure was repeated with ferrous ammonium sulphate in place of vanadyl sulphate, and 0·1M-acetate buffer (pH 6·0).

Formula of Thiazolidine–VO^{2+} Chelate Compound by Continuous Variations. [8]—A series of solutions was prepared within the range, 4×10^{-4}M-thiazolidine + 10^{-4}M-$VOSO_4$ to 10^{-4}M-thiazolidine + 4×10^{-4}M-$VOSO_4$. Phosphate buffer (0·1M; pH 6·0) was used as diluent.

Aliquot parts of thizolidine for the above mixtures were taken from a solution containing cysteine (5×10^{-3}M) and pyridoxal phosphate (10^{-3}M) in phosphate buffer (0·1M; pH 6·0) which had been allowed to equilibrate for 45 min. under nitrogen before the above mixtures were prepared. $E_{310}^{0\cdot2}$ values on the mixed solutions were measured and control values ($E_{310}^{0\cdot2}$ readings which would have been obtained had no chelation taken place) subtracted to give $\bar{D}_{310}$. A plot of $\bar{D}_{310}$ as ordinate against [Vanadyl sulphate]/([vanadyl sulphate] + [thiazolidine]) had a maximum $\bar{D}_{310}$ value of 0·05, corresponding to an abscissa of 0·5.

Specificity.—The results listed in Table 2 were obtained from measurements by Bray's method [15] or by a modification of this procedure. In the latter, a reaction vessel as shown in

[13] Morrison and Long, *J.*, 1958, 211.
[14] Schubert, *J. Biol. Chem.*, 1936, **114**, 341; Soloway, Kipnis, Ornfelt, and Spoerri, *J. Amer. Chem. Soc.*, 1948, **70**, 1667.
[15] Bray, *Analyst*, 1958, **83**, 987.

Fig. 7 was used; the lower bulb had a capacity of 20 ml., and the upper of 1 ml.; the apparatus was held at a slight angle to horizontal with the large bulb at the bottom. With the vessel clamped horizontally, metal solutions and mixtures of pyridoxal phosphate and substrate, respectively, were incubated in the separated bulbs for 15 min., and a stream of nitrogen (saturated with water vapour) was passed through the apparatus (to give anaerobic conditions). After the reactants had been mixed, the vessel was clamped in a vertical position, the contents being stirred by the gas stream, and the hydrogen sulphide evolved was flushed into aliquot parts of a solution of zinc acetate dihydrate (100 g.) and sodium acetate trihydrate (25 g.) in water (500 ml.). Aliquot parts (0·2 ml.) of the zinc acetate solution were taken for colour development.[15] Yields of hydrogen sulphide, determined by the two methods, were in good agreement. The modified method was more convenient for rate measurements than Bray's, since many observations could be made on a single sample merely by passing the gas into fresh zinc acetate solution at measured time intervals.

The results of Table 2 were not assembled in an uninterrupted series of observations, and there were slight variations in reactant concentrations in some cases. Concentrations were mainly: substrate $1{\cdot}2 \times 10^{-4}$M, NH_4VO_3 $2{\cdot}4 \times 10^{-5}$M, pyridoxal phosphate $2{\cdot}4 \times 10^{-5}$M. However, a cysteine standard was always run alongside the test determination and the results related to a common baseline for comparison. Measurements were at 37°. In the case of urease (B.D.H.), 0·2 ml. of a 6·2% w/v suspension of the protein in acetate buffer (0·1M; pH 6·0) acted as substrate.

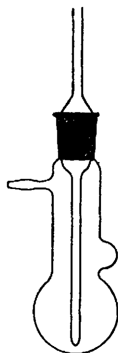


FIG. 7. Diffusion apparatus.

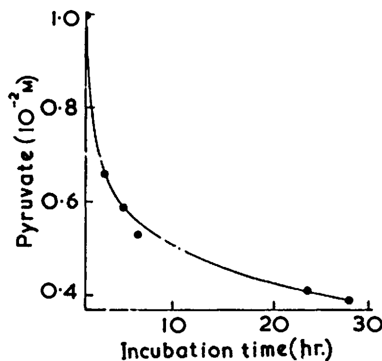


FIG. 8. Anaerobic incubation of pyruvate at 37° in presence of vanadyl sulphate. Pyruvate 0·01M; $VOSO_4$ 0·01M; acetate buffer (0·1M; pH 6·0).

Determinations with cysteine ethyl ester and DL-cysteinyl-D-valine were carried out after preliminary reduction of their disulphides by an equivalent quantity of sulphite. The disulphide and sodium sulphite, both $7{\cdot}5 \times 10^{-4}$M, were incubated together at 20° in phosphate buffer (0·1M; pH 6·0) under nitrogen. The extent of reduction at this stage (checked by assay with *p*-chloromercuribenzoate [16]) corresponded to the theoretical yield, $R_2S_2 + NaHSO_3 \rightleftharpoons RS{\cdot}SO_3Na + RSH$.[17] After 20 min., this solution (2 ml.) was submitted to the standard test procedure at 37° (pyridoxal phosphate, NH_4VO_3, $2{\cdot}4 \times 10^{-4}$M). The resultant curves for hydrogen sulphide were compared with those obtained with a solution of cystine in the same process; ~85% of the reduced disulphide was available for formation of hydrogen sulphide.

Tryptophan Synthetase Model.—Reactants were incubated as follows (total concns.): (i) indole (5×10^{-2}M) + serine (5×10^{-2}M) + alum (2×10^{-3}M) + pyridoxal or its phosphate (2×10^{-2}M); (ii) indole (5×10^{-2}M) + serine (5×10^{-2}M) + NH_4VO_3 (2×10^{-3}M) + pyridoxal or its phosphate (2×10^{-2}M).

In each case the total volume was 1 ml. (including 0·2 ml. of acetone). The solutions were heated on a boiling-water bath for 30 min. or kept at 20° ± 2° for 10 days. The mixtures were chromatographed on Whatman paper (No. 1) in butanol–acetic acid–water (60 : 15 : 25), and the tryptophan contents determined by the method described below.

[16] Boyer, *J. Amer. Chem. Soc.*, 1954, **76**, 4331.
[17] Swan, in "Sulfur in Proteins," ed. Benesch *et al.*, Academic Press, New York, 1959, p. 5.

Redox Effect (Fig. 2).—The total cysteine concentration was $7{\cdot}7 \times 10^{-3}$M throughout. In Bray's apparatus cysteine–cystine and pyridoxal phosphate ($3{\cdot}8 \times 10^{-4}$M) were incubated under nitrogen in the lower bulb, and ammonium vanadate ($3{\cdot}8 \times 10^{-4}$M) in the top. After 10 min. (to allow for temperature equilibration) the solutions were mixed and rolling commenced at 37° for 10 min. Hydrogen sulphide was measured in the usual way.[15]

Comparison of Activities of Vanadium Solutions (Fig. 3).—Solutions of V^{V} and V^{IV} were prepared from ammonium vanadate and vanadyl sulphate, respectively, and V^{III} by Stevens's method.[18] The V^{III} solution was standardised volumetrically.[19]

In the modified diffusion apparatus (Fig. 7), cysteine ($1{\cdot}2 \times 10^{-3}$M) and pyridoxal phosphate ($1{\cdot}2 \times 10^{-2}$M) were incubated together at 37° in the lower bulb, and the metal solution ($1{\cdot}2 \times 10^{-3}$M) in the upper: the total volume of mixed solutions was 2·5 ml. in acetate buffer (0·1M; pH 5·9).

Analysis.—Most of the analytical work was carried out on the following solution which was incubated at 37° under nitrogen, the hydrogen sulphide evolved being passed into a solution of zinc acetate: cysteine 0·05M; pyridoxal phosphate 0·01M; vanadium salt 0·01M; buffer 0·1M, pH 6·0.

Paper chromatography. The following solvent systems were used as routine: (1) ethanol–butan-1-ol–water–propionic acid (10 : 10 : 5 : 2); (2) butan-1-ol–acetic acid–water (12 : 3 : 5); (3) 80% phenol–water–ethanol–ammonia (*d* 0·880) (120 : 40 : 40 : 1); (4) butan-1-ol–acetic acid–water (4 : 1 : 1); (5) butan-1-ol–formic acid–water (15 : 3 : 2).

Two-dimensional chromatography was carried out by using the combinations 1, 2 (Fig. 4a), and 2,3 (Fig. 4b). R_F values are listed in Table 7. The mixture (~25 µl.) was applied to the

TABLE 7.

Chromatographic results (R_{ala} refers to alanine).

Solvent system:	1		2	3	4	5
	R_F	R_{ala}	R_F	R_F	R_F	R_{ala}
Lanthionine	0·05	0·08			0·02	0·1
Cystine	0·05	0·08			0·05	0·1
Lanthionine, sulphone of		0·02	0	0·20	0·01	0·02
Cysteic acid		0·20	0·08	0·10	0·06	0·15
Serine	0·21	0·60	0·20	0·36	0·20	0·41
Alanine	0·32	1·0	0·33	0·47	0·36	1·0
Cysteinesulphinic acid		0·20			0·06	0·15
Cysteine–NEM *	0·44					
Cysteine	0·32					
Pyridoxamine phosphate	0·10	0·40				

* *N*-Ethylmaleimide.

paper and, when this had dried, 100-vol. hydrogen peroxide (10 µl.) was added in two portions, the paper being allowed to dry in the atmosphere between applications. The positions of the amino-acids after chromatography were revealed by dipping the papers in a 0·2% v/v solution of ninhydrin in acetone and heating them for 3 min. at 90—100°. Basic solvent systems gave less efficient separation than did acidic solvents.

Quantitative. Columns of Dowex 50 × 8 ion-exchange resin (30 × 1 cm.) were prepared as described by Moore and Stein.[20] The columns were equilibrated with citrate buffer of pH 3·42 (as specified by Moore and Stein, but without thiodiglycol). Aliquot parts of the equilibrated mixture were adjusted to pH $\not>$2·5 and applied to the column. Elution was carried out with the same buffer. Colour development with ninhydrin was by Moore and Stein's procedure.[21] Alanine was eluted in a single band with cystine, and serine with lanthionine (Fig. 5). The alanine and serine concentrations, together with that of residual cysteine, were estimated from paper chromatograms as follows: The amino-acid mixture (2 ml.) was mixed with 0·5M-ethanolic *N*-ethylmaleimide (0·5 ml.), and after 30 min. at 20° ± 2° the

[18] Stevens, *Analyt. Chim. Acta*, 1956, **15**, 51.
[19] Vogel, " A Text Book of Quantitative Inorganic Analysis," Longmans, Green and Co., London, 1948, p. 396.
[20] Moore and Stein, *J. Biol. Chem.*, 1951, **192**, 663.
[21] Moore and Stein, *J. Biol. Chem.*, 1954, **211**, 907.

solution (25 μl.) was spotted in triplicate on Whatman No. 4 paper, and a descending chromatogram was run overnight. The amino-acid spots were revealed with ninhydrin–copper sulphate, and eluted with methanol, and the E_{570} values were measured by Lucy's method.[22] Pyridoxamine phosphate was estimated similarly, except that elution was with 2 : 1 v/v methanol–water and optical densities were measured at 470 mμ.

Ammonia was determined by Conway's method.[23]

Pyruvic acid (measured by Friedman and Haugen's method [24]) is lost on heating under nitrogen in the presence of vanadium salts, and the figures for this constituent in the mixtures of Table 4 were all low. Fig. 8 shows the effect of incubating 0·01M-pyruvic acid and 0·01M-vanadyl sulphate in acetate buffer (0·1M; pH 5·0) at 37° under nitrogen.

Indophenol Titrations.—Spectrophotometric work was carried out in a 1 cm. cell similar to that designed by Lazarow and Cooperstein.[25] The concentrations of reagents are shown in Table 6. Ammonium vanadate and indophenol were stored in the side arm; the cell contained pyridoxal phosphate, thiol, and buffer or, alternatively, aldehyde, thiazolidine, cysteine, and buffer. The whole was allowed to equilibrate in a thermostat-controlled Unicam cell-housing at 24° for 10 min. before removal of the nitrogen supply and mixing of the solutions. The rate of change of E^{1}_{600} was then measured.

This investigation has been supported by grants to this Institute from the Medical Research Council, the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund, and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

Chester Beatty Research Institute,
Fulham Road, London, S.W.3.
[*Received, August 9th,* 1961.]

[22] Lucy, Ph.D. Thesis, London, 1955, p. 66.
[23] Conway, "Microdiffusion Analysis and Volumetric Error," Crosby, Lockwood and Son, Ltd., London, 1957, p. 98.
[24] Friedman and Haugen, *J. Biol. Chem.*, 1943, **147**, 415.
[25] Lazarow and Cooperstein, *Science*, 1954, **120**, 674.