The Pyridoxal- and Pyridoxal 5'-Phosphate-Catalysed Non-Enzymic Degradations of L-Serine O-Sulphate and Related Compounds

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1. L-Serine O-sulphate and L-threonine O-sulphate are degraded in the presence of pyridoxal 5'-phosphate to yield equimolar amounts of the corresponding keto acid, ammonia and sulphate. 2. Pyridoxal catalyses the same reactions at a faster rate. 3. One of a number of bi- or ter-valent metal ions must be present for these degradations to proceed. The reaction rates are dependent on a number of factors including pH and the nature of the metal ion used. 4. Studies with related sulphate esters indicate that the α -hydrogen atom and the amino group are essential for activity. 5. Spectral changes during the pyridoxal and pyridoxal 5'-phosphate catalysis of L-serine O-sulphate breakdown suggest the formation of a Schiff base. 6. The mechanism for these reactions appears to be in accordance with the general mechanism proposed for pyridoxal-catalysed $\alpha\beta$ -elimination reactions.

The non-enzymic deamination of serine to pyruvate and ammonia, catalysed by pyridoxal and metal salts, was described by Metzler & Snell (1952a). This reaction is typical of a number of similar $\alpha\beta$ -elimination reactions that occur with appropriate β -substituted amino acid derivatives. Among compounds that undergo these reactions are threonine, cysteine, β -chloroalanine, serine O-phosphate, O-carbamoylserine, azaserine, threonine O-phosphate and S-aminoethylcysteine (Metzler & Snell, 1952a; Gregerman & Christensen, 1956; Longenecker & Snell, 1957a; De Marco, 1964). For most of the substrates listed a corresponding reaction catalysed by a pyridoxal 5'-phosphate-containing enzyme is known, and it has been suggested (Metzler, Ikawa & Snell, 1954b) that the enzymic and the non-enzymic reactions may proceed by closely related mechanisms.

Thomas & Tudball (1967) in a study on the enzymic degradation of L-serine O-sulphate to pyruvate, ammonia and SO_4^{2-} also reported that pyridoxal 5'-phosphate and metal ions catalyse what appears to be the same reaction. The work described in the present paper indicates that this non-enzymic degradation of L-serine O-sulphate and related compounds operates by the mechanism proposed by Metzler *et al.* (1954b) for pyridoxal-catalysed $\alpha\beta$ -elimination reactions.

MATERIALS AND METHODS

Chemicals. Pyridoxal hydrochloride and pyridoxal 5'-phosphate were purchased from Sigma (London) Chemical

Co. Ltd., London, S.W. 6. The metal salts used throughout were of A.R. grade with the exception of InCl_3 , a general-purpose reagent purchased from British Drug Houses Ltd., Poole, Dorset.

$\text{Al}_2(\text{SO}_4)_3$, K_2SO_4 , $24\text{H}_2\text{O}$, $(\text{NH}_4)_2\text{SO}_4$, FeSO_4 , $6\text{H}_2\text{O}$, CdCl_2 , $2\frac{1}{2}\text{H}_2\text{O}$, CoCl_2 , $6\text{H}_2\text{O}$, CrCl_3 , $6\text{H}_2\text{O}$, CuCl_2 , $2\text{H}_2\text{O}$, FeCl_3 , $6\text{H}_2\text{O}$, MgCl_2 , $6\text{H}_2\text{O}$, MnCl_2 , $4\text{H}_2\text{O}$, NiCl_2 , $6\text{H}_2\text{O}$ and ZnSO_4 , $7\text{H}_2\text{O}$ were all purchased from British Drug Houses Ltd. $\text{Ga}(\text{NO}_3)_3$ was obtained from Alfa Inorganics Inc., Beverly, Mass., U.S.A.

The O-sulphate esters of L-serine, D-serine, DL-homoserine, DL- α -methylserine, ethanolamine and 3-hydroxypropionic acid were prepared as potassium salts by sulphation of the parent compounds with conc. H_2SO_4 under the conditions described by Tudball (1962) for the O-sulphation of L-serine. Potassium L-threonine O-sulphate was prepared by the directions of Dodgson, Lloyd & Tudball (1961). Potassium glycyl-L-serine O-sulphate and potassium L-serylglycine O-sulphate were prepared by the methods outlined by Tudball, Noda & Dodgson (1964, 1965). L-Serine O-phosphate was purchased from Sigma (London) Chemical Co., O-methyl-DL-serine was from Mann Research Laboratories Inc., New York, N.Y., U.S.A., and O-acetyl-L-serine and O-acetyl-L-threonine were from Miles Laboratories Inc., Elkhart, Ind., U.S.A.

All experiments were performed with deionized water. Aqueous solutions (0.05 M) of pyridoxal, amino acids and sulphate esters were stored at 4°. Pyridoxal 5'-phosphate solutions were freshly prepared before use; all other solutions were used within a few days of preparation, except for pyridoxal solutions, which gave no evidence of deterioration.

Analytical methods. SO_4^{2-} was measured by the method of Dodgson (1961), the spectrophotometric readings being determined at 500 m μ rather than 360 m μ to eliminate absorption by pyridoxal 5'-phosphate. Keto acids were determined by the procedure of Friedemann & Haugen (1943) as modified by Metzler & Snell (1952b) and NH_3 by the boric acid-HCl microdiffusion method of Conway (1962). Pyridoxal and pyridoxal 5'-phosphate were

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Table 1. *Degradation of L-serine O-sulphate and L-threonine O-sulphate by pyridoxal or pyridoxal 5'-phosphate*

The substrates (final concn. 10mM) were incubated at 100° for 15 min. with CuCl₂ (1 mM) and pyridoxal (2mM) or pyridoxal 5'-phosphate (2mM) as indicated. Reaction mixtures were buffered at pH 7.0 with 0.02M-maleate adjusted with 0.02N-NaOH.

Substrate	Initial concn. of catalysts (mM)			Concn. of reaction products (mM)		
	Pyridoxal	Pyridoxal 5'-phosphate	CuCl ₂	Keto acid	SO ₄ ²⁻	NH ₄ ⁺
L-Serine O-sulphate	2.0	0	1.0	7.70	7.85	7.51
	2.0	0	0	0.53	0.55	0.50
	0	2.0	1.0	4.20	4.12	3.85
	0	2.0	0	0.28	0.30	0.30
	0	0	1.0	0.00	0.00	0.00
L-Threonine O-sulphate	2.0	0	1.0	1.20	1.20	1.13
	2.0	0	0	0.21	0.20	0.15
	0	2.0	1.0	0.65	0.69	0.52
	0	2.0	0	0.08	0.08	0.07
	0	0	1.0	0.00	0.00	0.00

determined by the methods described by Metzler & Snell (1952b).

Experimental procedure. Reaction mixtures were usually composed of substrate, metal ion, and pyridoxal or pyridoxal 5'-phosphate, buffered at pH 7.0 with a suitable buffer. Portions (1 ml.) of the reaction mixtures were incubated at 37° or 100° in sealed Pyrex tubes (8 cm. × 1 cm.). Tubes were withdrawn at appropriate time-intervals and cooled rapidly to 0°, and the contents were suitably diluted for analysis.

Spectrophotometry. Spectra were recorded by using an Optica CF4R recording spectrophotometer with a thermostatically controlled cell housing.

RESULTS

Pyridoxal- and pyridoxal 5'-phosphate-catalysed degradations of L-serine O-sulphate and L-threonine O-sulphate. The effect of pyridoxal and pyridoxal 5'-phosphate in catalysing the degradation of L-serine O-sulphate and L-threonine O-sulphate is summarized in Table 1. Equivalent amounts of keto acid, ammonia and SO₄²⁻ ions were liberated from both sulphate esters. Pyridoxal and pyridoxal 5'-phosphate remained unchanged at the end of the reaction in all cases. Neither substrate was attacked in the absence of pyridoxal or pyridoxal 5'-phosphate and the reactions were relatively slow in the absence of Cu²⁺ ions. The keto acids resulting from the degradation of L-serine O-sulphate and L-threonine O-sulphate were identified as pyruvate and 2-oxobutyrate respectively by the procedures outlined by Thomas & Tudball (1967). The comparative rates of sulphate production from L-serine O-sulphate and L-threonine O-sulphate (Fig. 1) show that the degradation of L-serine O-sulphate by either pyridoxal or pyridoxal 5'-phosphate was significantly more rapid than

Fig. 1. Rates of liberation of SO₄²⁻ ions from L-serine O-sulphate and L-threonine O-sulphate. The substrates (final concn. 10mM) were incubated at 100° with CuCl₂ (1mM) and pyridoxal (2mM) or pyridoxal 5'-phosphate (2mM). The reaction mixtures were buffered at pH 7.0 with 0.02M-maleate and contained: ○, L-serine O-sulphate and pyridoxal; △, L-serine O-sulphate and pyridoxal 5'-phosphate; □, L-threonine O-sulphate and pyridoxal; ●, L-threonine O-sulphate and pyridoxal 5'-phosphate.

that of the L-threonine ester, just as the deamination of serine occurs more readily than that of threonine (Metzler & Snell, 1952a). The rates observed with pyridoxal were significantly greater than those obtained in experiments with pyridoxal 5'-phosphate. The pyridoxal-catalysed breakdown of L-serine O-sulphate proceeded rapidly at pH values above 3.0, the process being accelerated by increasing the pH up to pH 10, above which base-catalysed degradation of the substrate occurred.

Table 2. *Effects of various metal ions on the degradation of L-serine O-sulphate by pyridoxal at 37° and at 100°*

The reaction mixtures were composed of 10 mM-L-serine O-sulphate, 2 mM-pyridoxal and 1 mM-metal ion, buffered at pH 7.0 with 0.02 M-maleate buffer. Incubations were for 15 min. at 100° and for 3 hr. at 37°.

Metal ion	Temp. ...	Keto acid produced (mm)	
		37°	100°
Cu ²⁺		3.45	8.50
Al ³⁺		1.55	6.57
Ga ³⁺		3.00	5.83
Fe ²⁺		2.75	5.30
Fe ³⁺		2.50	5.21
Mn ²⁺		0.15	4.38
Co ²⁺		0.25	3.57
Zn ²⁺		0.10	2.90
Ni ²⁺		0.20	2.84
None, Cd ²⁺ , Cr ³⁺ , In ³⁺ or Mg ²⁺		0.05-0.10	0.12-0.15

Table 3. *Pyridoxal- and metal ion-catalysed degradation of L-serine O-sulphate and related compounds*

Reaction mixtures consisted of the substrate (final concn. 10 mM), 2 mM-pyridoxal and 1 mM-CuCl₂, buffered at pH 7.0 with 0.02 M-maleate buffer. Incubation was for 15 min. at 100°.

Substrate	Concn. of product liberated (mm)
L-Serine O-sulphate	8.15
D-Serine O-sulphate	8.13
Glycyl-L-serine O-sulphate	0.00
L-Serylglycine O-sulphate	3.45
DL- α -Methylserine O-sulphate	0.10
DL-Homoserine O-sulphate	0.00
Ethanolamine O-sulphate	0.08
3-Hydroxypropionic acid sulphate	0.00
L-Threonine O-sulphate	1.19
O-Acetyl-L-threonine	1.17
O-Acetyl-L-serine	7.72
O-Methyl-DL-serine	0.94
L-Serine O-phosphate	4.05
L-Serine	0.98

Effect of metal ions on the pyridoxal-catalysed degradation of L-serine O-sulphate. Longenecker & Snell (1957a,b) demonstrated that the pyridoxal-catalysed liberation of PO₄³⁻ from serine O-phosphate was accelerated by a wide variety of bi- and ter-valent metal ions and showed that the amount of PO₄³⁻ liberated varied with the metal ion used. The catalytic activities at 37° and 100° of various metal ions in promoting the reaction of pyridoxal with L-serine O-sulphate were therefore compared (Table 2). Cu²⁺, Ga³⁺, Fe²⁺, Fe³⁺ and Al³⁺ were found to be highly active catalysts at both temperatures. Ni²⁺, Co²⁺, Zn²⁺ and Mn²⁺, which were only slightly effective at 37°, showed appreciable activity at 100°. Cd²⁺, Mg²⁺, Cr³⁺ and In³⁺ were essentially inactive at both temperatures.

Effect of pyridoxal and Cu²⁺ ions on compounds related to L-serine O-sulphate. The ability of pyridoxal to catalyse the degradation of a number of L-serine O-sulphate derivatives and related compounds was examined in an attempt to elucidate the mechanism of the pyridoxal-catalysed breakdown. If the pyridoxal-catalysed degradation of L-serine O-sulphate occurs in a manner analogous to that proposed by Metzler *et al.* (1954b) for the $\alpha\beta$ -elimination of amino acids, blocking of the amino group of L-serine O-sulphate should prevent Schiff-base formation and hence breakdown of the ester. To promote an $\alpha\beta$ -elimination reaction it is necessary to displace the electron pair binding the hydrogen to the α -carbon atom, a process that is favoured by the presence of an electronegative group on the β -carbon. Such a displacement would not be expected to proceed if either the α -hydrogen or the electronegative group were replaced with, for

example, a methyl group. The results obtained are summarized in Table 3. The activity towards the sulphate esters was determined by measuring the amount of SO₄²⁻ ion liberated, except with DL-homoserine O-sulphate, when the amount of keto acid liberated was determined. This was necessary as homoserine O-sulphate forms an insoluble barium salt that interferes with the determination of SO₄²⁻. When non-sulphated substrates were used, the activity was determined by measuring the liberated keto acid.

The dipeptide L-serylglycine O-sulphate was attacked, but the blocking of the amino group of the serine residue in the dipeptide glycyl-L-serine O-sulphate resulted in a complete loss of activity. The necessity for a free amino group was also reflected by the lack of activity towards the sulphate ester of 3-hydroxypropionic acid. The inability of ethanolamine O-sulphate to serve as a substrate in the system implies that the carboxyl group also contributes to the labilization of the α -hydrogen and the consequent liberation of the electronegative β -substituent. The necessity for an unblocked α -hydrogen atom to be present before degradation could proceed is well illustrated by the inertness of α -methylserine O-sulphate in the system.

Longenecker & Snell (1957a) pointed out that, in any given series of compounds, the more electronegative the β -substituent, the more rapid the elimination of that substituent. Thus β -chloro-L-alanine, by loss of chloride, yields pyruvate and ammonia much more rapidly than does serine (Greggerman & Christensen, 1956). Similarly the ester groups of O-carbamoylserine, azaserine, serine O-phosphate and threonine O-phosphate are more

Fig. 2. Comparative rates of formation of keto acid from L-serine *O*-sulphate, L-serine *O*-phosphate and L-serine at 100°. Reaction mixtures contained 10 mM-substrate, 2 mM-pyridoxal, 0.2 mM-CuCl₂ and 0.02 M-malate buffer, pH 7.0. ○, L-Serine *O*-sulphate; △, L-serine *O*-phosphate; □, L-serine.

electronegative than the hydroxyl groups of the parent amino acids, and the reactions with these compounds occur more readily than with serine and threonine (Longenecker & Snell, 1957a). With the sulphate ester of serine a similar result was obtained; the presence of the electronegative sulphate group increased the rate of degradation to nearly ten times that observed for L-serine (Fig. 2). Similarly, esterification of the hydroxyl group of serine with acetate markedly increased the rate of degradation. The replacement of the hydroxyl group of serine with a methoxyl group did not alter the rate of degradation (Table 3).

Spectral changes during pyridoxal and pyridoxal 5'-phosphate catalysis of L-serine *O*-sulphate breakdown. When solutions of L-serine *O*-sulphate, pyridoxal and cupric chloride were mixed, a new extinction band appeared with a maximum at 385 mμ (Fig. 3). The intensity of this band increased with time up to 120 min. when the system was incubated at 37°, and was paralleled by a corresponding decrease of the extinction maximum of pyridoxal at 315 mμ. These spectral changes are similar to those reported by Davis, Roddy & Metzler (1961) and by Cennamo (1967) after the incubation of pyridoxal with an amino acid in the presence of a cupric salt, and are consistent with the formation of a cupric chelate of a Schiff base between pyridoxal and L-serine *O*-sulphate. Breakdown of the Schiff base, as represented by a decrease in extinction at 385 mμ, could be detected if the incubation periods were prolonged. Fig. 4 shows that the extinction

Fig. 3. Spectral changes occurring in a solution containing 5 mM-L-serine *O*-sulphate, 0.2 mM-pyridoxal and 0.2 mM-CuCl₂ in 0.02 M-Na₂HPO₄-NaH₂PO₄ buffer, pH 7.0, at 37°. Curve 1 was recorded immediately after the mixing of the reactants. The remaining curves up to curve 2 were recorded 10, 20, 30, 45, 60, 90, 120 and 150 min. later. Measurements were made with an Optica CF4R spectrophotometer (light-path 10 mm.).

Fig. 4. Spectral changes occurring in a solution containing 5 mM-L-serine *O*-sulphate, 0.2 mM-pyridoxal 5'-phosphate and 0.2 mM-CuCl₂ in 0.02 M-Na₂HPO₄-NaH₂PO₄ buffer, pH 7.0, at 50°. Curve 1 was recorded immediately after the mixing of the reactants. The remaining curves up to curve 2 were recorded 10, 20, 30, 45, 60, 90, 120 and 150 min. later. The broken line shows the absorption spectrum of 0.2 mM-pyridoxal 5'-phosphate and 0.2 mM-CuCl₂ in 0.02 M-Na₂HPO₄-NaH₂PO₄ buffer, pH 7.0. Measurements were made with an Optica CF4R spectrophotometer (light-path 10 mm.).

maximum at 385 mμ of pyridoxal 5'-phosphate, measured in the presence of cupric chloride, increased in intensity when L-serine *O*-sulphate was added and the reaction mixture was subsequently incubated at 50°. On prolonging the time of incubation this extinction at 385 mμ decreased.

The extinction maximum at 290–310m μ that was present initially in the spectrum of pyridoxal 5'-phosphate was displaced to a shorter wavelength (260–280m μ) on the addition of L-serine O-sulphate. Spectral changes of this type were observed by Matsuo (1957) with systems of a number of amino acids, pyridoxal 5'-phosphate and certain metal ions, and were attributed to the formation of the metal chelates of pyridoxal 5'-phosphate-amino acid Schiff bases. In the absence of metal ions the absorption spectrum of the complex of pyridoxal 5'-phosphate and L-serine O-sulphate exhibited a maximum at 390–410m μ , which is characteristic of a Schiff base (Matsuo, 1957; Finseth & Sizer, 1967). These changes (Fig. 4) may thus be ascribed to the formation and subsequent breakdown of a cupric chelate of the Schiff base formed between pyridoxal 5'-phosphate and L-serine O-sulphate.

DISCUSSION

A general mechanism for pyridoxal-catalysed $\alpha\beta$ -elimination reactions of amino acids has been proposed by Braunstein & Shemyakin (1953) and by Metzler *et al.* (1954b). Basically the catalytic effectiveness of pyridoxal (or pyridoxal 5'-phosphate) and a metal ion is due to the intermediate formation of the metal chelate of a Schiff base formed between pyridoxal and the amino acid. In such a complex the amino acid can undergo a variety of reactions dependent on its structure and environment, but in all cases the essential factor is the displacement of an electron pair from the α -carbon atom of the amino acid. Displacement of the electron pair binding the hydrogen atom to the α -carbon atom permits the elimination of the β -substituent as the anion. Hydrolysis of the Schiff base of the aminoacrylic acid so formed gives a keto acid, ammonia and pyridoxal. The reactions involving L-serine O-sulphate and related compounds studied here would appear to be in accordance with this mechanism.

In their study of the non-enzymic dehydrochlorination of β -chloro-L-alanine, Gregerman & Christensen (1956) reported the formation of a condensation product between pyridoxal and the substrate, whereby chloride was released but pyruvate and ammonia were retained. No evidence for the existence of such a stable by-product was obtained in the present studies, as equivalent amounts of SO₄²⁻, pyruvate and ammonia were liberated from L-serine O-sulphate under all the conditions examined.

Metzler, Longenecker & Snell (1954a) noted that, with free serine, cleavage to yield glycine and formaldehyde can occur in the presence of pyridoxal and a catalytic metal ion as a competing reaction to the $\alpha\beta$ -elimination. However, no formation of

either formaldehyde or glycine was observed with L-serine O-sulphate; the introduction of the more electronegative sulphate group into the molecule thus prevents the non-enzymic cleavage of the carbon chain of serine. Serine transaminates slowly under the conditions used in this study (Metzler & Snell, 1952b), but no evidence for the formation of the sulphate ester of 3-hydroxypyruvate was obtained.

These studies indicate that it is not unlikely that a pyridoxal 5'-phosphate-dependent enzyme capable of catalysing the degradation of L-serine O-sulphate may occur in biological systems. Certainly the chemical activity of pyridoxal and its 5'-phosphate ester is compatible with the existence of such an enzyme. The demonstration (Thomas & Tudball, 1967) that the α -hydrogen atom and the amino group of L-serine O-sulphate are essential for the enzymic degradation adds weight to this argument. The evidence obtained to date, however (Thomas & Tudball, 1967), would seem to point to the view that the rat liver enzyme catalysing the degradation of L-serine O-sulphate does not require pyridoxal 5'-phosphate for activity. Thus addition of pyridoxal 5'-phosphate had no observable effect on enzyme activity, nor has any definite evidence been provided as to its association with the enzyme protein (Thomas, 1966; Thomas, Tudball & Fowler, 1968). In this context it is noteworthy that enzymic breakdown of L-serine O-phosphate results not from a pyridoxal 5'-phosphate-catalysed elimination reaction but from a hydrolytic and transfer reaction catalysed by serine O-phosphate phosphatase (phosphoserine phosphohydrolase, EC 3.1.3.3) (Byrne, 1961).

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