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The Enzymic Degradation of L-Serine *O*-Sulphate

MECHANISM OF THE REACTION

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1. During the enzyme-catalysed degradation of L-serine *O*-sulphate no exchange occurs between the hydrogen atom attached to the α-carbon atom of the substrate and the tritiated water of the incubation medium. 2. The participation of an intermediate carbanion has been demonstrated in the degradation by performing the reaction in the presence of tetranitromethane. 3. Photo-oxidation of the enzyme in the presence of Rose Bengal led to a rapid inactivation of enzyme with the concomitant loss of four histidine residues/molecule. 4. Rose Bengal was also a non-competitive inhibitor of the enzyme.

Previous studies on the enzymic degradation of L-serine *O*-sulphate by a specific system from pig liver have revealed that the αβ-elimination reaction catalysed by the enzyme involves the removal of the β-substituent through the scission of the C–O bond (Tudball & Thomas, 1972). C–O bond cleavage also occurs when the substrate is subjected to non-enzymic breakdown in the presence of either pyridoxal or alkali. However, as might have been predicted, the degradation occurring in acid media proceeds via the scission of the S–O bond.

In addition, aminoacrylic acid was identified as one of the intermediates formed during the enzymic degradation, and the overall reaction was shown to be effectively irreversible. The main features of the reaction mechanism are thus probaby identical with those proposed by Metzler *et al.* (1954) for the pyridoxal metal ion-catalysed αβ-eliminations of amino acids, even though the specific L-serine *O*-sulphate-degrading enzyme does not require this cofactor for activity (Tudball *et al.*, 1969). Studies to determine more completely the sequence of events occurring during the degradation of substrate and to investigate alternative means of performing the proton-abstraction event in the absence of pyridoxal and Schiff-base involvement were thus initiated. The present paper deals with further investigations into the mechanism of the enzyme-catalysed degradation of L-serine *O*-sulphate.

Materials and Methods

Chemicals

Pyridoxal 5′-phosphate and tetranitromethane were purchased from Sigma (London) Chemical Co., London S.W.6, U.K. Rose Bengal was obtained from Eastman Organic Chemicals, Rochester, N.Y., U.S.A. ^3H_2O was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Triton X-100 was obtained from British Drug Houses Ltd., Poole, Dorset, U.K., and 2,5-diphenyloxazole from Beckman Instruments Inc., Fullerton, Calif., U.S.A.

Potassium L-serine *O*-sulphate was prepared by the method of Tudball (1962) and β-chloro-L-alanine by the method of Fischer & Raske (1907).

Enzyme and assay

Source of enzyme. In all experiments a mixture of the isoenzymes of the pig liver L-serine *O*-sulphate-degrading system was employed, corresponding with the stage 8 preparation of Tudball *et al.* (1971). The specific activity of the enzyme preparation was 700 units/mg (unit defined by Thomas & Tudball, 1967).

Assay of enzyme activity. For those experiments involving tetranitromethane, the L-serine *O*-sulphate-degrading activity was assayed by the method of Thomas & Tudball (1967). In all other experiments, the coupled assay system of Tudball *et al.* (1971) was employed.

Experimental and Results

Attempted exchange of the α-proton of L-serine O-sulphate with ^3H_2O

Since the enzymic degradation of L-serine *O*-sulphate seems to occur by the mechanism described by Metzler *et al.* (1954), then the initial steps in the sequence should be the labilization and subsequent elimination of the α-hydrogen atom of the substrate as a proton (see Fig. 1). Moreover, if this process is rapid then proton exchange between the substrate and

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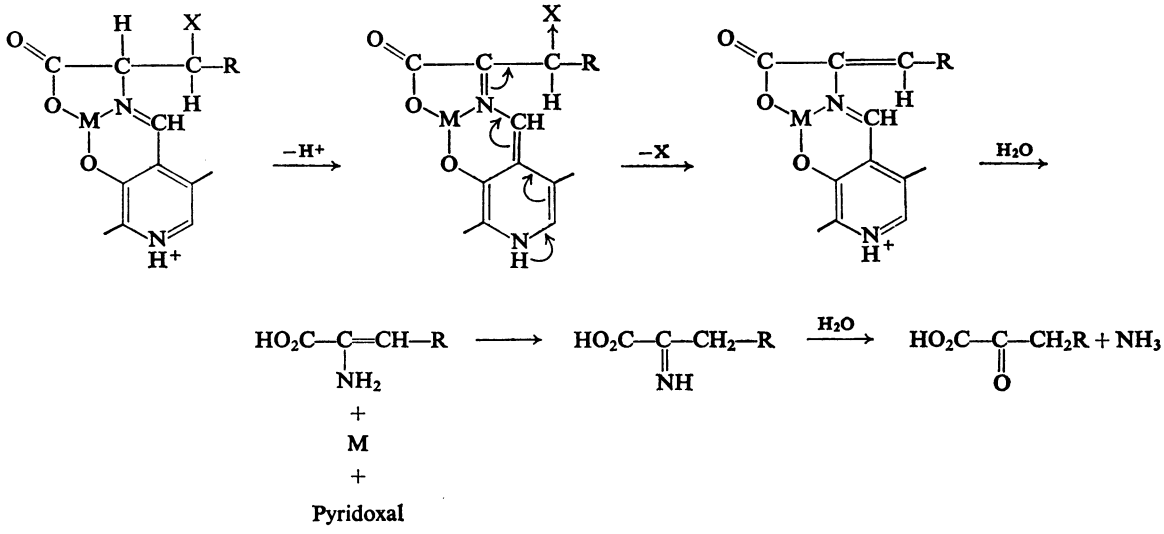

Fig. 1. *General mechanism for the αβ-elimination of an amino acid catalysed by pyridoxal and metal ion*

X represents the β-substituent (Metzler *et al.*, 1954).

the aqueous medium should occur. This possibility was examined as follows.

L-Serine *O*-sulphate (final concn. 0.05M) was incubated with enzyme (0.1mg) in 0.1M-tris–HCl buffer, pH7.0 (0.4ml), containing 1mCi of ^3H_2O. Incubations were performed at 20°C for 30min and the reactions terminated by immersing the reaction vessels in a boiling-water bath for 1min. Similar experiments were performed by using β-chloro-L-alanine, which is also a substrate for the enzyme (Thomas & Tudball, 1967). Control experiments with heat-inactivated enzyme were done simultaneously in all cases. Precipitated protein was sedimented by centrifuging and excess of ^3H_2O removed from the resulting supernatants by repeated distillation *in vacuo* at 37°C and dissolution in water until a constant value for the amount of radioactivity in the sample was recorded. For the purposes of assay, the residues obtained after distillation were dissolved in 1 ml of water and portions (0.1 ml) added to 10ml of scintillation fluid composed of Triton X-100 (500ml), toluene (1l) and 2,5-diphenyloxazole (5g). The amount of radioactivity in the samples was measured with a Beckman LS-100 liquid-scintillation counter.

There was no difference in radioactivity between test and control samples, indicating that no exchange between ^3H_2O and L-serine *O*-sulphate had occurred.

Formation of a carbanion intermediate during the enzymic desulphation of L-*serine O-sulphate*

The failure to detect exchange in the above experiments does not invalidate the proposed reaction mechanism and in theory it should prove possible to demonstrate proton removal indirectly. The removal of a proton from the α-carbon atom of the substrate should yield a detectable carbanion. Tetranitromethane has been employed on numerous occasions for this purpose (Christen & Riordan, 1968; Riordan & Christen, 1968). Reaction with carbanions and tetranitromethane results in the release of nitroform, which can be determined quantitatively by measuring the E_{350} of the test solution. Tetranitromethane also reacts with phenolic side chains and thiol groups in proteins (Vincent *et al.*, 1970; Riordan & Christen, 1968), also with the concomitant release of nitroform, so that care must be exercised in controlling experiments involving carbanion detection. The ability of tetranitromethane to react with phenolic side chains has been utilized to inhibit enzyme systems known to contain essential tyrosine residues (Christen & Riordan, 1970). Clearly the presence of an essential tyrosine residue is not compatible with the use of tetranitromethane for carbanion detection. Preliminary experiments under the conditions normally employed for carbanion detection showed that negligible inhibition of the L-serine *O*-sulphate-degrading activity occurred.

For carbanion detection all incubations and assays were performed at 37°C in 10mm light-path quartz cells (vol. 1ml). Changes in E_{350} were recorded by using a Hilger–Gilford Reaction-Kinetics Spectrophotometer fitted with a thermostatically controlled cell-holder. The final concentration of tetranitromethane employed was 0.84μM in all cases. L-Serine *O*-sulphate (final concn. 0.05M) was incubated with

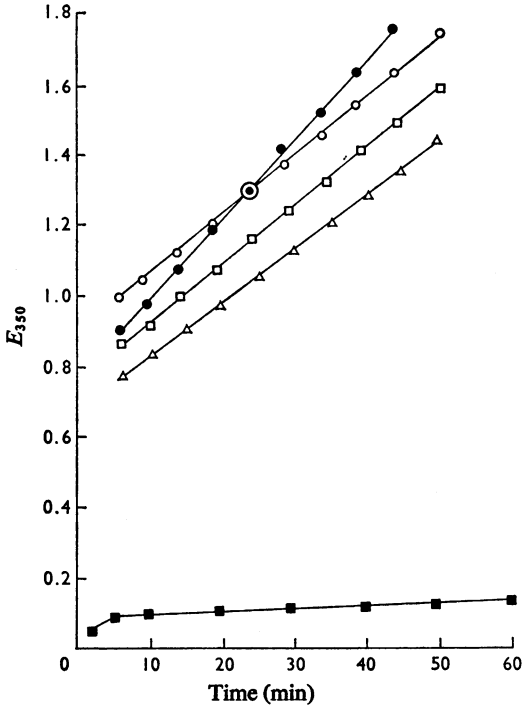

Fig. 2. *Time-course of intermediate carbanion formation in the enzyme-catalysed degradation of* L-*serine O-sulphate*

●, Native enzyme+0.05M substrate+0.84μM-tetranitromethane; ○, heat-denatured enzyme+0.05M substrate+0.84μM-tetranitromethane; □, heat-denatured enzyme+0.84μM-tetranitromethane; △, native enzyme+0.84μM-tetranitromethane; ■, 0.05M substrate+0.84μM-tetranitromethane. Experimental details are given in the text.

tetranitromethane in 0.1M-tris-HCl buffer, pH7.0 (0.9ml), and the reaction initiated by the addition of enzyme (0.02mg in 0.1ml of the tris buffer). Observations of the increase in E_{350} were made at 30s intervals. Control experiments were carried out in which the increase in E_{350} was recorded when tetranitromethane was incubated with native enzyme, heat-inactivated enzyme, heat-inactivated enzyme plus substrate, or substrate alone, under the above experimental conditions. The results of these experiments are presented in Fig. 2.

When native enzyme and substrate were incubated with tetranitromethane, the rate of nitroformate production was greater than was observed in control experiments. Even though high initial values for E_{350} were observed in all cases where protein was present, representing reaction with tetranitromethane, nitroformate production was accelerated only when the enzymic reaction was allowed to proceed. Though the magnitude of the effect is small ($0.16E_{350}$ unit/h), this would be expected since the stationary concentration of the presumed enzyme–carbanion complex would be extremely low under the prevailing expermental conditions.

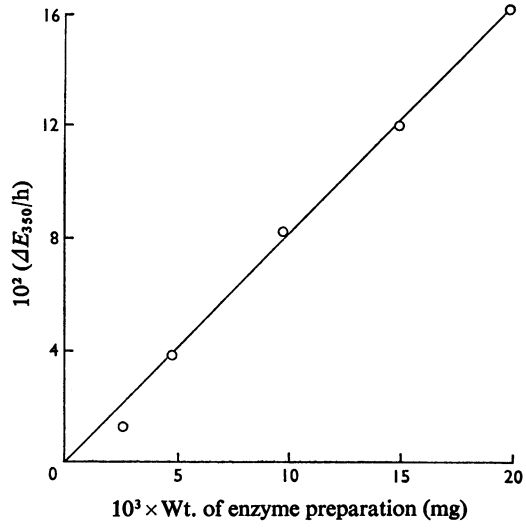

Fig. 3. *Effect of enzyme concentration on the nitration of the enzyme–substrate carbanion*

Various amounts of enzyme were incubated with 0.05M substrate in the presence of 0.84μM-tetranitromethane for 1h at 37°C. Experimental details are given in the text.

Effect of enzyme concentration on the nitration of enzyme–substrate carbanion. Various amounts of enzyme (0–0.02mg) were incubated with substrate (final concn. 0.05M) in the presence of tetranitromethane for 1h at 37°C as described above. The values for E_{350} at zero time and after 1h were recorded and compared with the corresponding control values obtained with the heat-denatured enzyme. The results were then recorded graphically by plotting the increase in E_{350}/h against enzyme concentration (Fig. 3).

The linear nature of the graph suggests that the rate of nitration is directly proportional to the enzyme concentration and therefore to the concentration of the enzyme–substrate carbanion.

Effect of variation of pH and substrate concentration on the nitration of enzyme–substrate carbanion. These experiments were conducted essentially as described above. The quantity of enzyme used throughout was 0.02mg. Experiments designed to study the effect of pH were carried out over the range 5.0–8.5, with substrate at a final concentration of 0.05M. Under

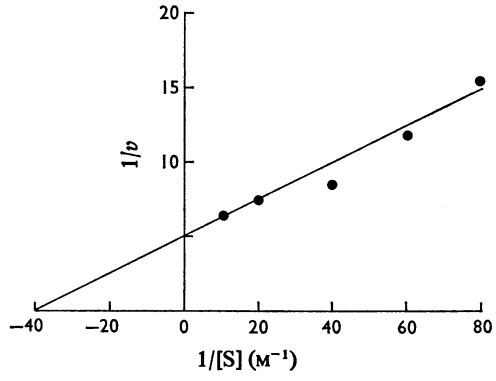

Fig. 4. *Effect of substrate concentration on the nitration of enzyme–substrate carbanion*

Incubations were performed at pH 7.0 in the presence of 0.02 mg of enzyme. Experimental details are given in the text.

the normal incubation conditions an optimum pH of 7.0 was recorded, in agreement with the value recorded by Tudball *et al.* (1971).

To study the effect of substrate concentration on the reaction a pH of 7.0 was used with substrate concentrations ranging between 6.25 mM and 0.1 M. The values obtained for $1/v$ [recorded as $1/(E_{350}/h)$] were plotted against 1/[S]. From this graph (Fig. 4) a value of 2.9×10^{-2} M was recorded for K_m, which agrees well with the value of 2.5×10^{-2} M reported by Tudball *et al.* (1971).

Evidence for an essential histidine residue in the L-*serine O-sulphate-degrading enzyme*

It is well known that histidine residues in proteins are sensitive to illumination in the presence of a photoactive dye. Recently this technique has been increasingly used for the oxidation of histidine residues by using the dye Rose Bengal, which preferentially brings about the photo-oxidation of histidine residues (Westhead, 1965).

The experimental procedure employed for studying the photo-inactivation of the L-serine *O*-sulphate-degrading system was similar to that described by Tudball & Thomas (1971). Rose Bengal (20 μg), enzyme (1 mg) and 0.1 M-tris–HCl buffer (2 ml) contained in a quartz cell of light-path 10 mm was irradiated at 16°C with light from a standard 1000 W lamp placed 10 cm away from the cell. Portions (100 μl) of the irradiated mixture were removed at intervals and used as the enzyme source in the standard coupled assay system. Control experiments were employed in which either Rose Bengal was

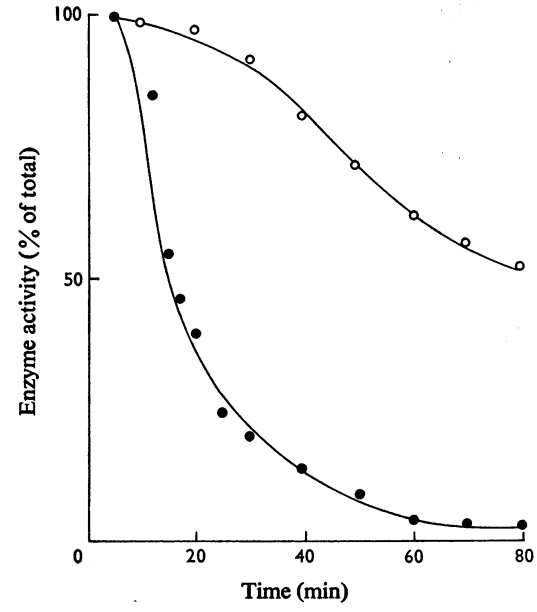

Fig. 5. *Time-course of the photo-oxidation of the* L-*serine O-sulphate-degrading enzyme in the presence of Rose Bengal*

The amount of enzyme activity is related to that at zero time, which was taken as 100%. ●, Enzyme+Rose Bengal; ○, enzyme+Rose Bengal+10 mM-isophthalic acid. Experimental details are given in the text.

omitted or the whole system was kept in the dark. Similar experiments were also performed in the presence of isophthalic acid (final concn. 10 mM), a known competitive inhibitor of the enzyme. The results of these experiments are presented graphically in Fig. 5. Considerable protection against photo-inactivation was afforded in the presence of isophthalate. The loss of enzyme activity in the absence of inhibitor exhibited first-order kinetics except over the first 10 min of irradiation, where little activity was lost.

The effect of pH on the photo-inactivation was also examined, since some information can be gained in this way about the nature of the amino acid residue(s) governing the photo-inactivation process.

Irradiations were performed in 0.05 M-sodium acetate–acetic acid buffer for the range pH 5.5–6.2 and in 0.1 M-tris–HCl buffer for the range pH 6.5–8.5. The results of these experiments are presented graphically in Fig. 6. The sigmoidal curve obtained is typical of that to be expected if histidine was the amino acid governing the photo-oxidative inactivation (Westhead, 1965).

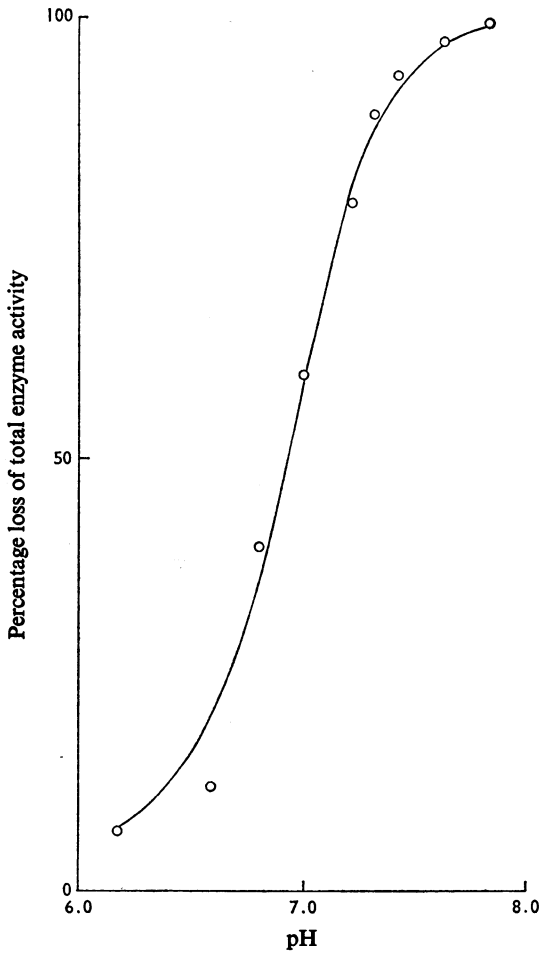

Fig. 6. *Effect of pH on the photo-oxidation of the* L-*serine O-sulphate-degrading enzyme in the presence of Rose Bengal*

Incubations were performed in 0.05M-sodium acetate–acetic acid buffer for pH values 5.5–6.2 and in 0.1M-tris–HCl buffer for pH values 6.5–8.5. Experimental details are given in the text.

Amino acid analysis of photo-inactivated enzyme. Photo-inactivation of the enzyme was carried out as described above and allowed to proceed until approx. 10% of the original activity remained (35min). A small quantity of charcoal was added to remove the Rose Bengal. After removal of the charcoal by centrifuging, the resulting supernatant was dialysed against five changes of water (total vol. 5l) and the non-diffusible material freeze-dried. The resulting preparation was dissolved in constant-boiling 6M-HCl (2ml) and hydrolysed *in vacuo* for 24h at 110°C. Excess of HCl was removed by repeated freeze-drying. For the subsequent determination of the amino acid composition, the freeze-dried samples were dissolved in 1.5ml of pH2.875 buffer (Technicon AutoAnalyzer Instruction Manual) and samples (0.5ml) analysed by using the Technicon Amino Acid Analyser. Control experiments were performed in an identical manner, only the irradiation step being omitted. The tryptophan content of the enzyme was measured before and after illumination by the method of Goodwin & Morton (1946). Amino acid analyses revealed that four histidine residues/molecule (mol.wt. 54000) had been destroyed during the photoinactivation. There was no evidence for the modification of any other amino acid residue.

Inhibition of enzyme activity by Rose Bengal in the dark. When the enzyme was maintained in the presence of Rose Bengal and the whole system kept in the dark, a marked decrease in activity occurred that could be immediately restored on addition of charcoal to remove the dye. The effect of the dye on enzyme activity was examined by using the coupled enzyme assay method. Dye was employed at concentrations of 5 and 10μg/ml and the substrate at concentrations between 6.25mM and 0.1M. The double-reciprocal plot of Lineweaver & Burk (1934) showed that the inhibition was non-competitive. Further, the spectrum of Rose Bengal in the presence of the enzyme was altered. The maximum peak of absorbance of the dye, normally at 548nm, shifted to 558nm with a concomitant decrease of 20% in its intensity. The inhibition of enzyme activity, coupled with the changes in the spectral properties of the dye, have been interpreted as a non-specific binding of the dye to the surface of the enzyme consequently bringing about a reversible conformational change in the protein. Similar effects have been observed by Bond *et al.* (1970) for 3-phosphoglyceraldehyde dehydrogenase (EC 1.2.1.9) and by N. Tudball & P. Thomas (unpublished work) for bovine glutamate dehydrogenase (EC 1.4.1.3).

Discussion

It is now clear that the mechanism of the enzymic degradation of L-serine *O*-sulphate follows the general scheme proposed by Metzler *et al.* (1954) for $\alpha\beta$-elimination reactions of amino acids catalysed by pyridoxal and metal ions. Since the above enzyme is not dependent on pyridoxal phosphate for its catalytic activity and, further, since there is no evidence for the formation of a Schiff base during the reaction (Tudball *et al.*, 1969), the proton-abstraction event must occur in a hitherto unreported manner.

The absence of exchange between ^3H of the aqueous medium and the substrate indicates that the removal of the hydrogen atom attached to the α-carbon atom, which is certainly the first chemical

event in the catalytic sequence, is also in all probability the rate-determining step for the reaction. The removal of the hydrogen as a proton is probably the only enzyme-catalysed step in the sequence. It is envisaged that the carbanion resulting from this elimination simply undergoes an electronic rearrangement leading to the expulsion of the β-substituent and the formation of aminoacrylic acid. The further hydrolysis of aminoacrylic acid occurs without the intervention of the enzyme, rendering the overall reaction effectively irreversible (Tudball & Thomas, 1972).

The chemical intermediates involved in the enzyme-catalysed degradation of L-serine *O*-sulphate have now been reasonably well-defined, but the role of the enzyme in this process is still somewhat ill-defined. Some explanation, however, might be forthcoming from the results obtained on the photo-inactivation of the enzyme. These results suggest but do not unequivocally demonstrate the involvement of an active-site histidine residue. In this context, however, it is noteworthy that imidazole is capable of bringing about the $\alpha\beta$-elimination of L-serine *O*-sulphate (N. Tudball, unpublished work), though at a far slower rate than the corresponding enzyme-catalysed breakdown. Histidine may thus be envisaged as bringing about a base-catalysed degradation of the substrate, resulting in the abstraction of a proton from the α-carbon atom. If this proves to be the case, it represents an unique example in enzyme catalysis.

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