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Involvement of the Superoxide Anion in Sulphoxidation

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Sulphoxidation of compounds capable of undergoing biological sulphoxidation has been demonstrated in a model system (NADH–phenazine methosulphate–O_2), known to generate superoxide anions (O_2^-). Addition of superoxide dismutase to this system results in complete inhibition, suggesting the involvement of O_2^- in sulphoxidation.

A number of thioethers that are used as therapeutic agents or insecticides are metabolized in animals to sulphoxides (Clare, 1947; March *et al.*, 1955; Burns *et al.*, 1957; Walkenstein & Seifter, 1959). In addition, biological sulphoxidation reactions such as the conversion of chlorpromazine into its sulphoxide have been reported (Gillette & Kamm, 1960). We have since reported the purification and properties of an enzyme from guinea-pig liver that catalyses the sulphoxidation of ethionamide (2-ethyl-4-thioisonicotinamide), an active tuberculostatic second-line drug (Prema & Gopinathan, 1972). Sulphoxidation is particularly significant in the case of ethionamide because the drug is converted readily into its sulphoxide on administration and the antituberculous activity is greater *in vivo* than *in vitro*. The present communication describes the sulphoxidation reaction carried out in a model system involving superoxide anion and its inhibition by superoxide dismutase.

Materials and methods

Phenazine methosulphate was from Sigma Chemical Co., St. Louis, Mo., U.S.A. NADH was from V.P. Chest Institute, New Delhi, India. Ethionamide was from May and Baker Ltd., Dagenham, Essex, U.K., and was recrystallized twice before use. Thioisonicotinamide and chlorpromazine were from May and Baker, Bombay, India. Melleril (thioridazine) was from Sandoz (India) Ltd., Bombay, India. Thiourea and thiosemicarbazide were from BDH Chemicals Ltd., Poole, Dorset, U.K. Silica gel for t.l.c. was from E. Merck A.G., Darmstadt, W. Germany.

The model system used for the production of superoxide anion (O_2^-) was similar to the one described by Prema Kumar *et al.* (1972*a*). Superoxide dismutase from sheep erythrocytes was purified up to the step of DEAE-cellulose chromatography by the method of McCord & Fridovich (1969).

Ethionamide sulphoxide formed in the reaction mixture was determined by extraction into chloroform and re-extraction into 0.1 M-HCl. After separation of the chloroform layer, the absorbance of the sample was recorded at 395 nm.

Chlorpromazine sulphoxide formed was determined by the method of Salzman & Brodie (1956).

Results

Conversion of ethionamide into ethionamide sulphoxide by the NADH–phenazine methosulphate–O_2 system. Table 1 shows a complete system required for

Table 1. *Ethionamide sulphoxidation in the model system*

The standard system contained, in a final volume of 1.0 ml: potassium phosphate buffer, pH 7.5, 40 μmol; NADH, 60.0 nmol; phenazine methosulphate, 100 nmol; ethionamide, 300 nmol. Incubations were carried out for 1 h at 28°C in the dark; the NADH was added in two instalments, at 0 and 30 min. The sulphoxide formed was extracted into chloroform and determined as described in the text under 'Materials and methods'. The superoxide dismutase was added just after the addition of substrate (120 μg of protein corresponds to 35 units of superoxide dismutase, a unit being defined as that amount of enzyme required to inhibit the rate of reduction of cytochrome *c* by 50% at 28°C).

Reaction mixture	Ethionamide sulphoxide formed (nmol)
Complete system	55
Minus phenazine methosulphate and NADH	0
Minus phenazine methosulphate	0
Minus NADH	0
Minus O_2	0
Plus dismutase (120 μg of protein)	35
Plus dismutase (240 μg of protein)	7

the sulphoxidation of ethionamide. Under anaerobic conditions, no sulphoxidation was observed, indicating an absolute requirement for O_2.

The product of the reaction was identified by t.l.c. on silica gels; the solvent system employed was butan-1-ol–acetic acid–water (4:1:5, by vol.) and the R_F values and absorption spectra of the product were compared with those of an authentic sample. The product can be detected on the chromatogram as a visible yellow spot and also after spraying with Dragendorff's reagent (containing bismuth subnitrite, tartaric acid and KI).

Addition of superoxide dismutase to the assay system resulted in inhibition of sulphoxidation, the extent of inhibition being approximately proportional to the concentration of enzyme added. At higher concentrations of enzyme there was complete inhibition. The results were similar with two independent preparations of superoxide dismutase.

Effect of varying the different components of the system on sulphoxidation. The effect of varying the different components of the reaction was studied; the sulphoxide formation was directly proportional to the amount of phenazine methosulphate included in the reaction mixture (Table 2). Increasing the concentration of both NADH and phenazine methosulphate to 5 times that of the standard reaction mixture resulted in 5-fold stimulation of the production of sulphoxide. At given concentrations of phenazine methosulphate and NADH, varying the ethionamide concentrations over a 10-fold range did not increase the formation of sulphoxide.

Sulphoxidation of other compounds. Other compounds, such as chlorpromazine, melleril, thiourea, thiosemicarbazide and thioisonicotinamide, which can undergo biological sulphoxidation, were examined in the model system. When chlorpromazine was used in place of ethionamide, chlorpromazine sulphoxide was formed; the product was identified after extraction into heptane–3-methylbutan-1-ol (100:1.5, v/v) and t.l.c. on silica gels. The other compounds, however, were tested indirectly by analysing their effectiveness as inhibitors of ethionamide sulphoxidation when included in the standard assay system. All these compounds competitively inhibited sulphoxidation of ethionamide, suggesting that they also undergo sulphoxidation.

Table 2. *Effect of varying the concentration of phenazine methosulphate on sulphoxidation*

The standard assay system described in Table 1 was employed, except that the concentration of phenazine methosulphate was varied as indicated.

Phenazine methosulphate added (nmol)	Product formed (nmol)
20	8.8
40	22.0
60	35.2
80	49.5
100	60.5
150	62.7
200	64.9

Discussion

The involvement of superoxide anions (O_2^-) in reactions catalysed by oxygenases has been postulated (King *et al.*, 1971; Symposium on Biological Hydroxylation Mechanisms, 1971). The possible participation of O_2^- in the reactions catalysed by intestinal tryptophan 2,3-dioxygenase (Hirata & Hayaishi, 1971) and liver cytochrome *P*-450 (Strobel & Coon, 1971) as well as in the hydroxylation of *p*-hydroxybenzoate (Strickland & Massey, 1971) and *p*-cresol (McCord *et al.*, 1971) has been described. Recently the mechanism of aromatic hydroxylation in microbial enzyme systems has been worked out, and this also involves superoxide anions (Prema Kumar *et al.*, 1972*a*,*b*).

The results presented in this communication show that compounds such as ethionamide and chlorpromazine can undergo sulphoxidation in the model system described by Prema Kumar *et al.* (1972*a*,*b*). We have recently purified to homogeneity a mono-oxygenase from guinea-pig liver that converts ethionamide into its sulphoxide (K. Prema & K. P. Gopinathan, unpublished work). This enzymic sulphoxidation as well as the non-enzymic sulphoxidation observed in the model system are inhibited by superoxide dismutase. These results therefore imply a role for superoxide anions in another class of biological reaction, namely sulphoxidation.

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Burns, J. J., Yu, T. F., Ritterband, A., Perel, J. M., Gutnan, A. B. & Brodie, B. B. (1957) *J. Pharmacol. Exp. Ther.* **119**, 418–426

Clare, N. T. (1947) *Aust. Vet. J.* **23**, 340–344

Gillette, R. & Kamm, J. (1960) *J. Pharmacol. Exp. Ther.* **130**, 262–267

Hirata, F. & Hayaishi, O. (1971) *J. Biol. Chem.* **246**, 7825–7826

King, T. E., Mason, H. S. & Morrison, M. (eds.) (1971) *Abstr. Int. Symp. Oxidases and Related Oxidation–Reduction Systems 2nd, Memphis*

March, R. B., Metcalf, R. L., Furuto, T. R. & Maxon, M. G. (1955) *J. Econ. Entomol.* **48**, 355–363

McCord, J. M. & Fridovich, I. (1969) *J. Biol. Chem.* **244**, 6049–6055

McCord, J. M., Beauchamp, C. O., Gosein, S., Misra, H. P. & Fridovich, I. (1971) *Abstr. Int. Symp. Oxidases and Related Oxidation–Reduction Systems 2nd, Memphis*, pp. 51–76

Prema, K. & Gopinathan, K. P. (1972) *Proc. Soc. Biol. Chem. India* **31**, 21

Prema Kumar, R., Ravindranath, S. D., Vaidyanathan, C. S. & Appaji Rao (1972*a*) *Biochem. Biophys. Res. Commun.* **48**, 1049–1054

Prema Kumar, R., Ravindranath, S. D., Vaidyanathan, C. S. & Appaji Rao (1972*b*) *Biochem. Biophys. Res. Commun.* **49**, 1422–1426

Salzman, P. & Brodie, B. (1956) *J. Pharmacol. Exp. Ther.* **118**, 46–54

Strickland, S. & Massey, V. (1971) *Abstr. Int. Symp. Oxidases and Related Oxidation–Reduction Systems 2nd, Memphis*, pp. 77–93

Strobel, H. W. & Coon, M. J. (1971) *J. Biol. Chem.* **246**, 7826–7829

Symposium on Biological Hydroxylation Mechanisms (1971) *Biochem. J.* **125**, 1P–11P

Walkenstein, S. S. & Seifter, J. (1959) *J. Pharmacol. Exp. Ther.* **125**, 283–286