Hydroxylation of Aromatic Compounds by Reduced Nicotinamide–Adenine Dinucleotide and Phenazine Methosulphate Requires Hydrogen Peroxide and Hydroxyl Radicals, but not Superoxide

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1. A mixture of NADH and phenazine methosulphate hydroxylates aromatic compounds at acidic pH values. 2. Hydroxylation is inhibited by catalase and by scavengers of the hydroxyl radical ($\cdot\text{OH}$) but not by superoxide dismutase. 3. It is concluded that neither $\text{O}_2^{\cdot-}$ nor $\text{HO}_2^{\cdot}$ is sufficiently reactive to hydroxylate aromatic rings.

A mixture of NADH and phenazine methosulphate generates the superoxide radical $\text{O}_2^{\cdot-}$ (Nishikimi *et al.*, 1972). This mixture is capable of hydroxylating added aromatic compounds at acid pH values, and it has been suggested that it is a suitable model system for the action of oxygenase enzymes (Prema Kumar *et al.*, 1972; Ashok Kumar *et al.*, 1975). Ravindranath *et al.* (1974) showed that hydroxylation by the NADH/phenazine methosulphate system was inhibited on addition of a superoxide dismutase preparation, and they therefore claimed that hydroxylation was due to a direct reaction of $\text{O}_2^{\cdot-}$ or its protonated form ($\text{HO}_2^{\cdot}$) with the aromatic ring.

However, Fee & Hildenbrand (1974) did not observe hydroxylation when $\text{O}_2^{\cdot-}$ dissolved in an organic solvent was added to an aqueous solution of an aromatic compound. Incubation of aromatic compounds with potassium superoxide ($\text{K}^+\text{O}_2^{\cdot-}$) in organic solvents did not bring about direct hydroxylation, although $\text{O}_2^{\cdot-}$ was found to be capable of the nucleophilic displacement of halide atoms from aromatic rings (Frimer & Rosenthal, 1976).

In view of the discrepancies between the above reports, I have reinvestigated the properties of hydroxylation by the NADH/phenazine methosulphate system.

Materials and Methods

NADH, phenazine methosulphate, catalase (type C-40), salicylic acid and 4-hydroxybenzoic acid were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Catalase (EC 1.11.1.6) was also obtained from Boehringer Corp., London W.5, U.K.; it had no significant contamination by superoxide dismutase (Halliwell, 1973). Catalase was assayed by the fall in A_{240} as H_2O_2 was destroyed; one unit is the amount that catalyses the

breakdown of $1\ \mu\text{mol}$ of $\text{H}_2\text{O}_2/\text{min}$ under the reaction conditions of Luck (1963).

Superoxide dismutase (EC 1.15.1.1) was purified as described by McCord & Fridovich (1969). One unit is the amount that inhibits the reduction of cytochrome *c* in a xanthine/xanthine oxidase system by 50%.

Incubation of aromatic compounds with NADH and phenazine methosulphate was carried out in the dark in a final volume of 1.00 ml as described by Ravindranath *et al.* (1974). The hydroxylated products were extracted into diethyl ether and assayed as described by Halliwell (1975).

Results

In agreement with published results (Prema Kumar *et al.*, 1972; Ravindranath *et al.*, 1974; Ashok Kumar *et al.*, 1975) a mixture of NADH and phenazine methosulphate was found to hydroxylate aromatic compounds at acidic pH values (Table 1). Both salicylic acid and 4-hydroxybenzoic acid were used as substrates. Hydroxylation was not inhibited by adding superoxide dismutase. This observation contradicts the results of Prema Kumar *et al.* (1972) and of Ashok Kumar *et al.* (1975), but these workers used a crude superoxide dismutase fraction obtained by acetone precipitation of a haemolysate. Table 1 shows that hydroxylation is strongly inhibited by small amounts of acetone, and it may be that their results were due to the presence of traces of acetone in the acetone powder. The superoxide dismutase used in the experiments described in the present paper had been purified as described by McCord & Fridovich (1969) and was highly active.

Cu^{2+} catalyses the breakdown of $\text{O}_2^{\cdot-}$ at acid pH values, with an overall rate constant approaching

Table 1. *Hydroxylation of aromatic compounds by the NADH/phenazine methosulphate system*

Incubations of aromatic compounds (either salicylic acid or 4-hydroxybenzoic acid) with NADH and phenazine methosulphate in a final volume of 1.00 ml were carried out at 30°C for 0.5 h in the dark as described by Ravindranath *et al.* (1974). The buffers used were 60 mM-citric acid/potassium citrate, pH 4.5, or 60 mM-KH₂PO₄/KOH, pH 5.5. After 0.5 h the diphenol produced was measured as described by Halliwell (1975). None of the reagents listed below interfered with colour development in the assay. The diphenols produced were 3,4-dihydroxybenzoic acid (from 4-hydroxybenzoate) or 2,3-dihydroxybenzoic acid (from salicylate).

Addition to reaction mixture	Concentration of compound added	Rate of hydroxylation (nmol of diphenol produced/30 min)			
		4-Hydroxybenzoic acid		Salicylic acid	
		pH 4.5	pH 5.5	pH 4.5	pH 5.5
None	—	73	87	37	56
None	—*	0	0	0	0
None	—†	0	0	0	0
Acetone	0.01 ml/ml of reaction mixture	35	35	—	—
	0.02 ml/ml of reaction mixture	23	31	16	22
EDTA	1.0 mM	66	—	36	—
	3.0 mM	68	—	36	—
H ₂ O ₂	0.2 mM	101	—	—	—
	0.37 mM	119	—	—	—
	0.9 mM	150	—	—	—
	1.9 mM	162	136	73	87
	1.9 mM*	0	0	0	0
	1.9 mM†	0	0	0	0
Superoxide dismutase	10 units	69	89	37	56
	20 units	72	88	38	56
	50 units	68	84	36	56
	100 units	67	82	38	58
	200 units	71	85	37	52
Catalase	25 units	54	—	—	—
	50 units	42	—	—	—
	75 units	25	—	—	—
	100 units	0	4	0	8
CuSO ₄	1.0 mM	70	—	25	—
MnCl ₂	1.0 mM	73	—	34	—

* NADH omitted.

† Phenazine methosulphate omitted.

that of superoxide dismutase (Rabani *et al.*, 1973). Mn²⁺ also reacts rapidly with O₂^{•-} (Halliwell & Ahluwalia, 1976; Kono *et al.*, 1976). However, 1 mM concentrations of Cu²⁺ or Mn²⁺ had little effect on hydroxylation by the NADH/phenazine methosulphate system, which further suggests that O₂^{•-} is not required.

In contrast, catalase was a powerful inhibitor of hydroxylation, indicating that H₂O₂ is needed. An equal amount of bovine serum albumin had no inhibitory effect. Ravindranath *et al.* (1974) claimed that catalase did not inhibit hydroxylation by the NADH/phenazine methosulphate system. I have no explanation for this discrepancy, but the results in the present paper have been obtained in at least six different experiments with batches of pure catalase obtained from different suppliers.

Further evidence that H₂O₂ is involved in the

hydroxylation was provided by the observation that hydroxylation was stimulated on addition of this compound. H₂O₂ did not induce any hydroxylation in the absence of either NADH or phenazine methosulphate (Table 1).

The effect of scavengers of the hydroxyl radical ([•]OH) on hydroxylation by the NADH/phenazine methosulphate system was examined. Mannitol, formate and thiourea (Anbar & Neta, 1967) were all powerful inhibitors; they also inhibited the increased rate of hydroxylation observed when H₂O₂ was added (Table 2). Thiourea has a much higher rate constant for reaction with [•]OH than does mannitol or formate (Anbar & Neta, 1967), and it was effective in inhibiting the hydroxylation when present at a much lower concentration. In contrast, urea or KCl, which do not react with [•]OH at significant rates (Anbar & Neta, 1967), did not inhibit hydroxylation.

Table 2. *Effect of hydroxyl-radical scavengers on hydroxylation of aromatic compounds by the NADH/phenazine methosulphate system*

In experiments (A) the reactions were carried out as described in the legend to Table 1. In experiments (B) reaction mixtures also contained 1.9mM-H₂O₂. None of the ·OH scavengers interfered with the assay used for measurement of diphenols (Halliwell, 1975).

Scavenger added (A)	Concentration (mM)	Rate of hydroxylation (nmol of diphenol produced/30min)			
		4-Hydroxybenzoic acid		Salicylic acid	
		pH4.5	pH5.5	pH4.5	pH5.5
None	—	80	87	37	56
Mannitol	10	50	—	—	—
	25	20	—	—	—
	50	18	—	—	—
	100	10	2	4	5
Sodium formate	10	34	—	—	—
	25	23	—	—	—
	50	13	—	—	—
	100	4	0	4	6
Thiourea	0.5	50	—	—	—
	1.0	40	—	—	—
	2.0	18	23	16	16
KCl	100	78	84	37	56
Urea	2.0	81	87	39	53
(B)					
None	—	180	136	73	87
Mannitol	100	33	8	8	9
Sodium formate	100	30	6	9	9
Thiourea	2.0	70	22	20	29
Urea	2.0	180	132	74	91
KCl	100	178	131	71	88

Discussion

The results described in the present paper show that hydroxylation by the NADH/phenazine methosulphate system requires both H₂O₂ and ·OH, but not O₂^{·-} or HO₂[·], since superoxide dismutase did not inhibit. It may well be that hydroxylation is brought about by ·OH generated in some way from H₂O₂. Although ·OH can probably be generated from O₂^{·-} by a mechanism that appears to involve H₂O₂ (Fridovich, 1975; Halliwell, 1976; Czapski, 1977), the lack of inhibition by superoxide dismutase shows that the ·OH is not derived from O₂^{·-} in the NADH/phenazine methosulphate system. Perhaps traces of metal ions (Mⁿ⁺) in the reagents interact with the H₂O₂ in a Fenton-type reaction:

The oxidized metal ion [M⁽ⁿ⁺¹⁾⁺] might be reduced to Mⁿ⁺ by NADH, or reduced phenazine methosulphate (generated by reduction of phenazine methosulphate by NADH) so allowing reaction (1) to continue. However, hydroxylation was not inhibited by EDTA (Table 1). Double-glass-distilled water was used throughout these experiments.

The results in the present paper are consistent with the chemical evidence (Fee & Hildenbrand, 1974; Frimer & Rosenthal, 1976), which suggests that O₂^{·-} or HO₂[·] are not sufficiently reactive directly to add on to and so hydroxylate aromatic rings. They also show that the NADH/phenazine methosulphate system is not a good model for enzyme-catalysed hydroxylation, since ·OH is unlikely to be involved in such enzyme-catalysed reactions (Halliwell, 1977).

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