Some Rate Constants for the Phenazine Methosulphate-Catalysed Oxidation of Reduced Nicotinamide-Adenine Dinucleotide

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Kinetic measurements have been made on the phenazine methosulphate-catalysed oxidation of NADH₂ and the effects of cyanide, EDTA, Fe²⁺, catalase and an unidentified mitochondrial factor on this reaction are examined. Chemical equations are formulated and values of the rate constants are determined. The data suggest the existence of an unreactive form of the reduced phenazine methosulphate; the stability and possible nature of such a compound are discussed.

Some kinetic measurements were made on the oxidation of NADH₂ by oxygen, catalysed by PMS*, in connexion with the preceding paper (Sowerby & Ottaway, 1966). Although the complex reaction of PMS with oxygen made elucidation of the results difficult, some points of interest emerged, and are recorded.

METHODS

All measurements were made on a Unicam SP.800 spectrophotometer, programmed to scan repeatedly the range 400–330 mμ, which includes the 387 mμ peak of PMS and the 338 mμ peak of NADH₂. The repeat time was approx. 30 sec., and was timed with a stop-watch. The concentrations of NADH₂ and of PMS, and thus of PMSH₂, were calculated from the traces by correcting for the off-peak absorption of each compound at the other's maximum.

The reaction mixture contained 1 ml. of 0.2 M-phosphate buffer (pH 7.7 unless otherwise specified), NADH₂ (approx. 0.13 mM), and any of the reagents specified below, in a volume of 2.8 ml. PMS (usually 67 mμmoles) was added at zero time in a volume of 0.2 ml. All experiments were carried out at 27°.

The O₂ concentration at any time was estimated by subtracting the calculated O₂ equivalent of (NAD formed—PMSH₂ present) from the initial concentration of O₂ (0.240 mM at 27°). It was assumed that the only product was H₂O₂ unless otherwise stated. Although this may not be true in all circumstances, O₂ was present in reasonable excess, and the error is not likely to be great.

Catalase was obtained from L. Light and Co. Ltd., Colnbrook, Bucks. All other reagents were obtained from the sources given by Sowerby & Ottaway (1966).

RESULTS

Experiments were carried out at pH 6.8 and at pH 7.7 without further additions, and also at pH 7.7

* Abbreviations: PMS, phenazine methosulphate; PMSH₂, reduced phenazine methosulphate.

to test the effects of cyanide, EDTA, Fe²⁺, catalase and the mitochondrial factor described by Sowerby & Ottaway (1966).

From the original traces (Fig. 1b) the concentrations of NADH₂ and of PMSH₂ at approx. 30 sec. intervals were obtained and plotted against time, for each experiment. Two such sets of curves are shown in Figs. 2(a) and 2(b). From these curves values for d[NADH₂]/dt and d[PMSH₂]/dt at various times during the reaction were obtained graphically. Usually, three well-spaced times in the period 30–120 sec. after the addition of PMS to the reaction mixture were chosen, since it was desired to find values for the three rate constants governing the reactions of NADH₂ with PMS, of NAD with PMSH₂ and of PMSH₂ with oxygen. A set of chemical equations was then formulated for each experiment, from which equations in terms of d[NADH₂]/dt, d[PMSH₂]/dt, [NADH₂], [PMSH₂], [NAD], [PMS] and the rate constants *k*₁, *k*₂ and *k*₃ could be deduced. The latter were then solved for *k*₁, *k*₂ and *k*₃ after inserting suitable values for the experimental variables. The chemical equations were rejected if any of the values found for the constants were negative. If they were positive, the calculations were repeated with three more sets of variables, at least one set being obtained for a time when the reaction was almost complete. If the replicate values calculated for *k*₁, *k*₂ and *k*₃ were not constant, the chemical equations were again rejected, and new formulations were tried until a set of equations was found to fit the experimental curves.

A simple formulation of the PMS-catalysed oxidation of NADH₂ is:

Fig. 1. Absorption spectra of NADH₂ and PMS. (a) Absorption spectra of NADH₂ (approx. 0.13 mm) (A) and of PMS (approx. 22 μm) (B). (b) Absorption spectra of cuvette containing 0.13 mm-NADH₂ at pH 7.7, before (A) and then after the addition of 67 μmoles of PMS. The traces were made at approx. 30 sec. intervals.

Fig. 2. Changes in concentration of NADH₂ (●) and PMSH₂ (○), after the addition of 67 μmoles of PMS to 0.4 μmole of NADH₂ at pH 7.7. Both solutions contained cyanide (1 mm). (a) No other addition; (b) solution contained 3 mg. of mitochondrial factor. The final volume was 3 ml. in each case.

We then have:

$$\frac{d[\text{NADH}_2]}{dt} = -k'_1[\text{NADH}_2][\text{PMS}] + k_2[\text{NAD}][\text{PMSH}_2]$$

$$\frac{d[\text{PMSH}_2]}{dt} = k'_1[\text{NADH}_2][\text{PMS}] - k_2[\text{NAD}][\text{PMSH}_2] - k_3[\text{PMSH}_2][\text{O}_2]$$

The only experimental results that were fitted by these equations were those in which the reaction mixture contained cyanide, either 10 or 1 mm (Table 1). Experiments in which the reaction mixture contained cyanide (1 mm) and either catalase (50 μg./3 ml.) or 3 mg. of the mitochondrial factor (described by Sowerby & Ottaway, 1966), which is

Table 1. Rate constants for oxidation of NADH₂ in the presence of cyanide, catalase and the mitochondrial factor

The reaction mixture contained 1 ml. of 0.2 M-phosphate buffer, pH 7.7, approx. 0.4 μmole of NADH₂, and either 3 or 30 μmoles of cyanide, in a total volume of 2.8 ml.; 67 μmoles of PMS in 0.2 ml. of water were added at zero time. Other additions are described in the text. Values are expressed l. m.-mole⁻¹ min.⁻¹. $k'_1 = k_1[\text{H}^+]$.

Concn. of cyanide (mm)	Addition	k'_1	k_2	k_3
10	—	67	34	67.5
1	—	73	34	37
1	Catalase	63		32.5*
1	Mitochondrial factor	99		31.5*

* Assuming no H₂O₂ present at any stage.

rich in catalase, gave results that could be described by the formulation:

The constants calculated by the use of these equations are also given in Table 1.

None of the other sets of observations could be fitted by equations derived from either reactions (1) and (2) or reactions (3) and (4). In most of the experiments at pH 7.7 the curves for the disappearance of PMSH₂ were much flatter than those in Fig. 2, although the initial (30sec.) concentration of PMSH₂ was not higher than that shown. One formulation of the overall reaction was found that fitted all these experimental results from the 30sec. observation until the oxidation was completed.

The kinetic equations that fitted the curves required that NADH₂ should be re-formed, by reduction of NAD, in a first-order or pseudo-first-order reaction, and that some of the PMSH₂ should be in a form that would not react with oxygen. The postulation of an NAD-PMSH₂ complex, reacting according to the following equations, enabled the kinetic equations to be deduced:

Other intermediate reactions may well occur, but it was not necessary to make use of them in order to fit the experimental data.

The kinetic equations derived from eqns. (5), (6) and (7) are:

$$\frac{d[\text{NADH}_2]}{dt} = -k'_1[\text{NADH}_2][\text{PMS}] + k_2[\text{NAD-PMSH}_2] \quad (8)$$

$$\frac{d[\text{NADH}_2]}{dt} + \frac{d[\text{total PMSH}_2]}{dt} = -k_5[\text{free PMSH}_2][\text{O}_2] \quad (9)$$

It is assumed that:

$$[\text{total PMSH}_2] = [\text{free PMSH}_2] + [\text{NAD-PMSH}_2]$$

By making use of this last relationship, the term [NAD-PMSH₂] can be eliminated from eqns. (8) and (9), and a single equation in terms of k'_1 , k_2 and k_5 can be obtained. By insertion of suitable values from the experimental data, three simultaneous equations can be written and solved for the three

Table 2. Values for some rate constants for the oxidation of NADH₂

The reaction mixture contained 1 ml. of 0.2M-phosphate buffer, pH 7.7, and approx. 0.4 μmole of NADH₂ in a total volume of 2.8 ml.; FeSO₄ and Na₃EDTA were added to give the concentrations stated, in 3 ml.; 67 mμmoles of PMS in 0.2 ml. of water were added at zero time. The constants are those of eqns. (8) and (9) of the text. All values refer to pH 7.7. The constants k'_1 and k_5 are expressed in l.m-mole⁻¹ min.⁻¹ and k_2 in min.⁻¹.

Addition	k'_1	k_2	k_5
None	93, 93	8.3, 6.4	58.5, 55
Fe ³⁺	96	8.5	57
EDTA (10 mM)	176	18.8	130
EDTA (1 mM)	95, 78	10.0, 18.2	39, 42
EDTA (1 mM) + mitochondrial factor	495	93	44*

* Assuming no H₂O₂ present at any stage.

constants. The values so calculated were checked as previously described, and are given in Table 2.

No equations that fitted all the observations could be found for the experiments in which catalase had been added in the absence of cyanide, or for the experiment in which the mitochondrial factor was present without other addition. The data from the experiments at pH 6.8 could be fitted either by reaction (3) followed by (2), or by reactions (5), (6) and (7). When the latter formulation was used, it could be calculated that [NAD-PMSH₂] would have been very large, and [free PMSH₂] very small, in relation to the [total PMSH₂] present at any stage. The values of k'_1 calculated on the basis of the two hypotheses were 90 and 148 l.m-mole⁻¹ min.⁻¹ respectively. Neither of these values is very different from that calculated for pH 7.7 (Table 2).

A certain amount of other evidence for the existence of an unreactive form of PMSH₂ was obtained by adding 0.05 μmole of NADH₂ to 0.25 μmole of PMS contained in 3 ml. of 0.03M-phosphate buffer, pH 7.7. The absorption peak at 340 mμ corresponding to NADH₂ completely disappeared within 60 sec. The 387 mμ peak corresponding to PMS also diminished but it did not return to its original value (when allowance had been made for dilution), even after 15 min. When the experiment was repeated with NADH₂/PMS ratios greater than 1, complete oxidation of NADH₂ took longer and the PMS peak slowly returned to its original value.

DISCUSSION

The data that could be obtained from spectrophotometric measurements provided no direct evidence for the existence of the postulated complex

of NAD and PMSH₂, nor could its behaviour be completely predicted, if its existence were assumed. Some calculations were, however, made which suggested that the breakdown of the complex to free PMSH₂ (eqn. 6) would not be a first-order reaction (whether this is so or not does not affect the validity of eqns. 8 and 9). One possibility, arising from the last experiments reported, is that the complex could be destroyed by collision with NADH₂ molecules, but that it would be stable in the absence of NADH₂. The kinetic data would require that this collision should not result in the oxidation of NADH₂. Another possibility suggested by analysis of the kinetic data is that the complex is destroyed by collision with free PMSH₂.

Either of these two possibilities would satisfactorily explain the results reported by Sowerby & Ottaway (1966). They consistently found that the PMA-mediated oxidation of glutamate by tetrazolium salts did not go to completion as predicted by the redox potentials of the components, although when pseudo-equilibrium had been reached all the components of the reaction mixture were still active. It would be expected that, when the oxidation of glutamate was more than half completed, the concentration of NADH₂ would fall to a very low level. The rate of decomposition of NAD-PMSH₂ would thus be very small and it is not improbable that the rate of oxidation of the remaining glutamate, catalysed by free PMS and free PMSH₂, would be negligibly small. Another point is that the present measurements showed that all the PMSH₂ present is reactive in the presence of cyanide, and Sowerby & Ottaway (1966) found that the reduction of tetrazolium salts by glutamate was much more efficient when cyanide was added.

Although the evidence for the existence of a NAD-PMSH₂ complex is fragmentary, it is well established that PMSH₂ can form binary complexes. Clemo & McIlwain (1934, 1935) described a series of compounds composed of one molecule of a dihydrophenazine and one molecule of a phenazine. They suggested that the molecules were held together by hydrogen-bonding between the ring nitrogen atoms. When *N*-methyl-dihydrophenazine was one component of the system, it was necessary that both it and the phenazine molecule should contain a charged (protonated) nitrogen atom, i.e. complex-formation would only occur in acid solution, and did not occur with free phenazine (Clemo & McIlwain, 1935). Complex-formation between *N*-alkyldihydrophenazines and other nitrogenous bases was also shown to occur, and it is reasonable to suppose that it could also take place with PMSH₂ and a nicotinamide nucleotide. If the same conditions are necessary as for complex-formation between phenazines and *N*-alkyldihydrophenazines, a complex could be formed between NAD and a protonated

form of PMSH₂, i.e. (I). Complex-formation would not, however, be possible between the uncharged (at neutral pH values) nitrogen atom of NADH₂ and PMSH₂, or between NAD and PMS in the neutral pH range, since neither of the species present would have a hydrogen atom attached to a nitrogen atom. Evidence for the existence of structure (I) at neutral pH is discussed below.

The constant k'_1 appearing in eqns. (1) and (5) is equal to $k_1[H^+]$, and it would therefore be expected that the value of k'_1 found at pH 6.8 would be some eight times that found at pH 7.7. This is certainly not so, no matter which of the two values calculated for k'_1 at pH 6.8 is chosen. This suggests strongly that at pH 7–8 the back reaction (2) increases in rate with increase in $[H^+]$, and moreover that both the protonated form (I) and the unprotonated form of PMSH₂ coexist.

A rather puzzling feature of the results is that the value of E'_0 at pH 7 for PMS/PMSH₂ in the presence of 1mM- or 10mM-cyanide, calculated from the values of k'_1 and k_2 in Table 1 and assuming that E'_0 for NAD/NADH₂ is -0.320 v (Clark, 1960), is -0.324 v. This is far lower than the value $+0.080$ v quoted by Dickens & McIlwain (1938), and it seems probable that cyanide reacts with one or more components of the system. It was shown by direct observation that the rate of formation of cyano-NAD was negligible at 1mM-cyanide concentration, although a slow reaction did occur at 10mM-cyanide. There was no spectrophotometric evidence for the formation of a compound of cyanide and PMS, although a reaction between these two compounds in dilute aqueous solution that results in a variety of compounds has been reported (McIlwain, 1937). The formation of an unstable compound between cyanide and PMSH₂ might explain the discrepancy.

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