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# ONE-ELECTRON REACTIONS IN BIOCHEMICAL SYSTEMS AS STUDIED BY PULSE RADIOLYSIS

## IV. OXIDATION OF DIHYDRONICOTINAMIDE-ADENINE DINUCLEOTIDE

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## SUMMARY

1. OH radicals oxidize NADH non-specifically, but weaker one-electron oxidizing agents made by reaction of OH with $Br^-$ or other solutes will oxidize NADH stoichiometrically to the $NAD^•$ semiquinone.

2. The spectrum of the semiquinone, its rate of dimerization and the spectrum and other properties of its dimer are identical to those of the semiquinone formed by reduction of $NAD^+$.

3. The semiquinone made either by oxidation of NADH or reduction of $NAD^+$ reacts with oxygen with $k = 2.0 \cdot 10^9$ $M^{-1} \cdot sec^{-1}$. Enzyme tests show that $NAD^+$ is produced.

4. No reaction could be demonstrated between $O_2^{-•}$ and NADH either in the absence or presence of $Mn^{2+}$. If electron transfer from NADH to $O_2^{-•}$ is possible, the rate constant is $\leqslant 27$ $M^{-1} \cdot sec^{-1}$.

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## INTRODUCTION

Although $NAD^+$ is often considered to operate biochemically through processes in which two electrons are transferred at the same time, the possibility of one-electro-mechanisms cannot be excluded[1]. The peroxidase–oxidase oxidation of NADH in particular may occur by a series of one-electron reactions[2], *via* the semiquinone $NAD^•$. $NAD^+$ can be converted into $NAD^•$ by irradiation[3] or electrolytically[4], but attempts to oxidize NADH to $NAD^•$ with radiolytically produced OH radicals have been only partly successful[3]. Similarly an earlier investigation had shown that OH radicals from Fenton's reagent can oxidize NADH, yielding, ultimately, $NAD^+$, but with a yield of only about 60 %[5]. The reason for these observations may be that OH radicals are unspecific, and can add to double bonds as well as abstract hydrogen atoms from various sites. By converting OH radicals into more selective oxidizing agents it has now been found possible to prepare $NAD^•$ specifically from NADH. Two further reactions which have been proposed[6,7] for the peroxidase–oxidase oxidation have also been studied, *viz.* the reaction of $NAD^•$ with oxygen and the reaction of $O_2^{-•}$ with NADH. As a result of these studies it has been found possible without using enzyme to convert NADH into $NAD^+$ in 100 % yield *via* free-radical reactions which have been proposed to explain peroxidase–oxidase action.

The principal system employed in this work has been an $N_2O$-saturated aqueous solution containing KBr at high concentration. Irradiation of such solutions leads to the formation of complexed bromine atoms ($Br_2^{\bullet -}$)[8–11].

## METHODS

$NAD^+$ and NADH were from Sigma. Water was redistilled from alkaline permanganate under argon. KBr and other chemicals were "AnalaR" grade. Solutions were deaerated where necessary by bubbling with argon from Air Products. $N_2O$ was medical grade from the British Oxygen Co.

The pulse radiolysis experiments were conducted with an 8–14 MeV electron linear accelerator (pulse length 0.005–5 μsec, doses up to 20000 rads) using cells of optical pathlength 1.6 or 2.5 cm, as in previous studies[12–15]. The optical absorption of the hydrated electron at 700 nm was used for dosimetry. For X-irradiation, solutions were contained in 100-ml flasks and were irradiated using a Siemens X-ray set operated at 300 kV. Doses were based on ionization measurements, taking into account the absorption of energy by the KBr in the solution by the photoelectric effect. The X-ray dose rate in the solutions containing 0.1 M KBr was 840 rads/min. In some of the other solutions the dose rate was up to 13 % less.

Absorption spectra were measured after irradiation using a Bausch and Lomb recording spectrophotometer. $NAD^+$ and NADH were determined by standard enzymic methods using yeast alcohol dehydrogenase and, at appropriate pH, ethanol and semicarbazide or acetaldehyde, respectively. Absorbances at 340 nm were measured with a Hilger spectrophotometer.

## RESULTS

Oxidation of NADH by OH was examined by X-irradiating $N_2O$-saturated NADH ($1.7 \cdot 10^{-4}$ M) buffered to pH 8.4. After a dose of about 9000 rads the loss of NADH corresponded to $G = 5.4$, the formation of enzymically inactive substance absorbing at 340 nm to $G = 1.1$ (assuming it is a dimer with $\varepsilon_{\max} = 6500$ (see refs. 3 and 16)) and the formation of $NAD^+$ to $G = 2.2 \pm 1$. In the X-irradiation of buffered NADH ($1.2 \cdot 10^{-4}$ M) to doses up to 15000 rads, the initial yield for loss of NADH was 3.2 in a deaerated solution and 2.2 in an oxygen-saturated solution. In the deaerated solution the initial yield for formation of enzymically inactive product absorbing at 340 nm was 1.8 and for $NAD^+$ was 0.2. In the $O_2$-saturated solution these yields were 0.4 and 1.4, respectively.

Pulse radiolysis of KBr ($10^{-1}$ M) in $N_2O$-saturated aqueous solution gave the species with an absorption maximum at 360 nm, attributed to $Br_2^{\bullet -}$. Under the conditions used (approx. 400 rad/pulse) the species decayed bimolecularly with half-life approx. 50 μsec. In the presence of NADH ($2 \cdot 10^{-4}$ M) the decay at 360 nm was much faster, $t_{1/2}$ approx. 6 μsec, and, concurrently, new changes in absorption were seen. After a few tens of μsec the changes were complete, and no further changes occurred over periods of hundreds of μsec. The change in absorption 80 μsec after pulse radiolysis of this solution is shown in Fig. 1a. The rate of appearance of the new absorption was first-order, $k_1 = 1.7 \cdot 10^5$ sec$^{-1}$, independent of wavelength in the range studied.

In order to find out whether the changes shown in Fig. 1a corresponded to the formation of NAD$^\bullet$, the maximum absorbance and rate of decay at 400 nm from $N_2O$-saturated KBr ($10^{-1}$ M) containing NADH ($2 \cdot 10^{-4}$ M) were compared with those obtained on pulse radiolysis of deaerated HCOONa ($10^{-1}$ M) containing NAD$^+$ ($5 \cdot 10^{-4}$ M).

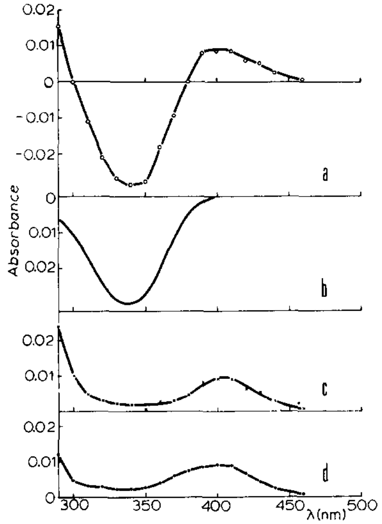


Fig. 1. (a) Absorption changes 80 μsec after pulse radiolysis of $N_2O$-saturated KBr ($10^{-1}$ M) + NADH ($10^{-4}$ M). (b) Absorption of NADH. (c) Difference between (a) (b). (d) Absorption of NAD$^\bullet$ radical[3] obtained by reduction of NAD$^+$ with $e^-_{aq}$ and $CO_2^{\bullet -}$.

In the latter case, $e^-_{aq}$ and $CO_2^{\bullet -}$ have previously been shown[3] to form NAD$^\bullet$, which has an absorption maximum at 400 nm and decays with a second-order rate constant $k = 5.6 \cdot 10^7$ M$^{-1} \times$ sec$^{-1}$. It was found that the maximum absorbance and second-order rate of decay were in fact the same for both solutions, consistent with formation of NAD$^\bullet$ *via* the reaction

$$Br_2^{\bullet -} + \text{NADH} \longrightarrow 2\,Br^- + H^+ + \text{NAD}^\bullet \qquad (1)$$

If Reaction 1 occurs, the transient change in absorbance shown in Fig. 1a would have to be the sum of the gain in NAD$^\bullet$ absorption and the loss in NADH absorption. The part of the change corresponding to loss of NADH can be calculated from known extinction coefficients assuming that at 400 nm the gain in absorbance is due to the formation of NAD$^\bullet$ from NADH. This part of the change is shown in Fig. 1b. Substraction of the absorbances of Fig. 1b from those in Fig. 1a yields Fig. 1c. The spectrum of an equal concentration of NAD$^\bullet$ prepared from NAD$^+$ (see ref. 3) is shown in Fig. 1d. Figs. 1c and 1d are the same except at wavelengths below 320 nm, where the various subtractions are most likely to cause error.

The changes in spectrum are therefore consistent with one-electron oxidation of NADH to NAD$^\bullet$ by $Br_2^{\bullet -}$. The first-order rate constant $k_1 = 1.7 \cdot 10^5$ sec$^{-1}$ leads to a rate constant for Reaction 1 of $9.0 \cdot 10^8$ M$^{-1} \cdot$sec$^{-1}$.

It was also found that addition of NADH to $N_2O$-saturated KCNS ($10^{-1}$ M) or

KI ($10^{-1}$ M) leads to an increase in the rate of decay of the transient absorptions attributed to $(CNS)_2^{\cdot -}$ (ref. 17) or $I_2^{\cdot -}$ (ref. 18). In the presence of NADH ($2 \cdot 10^{-4}$ M) the decays of the species at 500 nm and 370 nm respectively, were first-order, with rate constants $9.3 \cdot 10^4$ and approx. $10^4$ $sec^{-1}$. These values lead to second-order rate constants for Reactions 2 and 3

$$CNS_2^{\cdot -} + NADH \longrightarrow 2\ CNS^- + H^+ + NAD^{\cdot} \qquad (2)$$

$$I_2^{\cdot -} + NADH \longrightarrow 2\ I^- + H^+ + NAD^{\cdot} \qquad (3)$$

of $4.7 \cdot 10^8$ and $\sim 5 \cdot 10^7$ $M^{-1} \cdot sec^{-1}$, respectively.

The stable products of the action of $Br_2^{\cdot -}$ on NADH were examined using X-irradiation. $N_2O$-saturated solutions of NADH ($2 \cdot 10^{-4}$ M) containing KBr ($10^{-1}$ M) were given X-ray doses of up to 20000 rads. The pH of the solutions was initially 7 and increased on irradiation to about 8.5. The intensity of the absorption at 340 nm was found to decrease with dose, but there was no change in spectral shape. On adding acetaldehyde, pyrophosphate buffer (pH 8) and alcohol dehydrogenase to convert NADH into $NAD^+$, it was found that whereas the unirradiated solutions lost their absorption at 340 nm, the irradiated solutions retained a residual absorption. The solutions containing this absorbing species did not exhibit the characteristic fluorescence of NADH. These observations are consistent with the formation from $NADH^{\cdot}$ of the enzymically inactive, non-fluorescent NAD dimer with an absorption peak at 340 nm, which had previously[16] been obtained as a reduction product of $NAD^+$. The conversion was examined quantitatively by X-irradiating a solution of NADH ($10^{-4}$ M) containing KBr ($10^{-1}$ M) and pyrophosphate buffer ($10^{-2}$ M) at pH 8.6. The loss of NADH as a function of dose, and the corresponding formation of NAD dimer and $NAD^+$ is shown for $N_2O$-saturated solutions in Fig. 2. It can be calculated from the figure that the initial yield for loss of NADH is $G = 6.5$, and the initial yields for formation of NAD dimer and $NAD^+$ are $G = 2.5$ and $G = 1.9$, respectively. Since each molecule of dimer contains two units, the total yield of products equals $2 \times 2.5 + 1.9 = 6.9$ NAD units, in reasonable agreement with the loss of NADH. This shows that no products other than dimer and $NAD^+$ are formed in significant yield. By comparing the irradiation of solutions containing NADH ($2 \cdot 10^{-4}$ M) and KBr ($10^{-1}$ M) with and without $H_2O_2$ ($1 \cdot 10^{-4}$ M) it was found that the addition of $H_2O_2$ did not change the yields significantly.

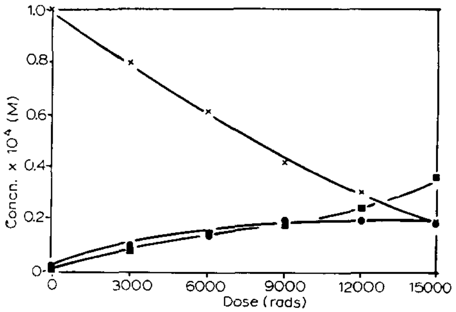


Fig. 2. Variation in concentration of NADH (×), $NAD^+$ (■) and $(NAD)_2$ (●) on X-irradiation of $N_2O$-saturated KBr ($10^{-1}$ M) + NADH ($10^{-4}$ M) + pyrophosphate buffer ($10^{-2}$ M), pH 8.6.

Fig. 2 also shows that the yield for loss of NADH falls off as the irradiation proceeds, as does the production of dimer, but the yield of $NAD^+$ appears to rise. This suggests that as the concentration of dimer rises, dimer may compete with NADH for oxidizing radicals, becoming oxidized further to $NAD^+$. Confirmation that this could happen was sought by first giving an X-ray dose of 70000 rads to an $O_2$-free solution containing $NAD^+$ ($10^{-3}$ M) and HCOONa ($5 \cdot 10^{-2}$ M), and then adding enough KBr to the solution to bring the concentration to 1 M, saturating the solution with $N_2O$ and giving an X-ray dose of 15000 rads. From the concentrations used, and the rate constants for the reactions of $e^-_{aq}$ and OH, the first irradiation[3] would lead to the production of dimer by reduction of $NAD^+$ by $e^-_{aq}$ and $CO_2^{\bullet -}$, and the second irradiation[9-11] should expose the dimer to oxidizing radicals. Tests with alcohol dehydrogenase provided qualitative confirmation of the conversion of dimer to $NAD^+$, although quantitative yields were not obtained.

The action on NADH of the radicals produced by the action of OH on solutes other than $Br^-$ was examined by X-irradiating buffered, $N_2O$-saturated solutions containing NADH ($1.6 \cdot 10^{-4}$ M) and $I^-$ or $CNS^-$ at $10^{-1}$ M. The yields for loss of NADH and formation of dimer and $NAD^+$ were the same within experimental error as in the solutions containing $Br^-$ at the same concentrations. Oxidation by $Tl^{2+}$ ions[19] was examined by X-irradiating a buffered, $O_2$-saturated solution of NADH ($1.6 \cdot 10^{-4}$ M) and $Tl_2SO_4$ ($5 \cdot 10^{-3}$ M). In this solution most of the OH would react with $Tl^+$ giving $Tl^{2+}$, whilst most of the $e^-_{aq}$ would react with oxygen. The yield for loss of NADH after a dose of approx. $10^4$ rads was only 1.35. Previous work[20] had shown that the radical formed from benzoate were not effective in reducing $NAD^+$. It therefore seemed possible that they might oxidize NADH. This possibility was tested by X-irradiating a buffered, deaerated solution containing NADH ($1.6 \cdot 10^{-4}$ M) and sodium benzoate ($10^{-2}$ M). After a dose of $\sim 10^4$ rads the yield for oxidation of NADH was found to be only 0.3.

The effect of $O_2$ on the transient absorption at 400 nm formed from NADH and $NAD^+$ under oxidizing and reducing conditions, respectively, was compared by pulse radiolysis of air-saturated solutions. In this case, $H_2O_2$ ($10^{-2}$ M), rather than $N_2O$, was used to convert $e^-_{aq}$ into OH and hence $Br_2^{\bullet -}$. It was found that the rate of decay at 400 nm on pulse radiolysis of an air-saturated solution containing NADH ($10^{-3}$ M), $H_2O_2$ ($10^{-2}$ M) and KBr ($10^{-1}$ M) was first-order, rate constant $5.0 \cdot 10^5$ $sec^{-1}$. In the case of air-saturated $NAD^+$ ($2 \cdot 10^{-3}$ M), HCOONa ($10^{-1}$ M), the corresponding first-order rate was $5.6 \cdot 10^5$ $sec^{-1}$. It seems possible that both these reactions may correspond to oxidation of $NAD^\bullet$ to $NAD^+$

$$NAD^\bullet + O_2 \longrightarrow NAD^+ + O_2^{-\bullet} \tag{4}$$

Assuming the concentration of oxygen to be $2.7 \cdot 10^{-4}$ M, the mean of the two results gives the rate constant for Reaction 4 to be $2.0 \cdot 10^9$ $M^{-1} \cdot sec^{-1}$, in agreement with the independent determination by WILLSON[21] ($1.9 \cdot 10^9$ $M^{-1} \cdot sec^{-1}$.)

The attack of oxidizing radicals on NADH in the presence of oxygen was also examined by X-irradiating oxygen-saturated NADH ($10^{-4}$ M) containing KBr ($10^{-1}$ M) and pyrophosphate buffer ($10^{-2}$ M) at pH 8.6. The effect of dose on the loss of NADH and the formation of dimer and $NAD^+$ are shown in Fig. 3. The yield for removal of NADH is $G = 3.0$. The yield for formation of $NAD^+$ is also $G = 3.0$. No dimer is formed. The presence of $MnSO_4$ ($10^{-5}$ M) during irradiation did not affect the yield. The attack of reducing radicals on $NAD^+$ in the presence of oxygen was examined by X-irradiating a solution of $NAD^+$ ($2 \cdot 10^{-4}$ M) containing HCOONa ($10^{-1}$ M) and pyrophosphate buffer ($10^{-2}$ M) at pH 8.5. Nitrogen containing 0.4% oxygen was bubbled through the solution during irradiation. No significant absorption developed at 340 nm, the absorbance (1-cm cells) after a dose of 50000 rads being less than 0.02. If oxygen had been absent the absorbance due to dimer formation, on a basis of a yield of reducing radicals of $G = 6$, would have been 1.0.

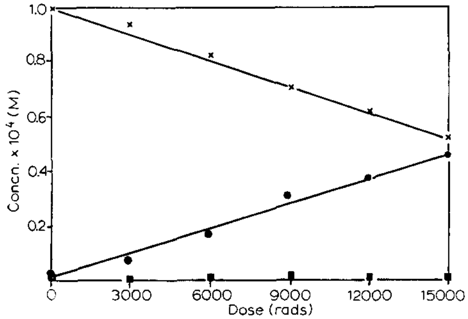


Fig. 3. Variation in concentration of NADH (×), $NAD^+$ (●) and $(NAD)_2$ (■) on X-irradiation of $O_2$-saturated KBr ($10^{-1}$ M) + pyrophosphate buffer ($10^{-2}$ M), pH 8.6.

Since the attack of oxidizing radicals on NADH in the presence of $O_2$ gives rise to $NAD^+$ in good yield, it seemed possible that traces of oxygen might be a partial cause of the formation of $NAD^+$ in the experiments whose results are given in Fig. 2. Several different checks were made of this possibility, and it was shown not to apply.

## DISCUSSION

Irradiation of $N_2O$-saturated NADH ($1.7 \cdot 10^{-4}$ M) in the absence of $Br^-$ would lead to oxidation of NADH by OH (total yield $G = 5.25$) and to a much smaller oxidation or reduction by H ($G = 0.55$). If the sole product of OH oxidation were $NAD^\bullet$, and other reactions could be neglected, $NAD^\bullet$ would form dimer with $G = 2.6$. The observed yield of only $G = 1.1$ shows that other reactions are occurring to a large extent, consistent with previous pulse radiolysis observations[3]. In an $O_2$-saturated solution of NADH ($1.2 \cdot 10^{-4}$ M) both $e^-_{aq}$ and H would react with $O_2$, giving, at the pH of the present experiments, $O_2^-$

$$e^-_{aq} + O_2 \longrightarrow O_2^{-\bullet} \qquad (5)$$

$$H + O_2 \longrightarrow H^+ + O_2^{-\bullet} \qquad (6)$$

but OH would still react with NADH. Little or no dimer is formed, presumably because $NAD^\bullet$ reacts with oxygen, possibly according to Reaction 4. Even in the absence of any reaction between $O_2^{-\bullet}$ and NADH these reactions would lead to the formation of $NAD^+$ with $G = 2.65$. The observed yield of $G = 1.4$ (53%) is similar to that observed with Fenton's reagent (60%)[5] and confirms still further that OH attacks NADH unspecifically.

Conversion of OH into complexed bromine atoms (represented as $Br_2^{\bullet -}$) enables NADH to be converted into a semiquinone, having the same rate of dimerisation

($5.6 \cdot 10^7$ $M^{-1} \cdot sec^{-1}$) and absorption spectrum (Fig. 1) as the semiquinone made by reduction of $NAD^+$ (ref. 3). The high rate constant for the formation of the radical ($9.0 \cdot 10^8$ $M^{-1} \cdot sec^{-1}$) is consistent with the reaction, being an electron transfer from NADH to the bromine atom. Theoretical calculations[22] have indicated that the radical formed by adding an electron to $NAD^+$ would differ from that made by removing an electron from NADH. The similarity of properties observed here suggests that the initial oxidized form of NADH may rapidly lose a proton

$$NADH^{+\cdot} \longrightarrow NAD^{\cdot} + H^+ \qquad (7)$$

or alternatively, that the semiquinone formed by reduction of $NAD^+$ rapidly protonates

$$NAD^{\cdot} \xrightarrow{H^+ \text{ or } H_2O} NADH^{+\cdot} \qquad (8)$$

ESR studies of pyridinyl radicals have been interpreted[23] in terms of an unprotonated form. This would suggest that the nicotinamide grouping of the stable form of the NAD radical is likewise unprotonated. If the radical is represented as $NAD^{\cdot}$ its formation in the overall Reaction 1 is essentially by electron transfer

$$Br^{\cdot-} + NADH \longrightarrow 2\ Br^- + NADH^+ \qquad (9)$$

followed by Reaction 7. $(CNS)_2^{\cdot-}$ and $I_2^{\cdot-}$ probably react similarly. Rate constants for these reactions are shown in Table I, together with other rate constants determined.

The yield for loss of NADH in the X-irradiation of $N_2O$-saturated solutions of NADH containing KBr, KCNS or KI at a concentration of $10^{-1}$ M, $G = 6.5$, is greater than would be expected from the yield of radicals in dilute solutions[24]. Presumably the high concentration of salt interferes with the radiation primary act, leading to higher yields. High yields were also noted in the radiolytic reduction of $NAD^+$ in the presence of ethanol at 0.5 M ($G = 6.6$) (see ref. 16) and of methylene blue in the presence of formate at 0.1 M ($G = 3.4$, *i.e.* 6.8 equivalents) (see ref. 12), no doubt for the

TABLE I

RATE CONSTANTS

| *Reaction* | *Rate constant* ($M^{-1} \cdot sec^{-1}$) |
|---|---|
| $Br_2^{\cdot-} + NADH \longrightarrow 2\ Br^- + NADH^+$ <br> $\downarrow$ <br> $NAD^{\cdot} + H^+$ | $9.0 \cdot 10^8$ |
| $CNS_2^{\cdot-} + NADH \longrightarrow 2\ CNS^- + NADH^+$ <br> $\downarrow$ <br> $NAD^{\cdot} + H^+$ | $4.7 \cdot 10^8$ |
| $I_2^{\cdot-} + NADH \longrightarrow 2\ I^- + NADH^+$ <br> $\downarrow$ <br> $NAD^{\cdot} + H^+$ | $\sim 5 \cdot 10^7$ |
| $NAD^{\cdot} + O_2 \longrightarrow NAD^+ + O_2^{-\cdot}$ | $2.0 \cdot 10^9$ |
| $O_2^{-\cdot} + NADH \xrightarrow{+H^+} H_2O_2 + NAD^{\cdot}$ | $\leqslant 27$ |

same reason. The high yield provides further confirmation that NAD$^\bullet$ radicals disappear by dimerisation rather than disproportionation

$$2\ \text{NAD}^\bullet \longrightarrow (\text{NAD})_2 \tag{10}$$

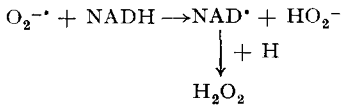
since disproportionation would diminish the net loss of NADH to $G \sim 3$, as with methylene blue. There is thus no evidence for the view[6,7] that NAD$^\bullet$ radicals can disproportionate.

The role of the small yield of H atoms is uncertain. One possibility is that they abstract H from NADH, yielding NAD$^\bullet$ and $H_2$. However, in no event can they account for more than 10 % of the changes observed.

If NADH is lost to give NAD$^\bullet$ with $G = 6.5$, then Reaction 10 should lead to dimer being formed with $G = 3.25$. The observed yield of $G = 2.5$, and the concurrent yield for formation of NAD$^+$ of $G$ about 1.9, may be due to further attack on dimer, as confirmed by the effect of prolonged irradiation (Fig. 2) and the effect of $Br_2^{\bullet -}$ on dimer formed by reduction of NAD$^+$.

The effect of $O_2$ on the rate of disappearance of NAD$^\bullet$ shows that NAD$^\bullet$ reacts rapidly with $O_2$. The enzyme experiments (Fig. 3) show that NAD$^+$ is produced, rather than a product such as an organic peroxide. The reaction may therefore be written with confidence as the electron transfer Reaction 4, in full confirmation of the proposal of YOKOTA AND YAMAZAKI[6]. The experiments of Fig. 3 show that conversion of NADH into NAD$^+$ is stoichiometric when $O_2$ is present (yield 100 %). This is consistent with the high rate constant for Reaction 4 which ensures that every NAD$^\bullet$ gives NAD$^+$. It should be noted that there is no uncertainty about the role of H atoms in this experiment, since owing to the high rate constant for their reaction with $O_2$ ($k = 2 \cdot 10^{10}\ M^{-1} \cdot sec^{-1}$) all H atoms will react according to Eqn. 6. The complete protection afforded by 0.4 % $O_2$ to the reduction of NAD$^+$ in the presence of formate is further proof of the efficiency of Reaction 4.

The experiments of Fig. 3 enable a limit to be calculated for the rate constant of the reaction

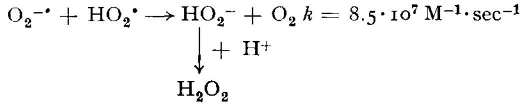


$$\begin{array}{l} O_2^{-\bullet} + \text{NADH} \longrightarrow \text{NAD}^\bullet + HO_2^- \\ \qquad\qquad\qquad\qquad\qquad \downarrow + H \\ \qquad\qquad\qquad\qquad\quad H_2O_2 \end{array}$$

(11)

$O_2^{-\bullet}$ is produced in the solution in a yield of about $G = 6$, so the rate of formation of $O_2^{-\bullet}$ is $8.7 \cdot 10^{-8}\ M \cdot sec^{-1}$. $O_2^{-\bullet}$ is lost *via* the reactions[25]

$$O_2^{-\bullet} + H^+ \leftrightarrows HO_2^\bullet \tag{12}$$



$$\begin{array}{l} O_2^{-\bullet} + HO_2^\bullet \longrightarrow HO_2^- + O_2\ k = 8.5 \cdot 10^7\ M^{-1} \cdot sec^{-1} \\ \qquad\qquad\qquad \downarrow + H^+ \\ \qquad\qquad\qquad H_2O_2 \end{array}$$

(13)

as well as possibly by Reaction 11. The p$K$ of the $HO_2^\bullet$ radical is 4.88. Recombination of $O_2^{-\bullet}$ proceeds at a negligible rate, whilst recombination of $HO_2^\bullet$ can be neglected at pH 8.6. Under certain circumstances $O_2^{-\bullet}$ can also disappear in a first-order reaction with impurities, but such a reaction could be neglected in the present instance since any impurities might be expected to be consumed rapidly after a few rads had been given. The rate of loss of $O_2^{-\bullet}$ by Reactions 12 and 13 is equal to $8.5 \cdot 10^{3,28}\ [O_2^{-\bullet}]^2$

$M \cdot sec^{-1}$. Reaction 11 would lead to a chain reaction consuming NADH, since $NAD^{\bullet}$ produced would give more $O_2^{-\bullet}$ by the fast Reaction 4. As there is no sign of a chain reaction it is quite safe to assume that the proportion of $O_2^{-\bullet}$ reacting according to Eqn. 11 is much less than 10 % of those disappearing by Eqns. 12 and 13. Since the rate of formation of $O_2^{-\bullet}$ must equal the rate of disappearance, we have

$$8.7 \cdot 10^{-8} \simeq 8.5 \cdot 10^{3.28} [O_2^{-\bullet}]^2 \qquad (14)$$

so that $[O_2^{-\bullet}] \simeq 10^{-5.6}$. Since the proportion of $O_2^{-\bullet}$ reacting according to Eqn. 11 is less than 10 %, we have

$$k \times 10^{-4} \times 10^{-5.6} \leqslant 0.1 \times 8.5 \times 10^{3.28} \times 10^{-11.2}$$

where $k$ is the rate constant for Reaction 11, from which we find $k \leqslant 27\ M^{-1} \cdot sec^{-1}$. In the solution containing $MnSO_4$, $O_2^{-\bullet}$ has the opportunity of oxidizing $Mn^{2+}$ (concentration $10^{-5}$ M) to give $Mn^{3+}$ rather than undergoing the slow sequence represented by Eqns. 12 and 13. $Mn^{3+}$ might be expected to oxidize NADH as in the mechanism proposed by Yamazaki and Piette[26] for the promotive effect of $Mn^{2+}$ on the peroxidase–oxidase reaction. The absence of any effect of manganese in the experiments reported here could be explained if $O_2^{-\bullet}$ reacts with $Mn^{2+}$ with a rate constant less than about 300 $M^{-1} \cdot sec^{-1}$, and/or if $Mn^{3+}$ does not undergo a fast electron-transfer reaction with NADH.

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