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Oxidation of Nicotinamide Adenine Dinucleotide by Hydroperoxyl Radical. A Flash Photolysis Study

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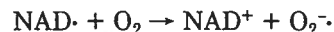
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The one-electron oxidation of nicotinamide adenine dinucleotide (NADH) by hydroperoxyl radical was studied over a broad range of reagent concentrations and pH values by the method of flash photolysis. Of the acid-base forms of the radical, $\text{HO}_2\cdot$ and $\text{O}_2^-\cdot$, only the first one is an active oxidant. The rate constant of the reaction $\text{HO}_2\cdot + \text{NADH} \rightarrow \text{H}_2\text{O}_2 + \text{NAD}\cdot$ is equal to $(1.8 \pm 0.2) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ at 20 °C. The temperature dependencies of the reaction rate at different pH values gave the activation energy of this reaction, $E_a = 5.5 \pm 0.5 \text{ kcal}$, as well as the standard enthalpy of the equilibrium $\text{HO}_2\cdot \rightleftharpoons \text{O}_2^-\cdot + \text{H}^+$, $\Delta H^\circ = 2.7 \pm 0.7 \text{ kcal}$.

Introduction

The mechanism of reaction of the coenzyme or substrate molecule, nicotinamide adenine dinucleotide (NADH), and its oxidized form NAD^+ have attracted widespread attention. Thus, in elegant stereochemical experiments, it was demonstrated that when NAD^+ acts as a coenzyme for dehydrogenases¹ a hydride ion is transferred stereospecifically from substrate to NAD^+ . However, as a substrate in peroxidase reactions,^{2,3} NADH is oxidized in a one-

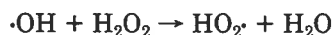
electron step to form the free radical $\text{NAD}\cdot$ which has the ability to undergo the reaction



The destruction of $\text{O}_2^-\cdot$, the superoxide anion, by superoxide dismutase was demonstrated in 1969.⁴ Since that time considerable controversy has arisen over the importance of $\text{O}_2^-\cdot$ as an oxidizing agent.^{5,6} This paper sheds further light on the relative importance of reactions of

superoxide anion and its protonated form, the hydroperoxyl radical HO_2^\cdot , using NADH as the primary reducing agent.

The flash photolysis of H_2O_2 was used as a source of O_2^\cdot or HO_2^\cdot . Irradiation of H_2O_2 with ultraviolet light leads to O-O bond rupture producing $\cdot\text{OH}$ radicals with a quantum yield of 0.82.⁷ Hydroxyl radicals react with H_2O_2 with a rate constant of $1.7 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ to generate hydroperoxyl radicals:⁸



In our experiments $[\text{H}_2\text{O}_2]_0$ ranged from 0.15 to 0.9 M and $[\text{NADH}]_0$ from 1×10^{-5} to $6 \times 10^{-5} \text{ M}$. Therefore, virtually every $\cdot\text{OH}$ was converted into HO_2^\cdot . Even if the reaction of $\cdot\text{OH}$ with NADH is diffusion controlled, its importance is nullified by keeping $[\text{H}_2\text{O}_2]/[\text{NADH}] > 10^3$.

Experimental Section

Materials and Methods. All the chemicals used were of reagent grade. Hydrogen peroxide, 30%, was from Fisher Scientific Co. and NADH, grade III, was obtained from Sigma. Buffer solutions for pH regions 4.2–5.3 and 6.2–7.5, respectively, were acetic acid–sodium acetate and potassium dihydrogen phosphate–sodium hydroxide. The ionic strength of the reaction solutions was 0.03 M. H_2O_2 and NADH concentrations were determined spectrophotometrically by using known molar absorptivities $\epsilon_{300} = 1 \text{ M}^{-1} \text{ cm}^{-1}$ and $\epsilon_{253.7} = 19.6 \text{ M}^{-1} \text{ cm}^{-1}$ for H_2O_2 ⁹ and $\epsilon_{340} = 6.2 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, $\epsilon_{366} = 3.3 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ for NADH.¹⁰

Experiments were performed on rapid reaction analyzer RA-601, obtained from Union Giken Co., 4018-1, Tsuda, Hirakata, Osaka 573-01, Japan, equipped with the flash photolysis mode and by using monochromatic flash light with $\lambda = 330 \text{ nm}$. Path lengths of the analyzing and photolyzing light were 1 cm each. Changes of absorbance at $\lambda = 340$ and 366 nm vs. time after a flash were recorded. The ratio of absorbance changes at both wavelengths was equal to the ratio of absorbances of NADH itself, showing that no product with absorption maxima at 340 nm was formed and, in agreement with Land and Swallow,¹¹ NAD-radical recombination reactions did not play any noticeable role under our experimental conditions.

The concentration of peroxide radicals formed per flash was measured by using the O_2^\cdot scavenger $\text{C}(\text{NO}_2)_4$.¹² The product of the one-electron reduction of tetranitromethane by O_2^\cdot is the nitroform anion $\text{C}(\text{NO}_2)_3^-$ with a molar absorptivity at 350 nm¹³ of $1.54 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. A solution containing H_2O_2 and $\text{C}(\text{NO}_2)_4$, at a pH low enough ($\text{pH} \approx 3$) for suppressing their direct reaction in the dark, was exposed to flash light and the absorbance change at $\lambda = 350 \text{ nm}$ was measured. Since the stoichiometry of the reaction $\text{O}_2^\cdot + \text{C}(\text{NO}_2)_4$ is 1:1¹⁴ the HO_2^\cdot (or O_2^\cdot) initial concentration after the flash would be measured. This value was compared with that obtained by using the expression $[\text{HO}_2^\cdot]_0 = 2.3/\epsilon_{330}[\text{H}_2\text{O}_2]\psi$, where ϵ_{330} is the molar absorptivity of H_2O_2 at the wavelength of the flash light (330 nm), l the optical path length in the direction of flash lights beam, ψ the quantum yield of H_2O_2 photolysis,⁷ and I_0 the number of quanta per unit of volume per flash, measured by the standard Hatchard and Parker's procedure.¹⁵ Both methods gave consistent results, the first one being more accurate and convenient. Calibration plots, i.e., dependencies of $[\text{HO}_2^\cdot]_0$ on $[\text{H}_2\text{O}_2]_0$ and voltage applied to the flash lamp are shown in Figure 1. The molar absorptivity of NADH at 330 nm is $5.8 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ at 20 °C and that of H_2O_2 is $0.11 \text{ M}^{-1} \text{ cm}^{-1}$. Therefore under some of our experimental conditions more light is being absorbed by NADH than by H_2O_2 . The NADH is known to fluoresce strongly¹⁶ with a lifetime of 4.5 ns.¹⁷ To the

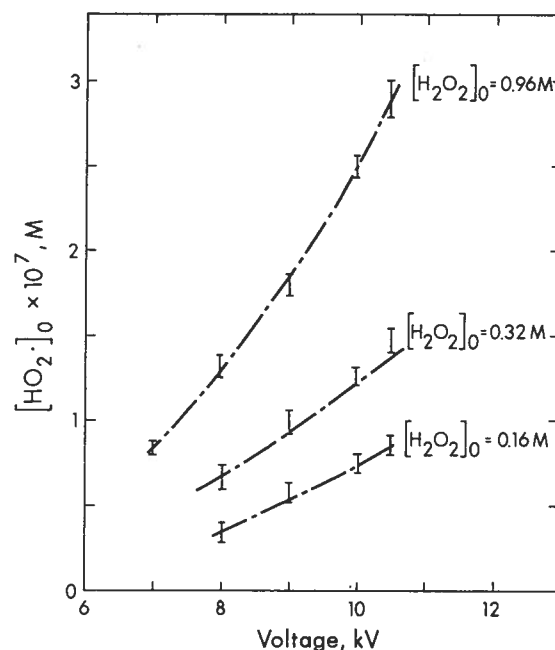


Figure 1. Calibration plot. The initial concentrations of superoxide radicals formed at different $[\text{H}_2\text{O}_2]$ vs. voltages applied to flash lamp.

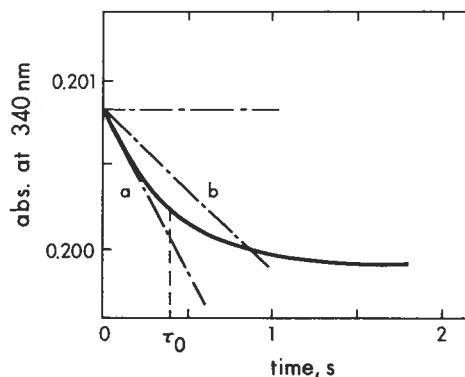


Figure 2. Absorbance change at $\lambda = 340 \text{ nm}$ vs. time after flash in the system $\text{NADH} + \text{H}_2\text{O}_2$. The procedure for obtaining the value of τ_0 is schematically shown. The broken line (a) corresponds to the initial slope of the kinetic curve, line (b) to the slope one-half that of (a). The value of τ_0 corresponds to the point of maximum distance of the kinetic curve from line (b) inside the angle between (a) and (b). At this point $(d[\text{NADH}]/dt)_{\tau_0} = 1/2 (d[\text{NADH}]/dt)_0$. $[\text{NADH}]_0 = 4.1 \times 10^{-5} \text{ M}$, $[\text{H}_2\text{O}_2]_0 = 0.32 \text{ M}$, 10.5 kV, pH 5.6, $t = 20^\circ \text{C}$.

best of our knowledge fluorescence is the sole result of the light absorption process by NADH. Flash experiments in the presence of NADH but absence of H_2O_2 led to recordings of absorbance vs. time at 340 nm which were constant within experimental error. Under the worst experimental conditions the signal-to-noise ratio was estimated to be 3, under the best 10.

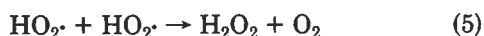
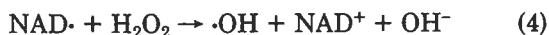
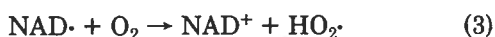
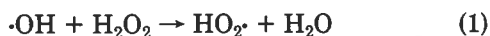
Results and Discussion

A set of flash photolysis experiments was performed at different concentrations of H_2O_2 , NADH, and H^+ . A typical run is illustrated by Figure 2. A table of results is available as supplementary material (see paragraph at end of text regarding supplementary material). The decrease of absorption at 340 nm corresponded to consumption of NADH in solution due to the photoinduced reaction. It could not be due to the action of primary hydroxyl radicals as was explained above. The dark interaction of NADH with H_2O_2 at $\text{pH} > 4$ was sufficiently slow to be neglected compared to the light induced reaction. From the following consideration one can see that

the interpretation of the entire trace as illustrated in Figure 2 is not simple. However, there is a simple interpretation of the initial slope and a quantity τ_0 , defined below.

The $\text{NAD}\cdot$ radical, product of the one-electron oxidation of NADH, is known as an extremely strong reducing agent, and in the presence of molecular oxygen dissolved in solution, it is oxidized by the reaction $\text{NAD}\cdot + \text{O}_2 \rightarrow \text{NAD}^+ + \text{O}_2\cdot^-$ with the rate constant equal to $2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$.¹¹ In addition H_2O_2 must also be a good oxidant for $\text{NAD}\cdot$ as was suggested elsewhere.³ Most of the hydroxyl radicals, produced by the reaction $\text{NAD}\cdot + \text{H}_2\text{O}_2 \rightarrow \text{NAD}^+ + \cdot\text{OH} + \text{OH}^-$, have to react with H_2O_2 under our experimental conditions (see above) giving $\text{HO}_2\cdot$ (or $\text{O}_2\cdot^-$).

Taking into consideration the recombination of $\text{HO}_2\cdot$ radicals, we obtain the following set of reactions ($\text{HO}_2\cdot$ here is used to designate whichever form of the superoxide radical which is reactive):



The rate constants characterizing $\text{NAD}\cdot + \text{O}_2$ and $\cdot\text{OH} + \text{H}_2\text{O}_2$ reactions are several orders of magnitude larger than that expected for $\text{HO}_2\cdot + \text{NADH}$ ^{3,11} ($k \leq 10^5 \text{ M}^{-1} \text{ s}^{-1}$ at $\text{pH} \geq 5.6$). Therefore $[\cdot\text{OH}]$ and $[\text{NAD}\cdot]$ are much smaller than $[\text{HO}_2\cdot]$, so reaction 5 is the only termination step of importance. Using the quasi-steady-state approximation,¹⁸ one obtains

$$d[\text{NAD}\cdot]/dt = 0 = k_2[\text{HO}_2\cdot][\text{NADH}] - k_3[\text{O}_2][\text{NAD}\cdot] - k_4[\text{H}_2\text{O}_2][\text{NAD}\cdot]$$

$$d[\cdot\text{OH}]/dt = 0 = k_4[\text{H}_2\text{O}_2][\text{NAD}\cdot] - k_1[\cdot\text{OH}][\text{H}_2\text{O}_2]$$

$$d[\text{HO}_2\cdot]/dt = k_3[\text{O}_2][\text{NAD}\cdot] + k_1[\cdot\text{OH}][\text{H}_2\text{O}_2] - 2k_5[\text{HO}_2\cdot]^2$$

Adding these equations, we have

$$d[\text{HO}_2\cdot]/dt = -2k_5[\text{HO}_2\cdot]^2$$

which upon integration yields

$$[\text{HO}_2\cdot] = \frac{[\text{HO}_2\cdot]_0}{1 + 2k_5[\text{HO}_2\cdot]_0 t}$$

where $[\text{HO}_2\cdot]_0$ is the initial concentration of $\text{HO}_2\cdot$ radicals, which can be obtained from the data of Figure 1.

Consumption of NADH corresponds to reaction 2 of the above scheme and is described by

$$\frac{d[\text{NADH}]}{dt} = k_2[\text{NADH}][\text{HO}_2\cdot] = k_2[\text{NADH}] \frac{[\text{HO}_2\cdot]_0}{1 + 2k_5[\text{HO}_2\cdot]_0 t} \quad (6)$$

The overall change of $[\text{NADH}]$ during a single experiment corresponded to only a few percent of the total $[\text{NADH}]$ so the latter can be considered as a constant in the interpretation of experimental data (Figure 2). The initial rate of NADH disappearance is given by a reduced form of eq 6 ($t = 0$)

$$-d[\text{NADH}]/dt|_{\text{initial}} = k_2[\text{NADH}][\text{HO}_2\cdot]_0 \quad (7)$$

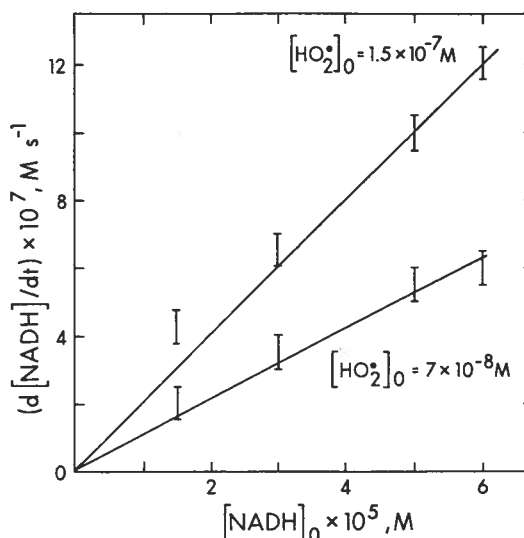


Figure 3. Initial rate of NADH oxidation plotted against $[\text{NADH}]_0$ at two different $[\text{HO}_2\cdot]_0$; $\text{pH} 4.9$, $t = 20^\circ \text{C}$.

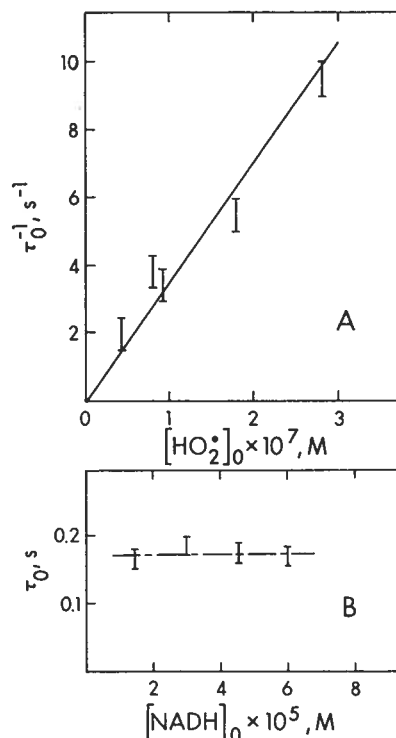


Figure 4. (A) The dependence of τ_0^{-1} on $[\text{HO}_2\cdot]_0$; $[\text{NADH}]_0 = 3.8 \times 10^{-5} \text{ M}$, $\text{pH} 4.9$, 20°C . (B) τ_0 plotted vs. $[\text{NADH}]$ at constant $[\text{HO}_2\cdot]_0$; $[\text{HO}_2\cdot]_0 = 1.5 \times 10^{-7} \text{ M}$, $\text{pH} 4.9$, 20°C .

so that k_2 can be calculated from Figure 3. The time, τ_0 , in which the rate of disappearance of NADH is reduced by a factor of 2 from its initial value is given by

$$\tau_0 = \frac{1}{2k_5[\text{HO}_2\cdot]_0} \quad (8)$$

Thus τ_0 must not depend on $[\text{NADH}]$ and has to be inversely proportional to $[\text{HO}_2\cdot]_0$. Our results in Figure 4 are in agreement with both statements, and the value of k_5 is readily obtained from Figure 4A. Values of k_2 and k_5 obtained at different pH values are listed in Table I.

It is known that the $\text{HO}_2\cdot$ recombination process (eq 5) is pH dependent because of acid-base equilibrium:

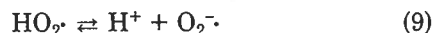
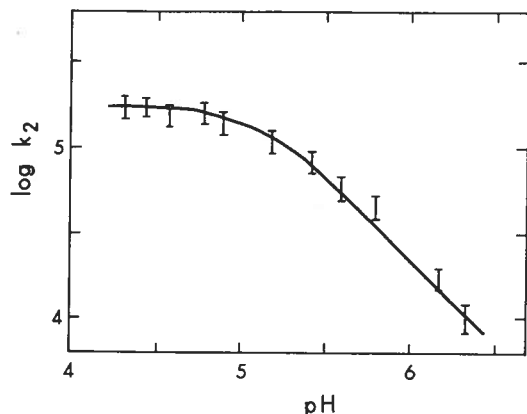


TABLE I: Values of k_2 , k_5 , and $k_{5, \text{calcd}}$ at Different pH Values ($t = 20^\circ \text{C}$)

| pH | $10^4 k_2$ $\text{M}^{-1} \text{s}^{-1}$ | $10^6 k_5$ $\text{M}^{-1} \text{s}^{-1}$ | $10^6 k_{5, \text{calcd}}$ $\text{M}^{-1} \text{s}^{-1}$ |
|------|---|---|---|
| 4.4 | 17 ± 2 | 15 ± 3 | 16.5 |
| 4.6 | 15 ± 2 | 17 ± 3 | 20.1 |
| 4.9 | 13 ± 2 | 22 ± 3 | 21.9 |
| 5.25 | 11 ± 1.5 | 19 ± 2 | 18.5 |
| 5.6 | 8.5 ± 1.5 | 14 ± 2 | 13.7 |
| 5.8 | 5.5 ± 1.5 | 7.5 ± 1.5 | 8.4 |
| 6.25 | 2 ± 1 | 3 ± 0.5 | 3.5 |

Figure 5. The dependence of $\log k_2$ on pH at 20°C , where k_2 is the apparent rate constant for the reaction of hydroperoxyl radical with NADH. The results indicate that $\text{O}_2^{\cdot -}$ is unreactive.

with $\text{p}K_9 = 4.88$.¹⁹ There are three possible ways of recombination

- $\text{HO}_2^{\cdot} + \text{HO}_2^{\cdot} \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$
- $\text{HO}_2^{\cdot} + \text{O}_2^{\cdot -} \rightarrow \text{HO}_2^- + \text{O}_2$
- $\text{O}_2^{\cdot -} + \text{O}_2^{\cdot -} \rightarrow \text{O}_2^{2-} + \text{O}_2$

with rate constants $k_a = 0.76 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, $k_b = 8.7 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, and $k_c \leq 0.3 \text{ M}^{-1} \text{ s}^{-1}$.^{19,20} At the pH's of our experiments (c) is insignificant and the rate of superoxide radical recombination is given by

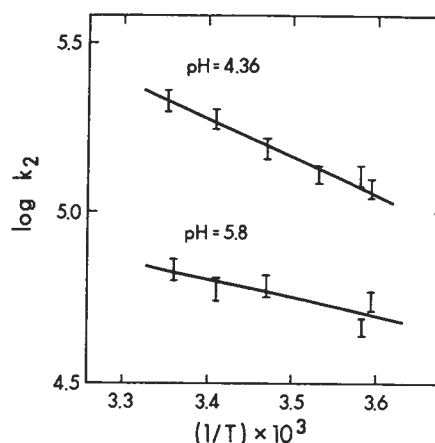
$$-\frac{d[\text{HO}_2^{\cdot}]}{dt} = 2 \frac{\left[k_a + k_b \frac{K_9}{[\text{H}^+]} \right]}{\left[1 + \frac{K_9}{[\text{H}^+]} \right]^2} [\text{HO}_2^{\cdot}]^2$$

where $[\text{HO}_2^{\cdot}]$ designates total radical concentration, in both acidic and basic forms, and

$$k_5 = \left(k_a + k_b \frac{K_9}{[\text{H}^+]} \right) / \left[1 + \frac{K_9}{[\text{H}^+]} \right]^2 \quad (10)$$

Using formula (10) and known values of k_a , k_b and K_9 , one can calculate k_5 for various pH values (see $k_{5, \text{calcd}}$ in the last column of Table I). Calculated and experimentally obtained values of k_5 practically coincided which is additional support for the above mechanism outlined in eq 1-5.

Figure 5 is a semilogarithmic plot of k_2 vs. pH. One can see that the rate constant is independent of pH at $\text{pH} \leq 5$ and is proportional to $[\text{H}^+]$ at higher pH. Since there are no acidic groups with $\text{p}K$'s close to 5 in the NADH molecule, the $\text{p}K$ for the acidic dissociation of HO_2^{\cdot} (4.88)¹⁹ is indicated by the results. Therefore the acidic form of the superoxide radical only is active in the oxidation of NADH. The elementary rate constant of the $\text{HO}_2^{\cdot} + \text{NADH}$ reaction, obtained from Figure 5, is equal to $(1.8$

Figure 6. The Arrhenius plots of $\log k_2$ vs. reciprocal temperature at different pH values.

$\pm 0.2) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. It is interesting to note that the effective rate constant for NADH oxidation by superoxide radical at pH 5.6, predicted by Yokota and Yamazaki from enzymatic studies,³ $6 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, is quite close to our value of $(8.5 \pm 1.5) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$. Similarly, the value of Land and Swallow,¹¹ $\leq 27 \text{ M}^{-1} \text{ s}^{-1}$ at pH 8.4, is roughly in accord with our results since $[\text{HO}_2^{\cdot}]$ is small at higher pH. The fact that only the HO_2^{\cdot} form of superoxide radical is active as oxidant can be explained by the better thermodynamic stability of H_2O_2 , the product of hydrogen atom addition to HO_2^{\cdot} , compared to HO_2^- , the product of $\text{O}_2^{\cdot -}$ reduction.

The temperature dependence of k_2 at different pH values yields the following information. Since NADH reacts with the acidic form of superoxide radicals only, the apparent second-order rate constant is given by the expression

$$k_{2, \text{app}} = \frac{k_2' [\text{H}^+]}{K_9 + [\text{H}^+]}$$

where k_2' is the elementary rate constant of the $\text{NADH} + \text{HO}_2^{\cdot}$ reaction and K_9 is defined above.

At $\text{pH} < \text{p}K_9$, $k_{2, \text{app}} = k_2'$ and $E_{\text{app}} = E'$. At $\text{pH} > \text{p}K_9$, $k_{2, \text{app}} = k_2' [\text{H}^+] / K_9$ and $E_{\text{app}} = E' - \Delta H_9^\circ$, where E designates activation energies and ΔH_9° is the standard enthalpy of equilibrium 9. Figure 6 shows standard Arrhenius plots of $\log k_{2, \text{app}}$ vs. $1/T$ at pH 4.36 and 5.8. From the slopes of these straight lines E' and ΔH_9° are estimated to be (5.5 ± 0.5) and $(2.7 \pm 0.7) \text{ kcal}$. Considering the important role of the molecular oxygen reduction products in various processes, their kinetic and thermodynamic properties²¹⁻²³ may be of some interest.

In conclusion, our major finding is that although superoxide anion is unreactive as an oxidant in the presence of NADH, its protonated form, the hydroperoxyl radical, is highly reactive. This finding, if it is also valid for other systems, may help eliminate much of the controversy which currently exists in the literature concerning the oxidizing ability and hence ease of reduction of $\text{O}_2^{\cdot -}$ and HO_2^{\cdot} .

Supplementary Material Available: Tables A and B containing concentration and pH dependencies of τ_0 and $-[d(\text{NADH})/dt]$, and temperature dependency data (2 pages). Ordering information is available on any current masthead page.

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