Lardy, H. A., and Phillips, P. H. (1945), *Arch. Biochem. 6*, 53.
Lardy, H. A., and Wellman, H. (1953), *J. Biol. Chem. 201*, 357.
Makman, R. S., and Sutherland, E. W. (1965), *J. Biol. Chem. 240*, 1309.
Manchester, K. L. (1970), *Biochem. J. 117*, 39P.
Mann, T. (1964), The Biochemistry of Semen and of the Male Reproductive Tract, New York, N. Y., Wiley.
Morton, B. E., and Lardy, H. A. (1967a), *Biochemistry 6*, 43.
Morton, B. E., and Lardy, H. A. (1967b), *Exp. Cell Res. 46*, 511.
Nakano, J., Oliver, R., and Ishii, T. (1970), *Pharmacology 3*, 273.
Namm, D. H., Mayer, S. E., and Maltbie, M. (1968), *Mol. Pharmacol. 4*, 522.
O'Dea, R. F., Haddox, M. K., and Goldberg, N. D. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 473.
Parisi, M., and Bentley, P. J. (1970), *J. Endocrinol. 48*, 117.
Pautard, F. G. E. (1962), *in* Spermatozoan Motility, Bishop, D. W., Ed., Washington, D. C., American Association for the Advancement of Science, p 189.
Pento, J. T., Cenedella, R. J., and Inskeep, E. K. (1970), *J. Animal Sci. 30*, 409.
Price, T. D., Ashman, D. F., and Melicow, M. M. (1967), *Biochim. Biophys. Acta 138*, 452.
Rasmussen, H. (1970), *Science 170*, 404.
Robison, G. A., Butcher, R. W., Oye, I., Morgan, H. E., and Sutherland, E. W. (1965), *Mol. Pharmacol. 1*, 168.
Robison, G. A., Butcher, R. W., and Sutherland, E. W. (1968), *in* Annual Review of Biochemistry, Vol. 37, Boyer, P. D., Ed., Palo Alto, Calif., Annual Reviews Inc., p 149.
Salisbury, G. W. (1962), *in* Spermatozoan Motility, Bishop, D. W., Ed., Washington, D. C., American Association for the Advancement of Science, p 59.
Solomon, S., Brush, J. S., and Kitabchi, A. E. (1970), *Science 169*, 387.
Sutherland, E. W., Robison, G. A., and Butcher, R. W. (1968), *Circulation 37*, 279.
Triner, L., Vulliemoz, Y., Schwartz, I., and Nahas, G. G. (1970), *Biochem. Biophys. Res. Commun. 40*, 64.
Voglmayer, K. J., White, I. G., and Quinn, P. J. (1969), *Biol. Reprod. 1*, 121.
Yanagimachi, R. (1969), *J. Reprod. Fert. 18*, 275.
Yanagimachi, R. (1970), *J. Reprod. Fert. 23*, 193.

Autoxidation of Reduced Pyridine Coenzymes and of Their Models Promoted by *N,N,N′,N′*-Tetramethyl-*p*-phenylenediamine*

E. J. H. Bechara† and G. Cilento‡

ABSTRACT: In the presence of *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine as a free-radical source, the reduced pyridine coenzymes and their models autoxidize by a free-radical mechanism (HO_2 chain). The main products are the pyridinium form of the substrate and hydrogen peroxide. In accord with the HO_2 chain process are: (i) the first-order dependence of the rate on the dihydronicotinamide concentration and the half-order dependence upon both the oxygen and catalyst concentration, (ii) the value 2.3 for the k_H/k_D ratio on substituting hydrogen by deuterium at C_4 of the dihydronicotinamide, (iii) the identical rate in D_2O and H_2O, (iv) the value of 10.0 kcal/mole for the activation energy, and (v) the pH behavior. The work supports the view that biochemical generation of perhydroxyl radicals depends on low redox potentials rather than on specific enzyme structure.

Tetramethyl-*p*-phenylenediamine,[1] presumably in the monoprotonated form, catalyzes the autoxidation of *p*-phenylenediamine and almost certainly its own autoxidation as well (Cilento and Zinner, 1967). TMPD has now been found to catalyze the autoxidation of reduced pyridine coenzymes and of their models.

Since TMPD is commonly used in studies of the respiration–phosphorylation chain this catalyzed autoxidation of 1,4-dihydronicotinamides has been thoroughly investigated.

Materials

1-Benzyl-1,4-dihydronicotinamide (mp 123°) was prepared according to Mauzerall and Westheimer (1955); to prepare the C-4 monodeuterio analog, D_2O was substituted for H_2O in the reaction mixture. 1-*n*-Propyl-1,4-dihydronicotinamide, prepared according to Suelter and Metzler (1960), was recrystallized from water (mp 195°). The reduced pyridine coenzymes and catalase were from Sigma Chemical Co., the alcohol dehydrogenase from C. F. Bohringer und Soehne.

TMPD·2HCl (BDH reagent) was purified by dissolving in absolute methanol; by cooling the saturated solution

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[1] Abbreviation used is: TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

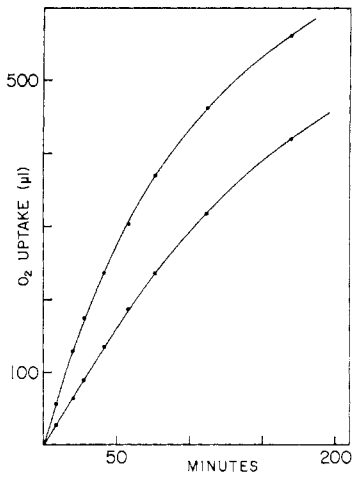


FIGURE 1: Oxygen uptake in the autoxidation of 2.0×10^{-2} M NADH catalyzed by 1.0×10^{-3} M TMPD in 0.2 M Tris buffer (pH 7.00). Lower curve: in presence of 5×10^{-7} M catalase.

colorless crystals were obtained. For comparative purposes, a batch was purified by dissolution in acetone–water and precipitation by adding more acetone. Identical catalytical behavior was observed with both samples.

p-Hydroquinone was recrystallized from water. Deuterium oxide (General Dynamics Corp.) was 99.5% pure.

Methods

The reaction has been studied at 25°, usually by following O_2 uptake in the Warburg manometer, occasionally by following the disappearance of the longer wavelength band of the coenzyme. In the latter cases, to assure a constant O_2 concentration, the reaction was performed in the Warburg and aliquots were removed and diluted at various intervals.

The solvents were Tris and phosphate buffers; in the case of the sparingly soluble 1-benzyl-1,4-dihydronicotinamide and often with NADH, methanolic buffers (1:1, v/v) were used. For solvent isotope effect experiments, all substrate and buffer solutions were freshly prepared in D_2O.

The reported pH values are as read on a Metrohm potentiometer. Spectra were taken on a Cary recording spectrophotometer.

Separation of the Pyridinium Cation. In the case of the model coenzymes, the Dowex 50W ion-exchange resin (dry mesh 200–400; 4% cross-linked) was used. An aliquot (0.5 ml) of the reaction mixture was slowly passed through the column (0.58 × 3.0 cm) in the NH_4^+ form. The column was exhaustively washed with water and the cations were eluted with a 5 M solution of highly pure NH_4Cl. From reaction mixtures in Tris buffer the 1-benzyl-3-carboxamidopyridinium cation was obtained in the first five or six 5-ml fractions; with the propyl model the cation was obtained in the first two 5-ml fractions. These pyridinium cations were easily identified by their ultraviolet spectrum. With the propyl model another cation can be eluted in later fractions; this matter is discussed in the accompanying paper (Bechara and Cilento, 1971).

Kinetics. The dependence of the rate of O_2 uptake upon the concentration of a reactant or catalyst was obtained from the slope of the straight line in a log–log plot of the initial rate *vs.* the concentration; in such studies the concentration of the other participants was kept constant.

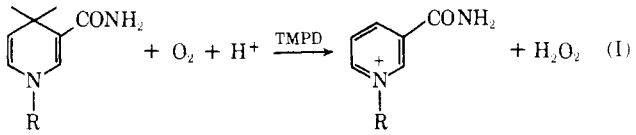
To ascertain whether the disappearance of the dihydronicotinamide absorption at constant oxygen concentration would follow pseudo-first-order kinetics, the log of the absorbancy was plotted as a function of time. In this process, the weak residual absorbancy was disregarded as in the later stages of the reaction a larger part of the catalyst is in the semiquinone form. The value of the rate constant, k, was obtained from the slope $-k/2.303$.

To determine the effect of temperature, the reaction was also run at 37° and 46°. The activation energy, E_A, was obtained from the slope $-E_A/2.303R$ in the plot of the logarithm of the initial velocity *vs.* $1/T$.

Reproducibility of kinetic runs was usually satisfactory, with initial rates diverging at most by 12%.

Results

Solutions of the pyridine coenzymes, NADH and NADPH, or of their model compounds 1-benzyl- and 1-*n*-propyl-1,4-dihydronicotinamide, autoxidize very slowly; yet, in the presence of TMPD, even in concentrations two orders of magnitude smaller the rate of O_2 uptake is greatly increased. A representative example is shown in Figure 1. Final oxygen consumption is stoichiometric or somewhat above for water formation. A rise in pH is also always observed. It will be shown that the main reaction which occurs is the oxidation of the dihydronicotinamide to the pyridinium cation followed by slow decomposition of the hydrogen peroxide (eq I).



(I)

Identification of Products. Spectrophotometrically the disappearance of the 340-mμ absorption band of NADH is followed by an increase of the 260-mμ peak. In the case of the benzyl model, the spectrum of the final reaction mixture in Tris buffer clearly showed the pyridinium absorption at 265 mμ and another absorption band at *ca.* 290 mμ, peculiar to the products resulting from saturation of the 5,6 double bond of 1,4-dihydronicotinamides (Schreier and Cilento, 1969). In the case of the propyl model in Tris only the latter absorption was discernible.

NAD^+ was identified and assayed enzymically (Ciotti and Kaplan, 1957). The yield was 80%. With the benzyl model, the pyridinium salt was isolated from Tris reaction mixtures in yields up to 85%. The substoichiometric yield is due, at least in the case of the model, to the fact that TMPD, presumably in the monoprotonated form, also catalyzes solvent addition to the 5,6 double bond of the dihydronicotinamide. This could be easily demonstrated by following, in anaerobiosis, the disappearance of the longer wavelength band of the dihydronicotinamide concomitantly to the development of the band of the 5,6-saturated product at *ca.* 290 mμ. Since the rate of the autoxidation process is a function of the oxygen concentration, this competing 5,6 solvent addition could be minimized by working in pure oxygen; almost quantitative yields of the benzylpyridinium cations were thus obtained.

The *n*-propyl model is very prone to 5,6 water addition; yet working in D_2O this addition is greatly slowed down (Kim and Chaykin, 1968) and the pyridinium salt forms in 90% yields.

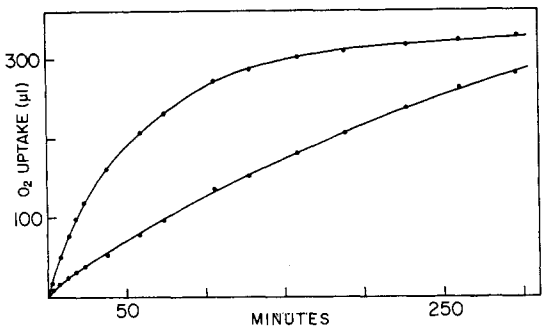


FIGURE 2: Oxygen uptake in a system of 1.5×10^{-2} M 1-*n*-propyl-1,4-dihydronicotinamide and 1.0×10^{-3} M TMPD in 0.2 M buffer (pH 6.8). Upper curve, in Tris; lower, in phosphate.

Formation of hydrogen peroxide could be demonstrated by adding catalase to a reacting system; the initial rate of O_2 uptake dropped by a factor of two (Figure 1), indicating that half of the O_2 taken up is returned by the enzymic decomposition of hydrogen peroxide. This result also indicates that hydrogen peroxide is an inert product of the reaction. As expected, the final reaction mixtures showed exactly the same pH.

Qualitative Observations. The benzyl model reacts somewhat slower and the propyl model somewhat faster than the coenzymes. The latter were studied in both 100% aqueous and 50% methanolic buffers but no great differences were observed.

The reaction proceeds equally well in phosphate as in Tris buffers. However in phosphate the 5,6 hydration of the dihydronicotinamide is much more serious (Alivisatos *et al.*, 1964, 1965; Anderson *et al.*, 1965) whereby rates tend to become lower and total O_2 consumption smaller. Yet with the propyl model a very interesting fact was observed; in phosphate despite complete hydration, O_2 consumption occurs (Figure 2). Because of its importance, this result was the starting point for the investigation related in the accompanying paper (Bechara and Cilento, 1971).

The effect of the pH has been studied with methanolic Tris buffer solutions of the benzyl model. The autoxidation becomes faster by lowering the pH to 6.49; further lowering to pH 6.21 and 6.03 does not affect the rate (Figure 3). Obviously at these low pH's Tris does not practically act as buffer, a fact which is irrelevant as far as initial rates are concerned.

TABLE I: Dependence of the Initial Rate of the TMPD-Catalyzed Oxidation of 1,4-Dihydronicotinamides upon the Concentration of the Dihydronicotinamide, [TMPD] = 1.0 mM, pH 6.88.

Solvent (M)	NADH (mM)	Benzyl-dihydro-nicotin-amide (mM)	Initial Rate (min) μl of O_2[a]	Order
Tris buffer (0.1)	10.0		3.28	
				1.0
	20.0		6.68	
Methanolic Tris buffer (0.1)		10.0	1.82	
		20.0	4.12	
		30.0	6.84	
				1.1
		40.0	8.40	

[a] Final volume, 3.1 ml.

TMPD could not be replaced by *p*-phenylenediamine. TMPD also catalyzes the autoxidation of *p*-hydroquinone at pH 6.7 but only slightly at pH 7.3 and not at pH 7.8. TMPD catalyzes the autoxidation of the 5,6-hydrated propyl model, provided phosphate is present (Bechara and Cilento, 1971).

No O_2 uptake occurred when TMPD was added to cytochrome *c* Fe^{2+}, thiophenol, xanthene, diphenylmethane, dihydrophenanthrene, tetralin, acetanilide, aniline, nitrobenzene, lactate, formate, or succinate solutions.

Kinetics. The rate of O_2 uptake by TMPD alone in concentrations much higher than catalytical is small. Therefore, the main role of TMPD is not to act cyclically through spontaneous oxidation to Wurster's blue semiquinone and reduction of the latter by the dihydronicotinamide. This cycle does, however, operate as the reaction mixture develops the blue color of the radical at the end of the reaction.

Data in Table I indicate that the rate of O_2 uptake bears a first-order dependence on the dihydronicotinamide concentration. This can also be seen from the pseudo-first-order disappearance of the NADH absorption (Figure 4); under the experimental conditions employed (pH 6.88; TMPD,

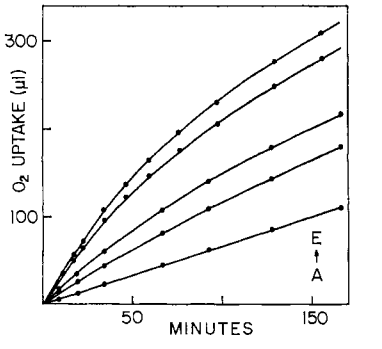


FIGURE 3: The effect of varying the pH upon the rate of autoxidation of 1.5×10^{-3} M 1-benzyl-1,4-dihydronicotinamide in 0.1 M methanolic Tris containing 1.0×10^{-3} M TMPD. Curve A, pH 7.82; B, pH 7.49; C, pH 7.14; D, pH 6.77; E, pH 6.49. The curves for pH 6.21 and 6.03 are very similar to curve E.

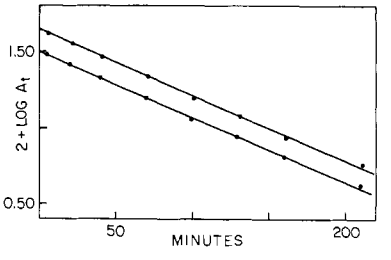


FIGURE 4: First-order disappearance of the NADH absorption in the autoxidation catalyzed by 1.0×10^{-3} M TMPD in Tris buffer, pH 6.88. Upper line, measurements at 345 mμ; lower line, at 360 mμ.

TABLE II: Effect of Air and Pure Oxygen upon the TMPD-Catalyzed Oxidation of 1,4-Dihydronicotinamides in 0.1 M Methanolic Tris Buffer (pH 6.87).

TMPD (mM)	NADH (mM)	Benzyl-dihydro-nicotin-amide (mM)	μl of O_2 (min)[a] Air	O_2	Order
0.2	15.0		1.58	4.40	0.6
1.02		15.0	2.60	5.64	0.5

[a] Final volume, 3.1 ml.

1.0 mM), k is 1.7×10^{-4} sec^{-1}. This first-order behavior was observed during three half-lives.

The rate of O_2 uptake in the autoxidation of NADH and of the benzyl model is increased by a factor of 2.7 and 2.2, respectively, on passing from air-saturated to pure O_2-saturated solutions; the order in the O_2 tension is therefore 0.6–0.5 (Table II). Regarding the effect of the catalyst concentration a half-order dependence is observed (Table III).

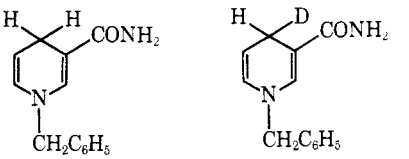
To study the influence of the temperature, NADH in entirely aqueous Tris buffer was used as substrate. Taking into account the variation of the oxygen solubility with temperature and the 0.6-order dependence upon the oxygen concentration, the energy of activation was found to be 10.0 kcal/mole. Clearly, the temperature may also affect the reaction through a change in the pK_a of the $HO_2\cdot$ radical and of the catalyst. In the latter case, however, the change in proportion of the free and protonated TMPD is presumably somewhat balanced by a similar effect upon the buffer, as also Tris in an amine.

TABLE III: Dependence of the Initial Rate of the TMPD-Catalyzed Oxidation of 1,4-Dihydronicotinamides upon the Concentration of the Catalyst (pH 6.86).

Solvent (M)	NADH (mM)	Benzyl-dihydro-nicotin-amide (mM)	TMPD (mM)	Initial Rate (min) (μl of O_2[a])	Order
Methanolic	15.4		0.05	0.94	
Tris buffer (0.1)	15.4		0.10	1.22	
					0.53
	15.4		0.20	1.82	
	15.4		0.40	2.58	
Tris buffer (0.2)	15.0		0.01	0.60	
	15.0		0.10	1.34	
					0.48
	15.0		1.00	4.36	
Methanolic		15.0	0.05	0.36	
Tris buffer (0.1)		15.0	0.10	0.56	
					0.62
		15.0	0.20	0.88	

[a] Final volume, 3.1 ml.

Since in the oxidation of the dihydronicotinamide to the pyridinium cation one of the C_4–H bonds is broken, the initial rate of autoxidation of the benzyl model was compared to that of the C_4-monodeuterio analog. The result was a decrease in rate.



relative rate 1.00 0.71

Therefore, considering that the rate is first order in the dihydronicotinamide concentration, one finds that k_H/k_D is 2.3.

Substituting water by D_2O in the autoxidation of NADH, no important changes were observed. Since pD = $pH_{measured}$ + 0.4 (Glasoe and Long, 1960; Srere *et al.*, 1961) the Tris buffer in D_2O was prepared so as to give a pH 6.6 whereas the buffer in H_2O had a pH of 7.0.

With the propyl model identical rates in D_2O and H_2O were only observed in the initial stage of the reaction, because, as already pointed out, extensive hydration occurs in H_2O.

Discussion

Mechanism. It is unlikely in view of the observed rate concentration dependences that the main process is a direct oxidation of the dihydronicotinamide by molecular oxygen as seems to happen when the autoxidation is catalyzed by 2,6-dihalogeno-4-aminophenols (Silva Araujo and Cilento, 1969). With the latter catalysts, the rate of NADH autoxidation is first order in the oxygen concentration whereas with TMPD a 0.6–0.5-order dependence in the oxidation of the coenzyme and of the benzyl model is observed. Further important differences are the low value for the k_H/k_D ratio in TMPD catalysis and the fact that TMPD also promotes the autoxidation of *p*-hydroquinone.

Whereas with the 2,6-dihalogeno-4-aminophenol catalysts a hydride ion seems to be transferred from the 1,4-dihidronicotinamide to O_2 (Silva Araujo *et al.*, 1970), with TMPD as catalyst a homolytic mechanism will be shown to be operative. Actually, since 1,4-dihydronicotinamides may be oxidized in two one-electron steps and as the pyridinyl radical is highly reactive toward oxygen (Kosower and Pozionek, 1964), a chain mechanism is very likely. Considering also that NADH can reduce Wurster's blue (Mustafa *et al.*, 1968), we suggest the following series of reactions.

$$B + O_2 \xrightarrow{k_1} S^+ + O_2^-$$

$$D + HO_2\cdot \xrightarrow{k_2} Py\cdot + H_2O_2$$

$$D + O_2^- \xrightarrow{k_2'} Py\cdot + HO_2^-$$

$$D + S\cdot^+ \xrightarrow{k_3} B + Py\cdot + H^+$$

$$Py\cdot + O_2 \xrightarrow{k_4} P^+ + O_2^-$$

$$O_2^- + HO_2\cdot \xrightarrow{k_5} O_2 + HO_2^-$$

In this scheme B is TMPD, D is the dihydronicotinamide, $S\cdot^+$ the semiquinone (Wurster's blue), Py· the pyridinyl radical, and P^+ the pyridinium cation. $HO_2\cdot$ is the perhydroxyl radical and O_2^-, its conjugate base, is the superoxide ion. The termination step is formulated according to the work of Behar *et al.* (1970).

The very reasonable assumption is made that step I is much slower than the others and that the only reaction between radicals which takes place is that between perhydroxyl radicals and superoxide ions. Then applying the steady state treatment we obtain eq 1, where K is the ionization constant of the perhydroxyl radical.

$$\text{rate} = k_1(\text{B})(\text{O}_2) + \left[k_2\left(\frac{(\text{H}^+)}{K^+}\right)^{1/2} + k_2'\left(\frac{K}{(\text{H}^+)}\right)^{1/2}\right]\left[\frac{2k_1(\text{B})(\text{O}_2)}{k_5}\right]^{1/2}(\text{D}) \quad (1)$$

Now, since the rate of autoxidation of the catalyst alone is generally much smaller than that of the complete system, the $k_1(B)(O_2)$ term can often be neglected. Under such conditions, the rate should bear a first-order dependence on the dihydronicotinamide and a half-order dependence on both the oxygen tension and the catalyst concentration. These dependences are indeed reasonably well observed. At constant oxygen concentration, the simplified form of eq 1 can be integrated to give a pseudo-first-order rate expression for the disappearance of the dihydronicotinamide as is indeed observed: $\log (D)_t = -(kt/2.303) + \log (D)_0$.

The proposed mechanism is also in accord with the k_H/k_D ratio, the magnitude of which, 2.3, indicates homolytic cleavage of one of the C_4–H bonds of the dihydronicotinamide. This is further supported by the value of the activation energy, 10.0 kcal/mole. This value is consistent with the fact that the main kinetic step involves hydrogen abstraction.

Also the observed pH behavior fits the scheme if one assumes that k_2 is 10–100 times larger than k_2'. Hence lowering the pH will increase the proportion of the $HO_2\cdot$ radicals ($pK_a = 4.8$; Behar *et al.*, 1970) and therefore the rate, until protonation of the catalyst will, by hampering the initiation step, counterbalance the increase in rate.

Going from H_2O to D_2O a change in K should occur and therefore there should also be a change, although smaller, in the rate. The similar rates observed at pH (pD) 7.0 suggest a balance of effects and, therefore, that at neutral pH's the O_2^- and $HO_2\cdot$ species contribute to the rate to a roughly similar extent.

Thermodynamic Considerations. In step 2, $D + HO_2\cdot \rightarrow Py\cdot + H_2O_2$, a C–H bond is replaced by a O–H bond.

Considering that the bond dissociation energy of the second hydrogen in H_2O_2 is 90 kcal/mole (Benson, 1965) and of the C–H bond in trimethylmethane is 91 kcal/mole (Benson, 1965), ΔH should be practically zero; nevertheless ΔH will be negative because the pyridinyl radical is resonance stabilized.

On the other hand, step 4, $Py\cdot + O_2 \rightarrow P^+ + O_2^-$, is certainly exothermic. For an H· atom-transfer case such as the autoxidation of the cyclohexadienyl radical, $-\Delta H$ is calculated to be about 23 kcal/mole from the estimated dissociation energy of the C–H bond in the radical (24 kcal/mole; Benson, 1965) and the bond dissociation energy of the first hydrogen in H_2O_2 (47 kcal/mole; Foner and Hudson, 1962).

Presumably the exothermicity of steps 2 and 4 and the instability of the hypothetical $PyO_2\cdot$ radical are responsible for the fact that the autoxidation of 1,4-dihydronicotinamides seems to proceed through the HO_2 mechanism. The situation contrasts sharply that of reduced flavins, which are known to originate intermediate hydroperoxides (Mager and Berends, 1966; Massey *et al.*, 1969). Moreover if a semiquinone is formed from the hydroperoxide as has been suggested in the case of the flavoprotein dehydrogenases (Massey *et al.*, 1969), the semiquinone will only slowly react with oxygen.

Biological Considerations. Despite the fact that the catalytical effect of TMPD upon the autoxidation of the pyridine coenzymes is still clearly detectable when TMPD is 10^{-5} M, it is unlikely that the effect is important in studies of the oxidation–phosphorylation chain, expecially because TMPD will transfer an electron to the cytochrome system, not to oxygen (Mustafa *et al.*, 1968).

The superoxide ion is known to be formed in some biochemical systems, for instance during the oxidation of reduced flavins by oxygen (Ballou *et al.*, 1969; Orme-Johnson and Beinert, 1969). It also participates in the oxidase activity of peroxidase (Yamazaki, 1966). In the latter reaction, free radicals of the donor are formed at the expense of hydrogen peroxide; the free radical activates oxygen to the perhydroxyl radical which maintains the chain reaction.

Our work is consistent with the view that biochemical generation of the superoxide ion depends on low redox potential rather than on specific enzyme structure (Nilsson *et al.*, 1969).

Appendix

From the proposed kinetics one obtains

$$\text{rate} = k_4(\text{Py}\cdot)(\text{O}_2)$$

$$\frac{d(S\cdot^+)}{dt} = k_1(\text{B})(\text{O}_2) - k_3(\text{D})(S\cdot^+) = 0 \quad (A1)$$

$$\frac{d(\text{Py}\cdot)}{dt} = k_2(\text{D})(\text{O}_2\text{H}) + k_2'(\text{D})(\text{O}_2^-) + k_3(\text{D})(\text{S}^+) - k_4(\text{Py}\cdot)(\text{O}_2) = 0 \quad (A2)$$

$$\frac{d(\text{O}_2\text{H} + \text{O}_2^-)}{dt} = k_1(\text{B})(\text{O}_2) + k_4(\text{Py}\cdot)(\text{O}_2) - k_2(\text{D})(\text{O}_2\text{H}) - k_2'(\text{D})(\text{O}_2^-) - k_5(\text{O}_2^-)(\text{O}_2\text{H}) = 0 \quad (A3)$$

From eq A2 and A3

$$2k_1(\text{B})(\text{O}_2) = k_5(\text{O}_2^-)(\text{O}_2\text{H}) \quad (A4)$$

From eq A2

$$(\text{Py}\cdot) = \frac{k_2(\text{D})(\text{O}_2\text{H}) + k_2'(\text{D})(\text{O}_2^-) + k_3(\text{D})(S\cdot^+)}{k_4(\text{O}_2)}$$

Hence

$$\text{rate} = k_1(\text{B})(\text{O}_2) + k_2(\text{D})(\text{O}_2\text{H}) + k_2'(\text{D})(\text{O}_2^-)$$

or more simply

$$\text{rate} = k_2(\text{D})(\text{O}_2\text{H}) + k_2'(\text{D})(\text{O}_2^-)$$

From eq A4 and from $(H^+)(O_2^-)/(HO_2) = K$

$$(HO_2\cdot) = \left(\frac{2k_1(B)(O_2)(H^+)}{k_5K}\right)^{1/2}$$

Similarly

$$(O_2^-) = \left(\frac{2k_1(B)(O_2)K}{k_5(H^+)}\right)^{1/2}$$

Hence

$$\text{rate} = \left[\frac{k_2(H^+)^{1/2}}{K^{1/2}} + \frac{k_2'K^{1/2}}{(H^+)^{1/2}}\right](D)\left(\frac{2k_1(B)(O_2)}{k_5}\right)^{1/2}$$

As a consequence of the square-root term the effect of changing the value of K (going from H_2O to D_2O solutions) will be less important than expected from the rate expression derived by a referee. Furthermore since both species, O_2H and O_2^-, react, the effect upon one reaction may be, in part or totally, balanced by an opposite effect on the other reaction.

(There is no doubt that k_2 must be much larger than k_2'. If one assumes that $k_2 \sim 100k_2'$, then at neutral pH's the perhydroxyl and the superoxide ion will contribute, to a roughly similar extent, to the rate.)

Going from H_2O to D_2O, K will decrease by a factor of about 3 (suggested by a referee); therefore one reaction will be slightly accelerated (because of the $1/(K)^{1/2}$ term) whereas the other will be slightly slowed down (because of the $(K)^{1/2}$ term).

Going from neutral pH's to lower pH's, the reaction will become faster because the O_2^- ions are being replaced by the more reactive O_2H radicals. However at sufficiently low pH's, the effect will be balanced by the protonation of the catalyst, that is, by hampering the initiation step (similar and opposite effects through the $(H^+)^{1/2}$ term and the $(B)^{1/2}$ term, since at acidic pH's $k_2'K^{1/2}/(H^+)^{1/2}$ can be disregarded relative to $k_2(H^+)^{1/2}/K^{1/2}$).

References

Alivisatos, S. G. A., Ungar, F., and Abraham, G. (1964), *Nature (London) 203*, 973.

Alivisatos, S. G. A., Ungar, F., and Abraham, G. J. (1965), *Biochemistry 4*, 2616.

Anderson, B. M., Reynolds, M. L., and Anderson, C. D. (1965), *Arch. Biochem. Biophys. 110*, 577.

Ballou, D., Palmer, G., and Massey, V. (1969), *Biochem. Biophys. Res. Commun. 36*, 898.

Bechara, E. J. H., and Cilento, G. (1971), *Biochemistry 10*, 1837.

Behar, D., Czapski, G., Rabani, J., Dorfman, L. N., and Schwarz, H. A. (1970), *J. Phys. Chem. 74*, 3209.

Benson, S. W. (1965), *J. Chem. Phys. 42*, 502.

Cilento, G., and Zinner, K. (1967), *Biochim. Biophys. Acta 143*, 93.

Ciotti, M. M., and Kaplan, N. O. (1957), *Methods Enzymol. 3*, 890.

Foner, S. N., and Hudson, R. L. (1962), *J. Chem. Phys. 36*, 2681.

Glasoe, P. K., and Long, F. A. (1960), *J. Phys. Chem. 64*, 188.

Kim, C. S. Y., and Chaykin, S. (1968), *Biochemistry 7*, 2339.

Kosower, E. M., and Pozionek, E. J. (1964), *J. Amer. Chem. Soc. 86*, 5515.

Mager, H. I. X., and Berends, W. (1966), *Biochim. Biophys. Acta 118*, 440.

Massey, V., Miller, F., Feldberg, R., Schuman, M., Sullivan, P. A., Howell, L. G., Mayhew, S. G., Matthews, R. G., and Foust, G. P. (1969), *J. Biol. Chem. 244*, 3999.

Mauzerall, D., and Westheimer, F. H. (1955), *J. Amer. Chem. Soc. 77*, 2261.

Mustafa, M. G., Cowger, M. L., Labbe, R. F., and King, T. E. (1968), *J. Biol. Chem. 243*, 1908.

Nilsson, R., Pick, F. M., and Bray, R. C. (1969), *Biochim. Biophys. Acta 192*, 145.

Orme-Johnson, W. H., and Beinert, H. (1969), *Biochem. Biophys. Res. Commun. 36*, 905.

Schreier, S., and Cilento, G. (1969), *Biochemistry 8*, 2140.

Silva Araujo, M., Bechara, E. J. H., Zinner, K., and Cilento, G. (1970), *Record Chem. Prog.* (in press).

Silva Araujo, M., and Cilento, G. (1969), *Biochemistry 8*, 2145.

Srere, P. A., Kosicki, G. W. and Lumry, R. (1961), *Biochim. Biophys. Acta 50*, 184.

Suelter, C. H., and Metzler, D. E. (1960), *Biochim. Biophys. Acta 44*, 23.

Yamazaki, I. (1966), *in* Biological and Chemical Aspects of Oxygenases, Block, K., and Hayaishi, O., Ed., Tokyo, Maruzen Co., p 433.