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Pyridine Coenzymes. VIII. Autoxidation of the Reduced Form Catalyzed by 4-Amino-2,6-diiodophenol*

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ABSTRACT: The reduced pyridine coenzymes are stoichiometrically and directly oxidized by molecular oxygen to the pyridinium form, under the catalytical effect of protonated 4-amino-2,6-diiodophenol. With the model coenzyme, 1-benzyl-1,4-dihydronicotinamide, two processes take place simultaneously: (i) the direct oxidation and (ii) addition to the 5,6-double bond of the dihydronicotinamide followed by autoxidation of the resulting product. The latter process is more important in phosphate than in Tris buffer and leads to a new cation, besides the pyridinium ion. The catalyst does not shuttle electrons between the dihydronicotinamide or its 5,6 derivatives, and oxygen; it specifically promotes the transfer of an hydride ion or of its elements to the O_2 molecules by preferential stabilization of the transition state through complexation and proton donation.

In the precedent paper (Schreier and Cilento, 1969) it was reported that *o*-halogenophenols, including thyroid hormones, catalyze the hydration of 1,4-dihydronicotinamides. We have now found that DIPAP[1] not only catalyzes solvent addition to 1-benzyl-1,4-dihydronicotinamide, a model coenzyme, but also the autoxidation of the resulting or intermediate products as well as of the model itself. In the case of the natural coenzymes, NADH and NADPH, only the autoxidation occurs with stoichiometric formation of NAD, and, almost so, of NADP. This work joins and significantly expands another line of research, that of nonclassical oxygen activation (Cilento and Zinner, 1966, 1967a–c). The important novel feature is that DIPAP, unlike other nonclassical oxygen activators, does not catalyze the autoxidation of compounds which autoxidize by one electron or H · atom transfer.

The present work appears therefore of considerable significance in connection with the transfer of the H^- ion or of its elements to oxygen.

Materials

DIPAP (mp 220°) was prepared according to Woollett *et al.* (1937), 2,6-diiodo-*p*-benzoquinone (mp 180°) according to Seifert (1883), and 1-benzyl-1,4-dihydronicotinamide (mp 123°) according to Mauzerall and Westheimer (1955). They were pure by both melting and spectral criteria. NADH, NADPH, and D-glucose 6-phosphate (disodium salt) were from Sigma Chemical Co.; the alcohol dehydrogenase and glucose 6-phosphate dehydrogenase from C. F. Bohringer & Soehne. All the other compounds were available in these laboratories from earlier work and were purified once more by routine procedures.

Methods

The reaction has been studied at 25° manometrically by following O_2 uptake in the Warburg apparatus and spectrophotometrically by following the disappearance of the long-wavelength band of the dihydronicotinamide. The solvents were Tris–CH_3OH (1:1 or 1:2, v/v) and phosphate–CH_3OH (1:1, v/v) buffers of 0.1 M concentration. Occasionally acetone was substituted

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[1] DIPAP = 4-amino-2,6-diiodophenol.

for methanol. The reported pH values are as read in a Methrom potentiometer.

The oxidized coenzymes formed in the reaction were determined by enzymically reducing a properly diluted solution of the final reaction mixture and reading the absorbancy at 340 mμ (ϵ_{max} 6220). NAD was reduced with ethanol (Ciotti and Kaplan, 1957); NADP with glucose 6-phosphate (Horecker and Kornberg, 1957).

The pyridinium salt formed in the reaction of the model coenzyme was isolated with the cation-exchange resin (Dowex 50X-4, 200–400 dry mesh). For that purpose, a reaction mixture 15 mM in the model coenzyme and 1.8 mM in DIPAP was prepared and the O_2 uptake followed up. At the end 1 ml was diluted to 50 ml with aqueous methanol and 3 ml of the diluted solution passed through the column (1 cm high; diameter, 6 mm) in the NH_4^+ form. The column was thoroughly washed with water and the cations eluted with a saturated solution of NH_4Cl. The pyridinium salt was obtained in the first and second 10-ml fractions and easily identified by its ultraviolet spectrum. Another cation came out in third fraction.

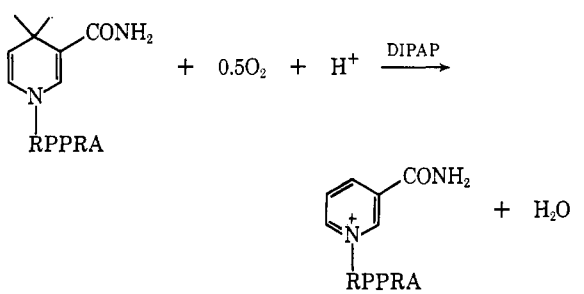
The order of reaction with respect to a reactant was ascertained from the slope of the straight line in a log–log plot of the initial rate *vs.* the concentration. The rates were measured as a function of the concentration of the reactant while keeping constant that of the other participants. The effect of oxygen pressure was studied by performing experiments with air saturated and pure oxygen-saturated solutions.

Reproducibility. Concerning product formation, the reproducibility was good except occasionally for the second cation; regarding initial rates it was excellent in the case of the natural coenzymes, fair in the case of the model.

Results

Autoxidation of the Reduced Coenzymes. A solution of NADH autoxidizes at a slow rate; yet in presence of DIPAP in concentration much smaller than stoichiometric, the rate of O_2 uptake is greatly increased (Figure 1). In Tris, O_2 consumption is close above the stoichiometric value for water formation (Figure 2; Table I); in phosphate, a somewhat smaller consumption is observed. At the end of the reaction, NADH has practically disappeared and a small or appreciable increase in pH is always observed. Similar results were obtained with NADPH.

The most obvious over-all reaction occurring is



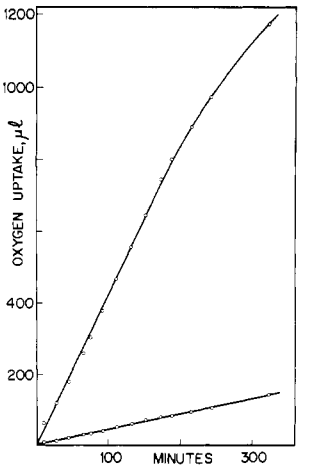


FIGURE 1: Oxygen uptake in the system of 40.1 mM NADH and 1.97 mM 4-amino-2,6-diiodophenol. Lower line, NADH alone. There is no absorption whatsoever by the catalyst alone. Solvent: 0.2 M Tris buffer–CH_3OH (1:1, v/v), pH 6.8.

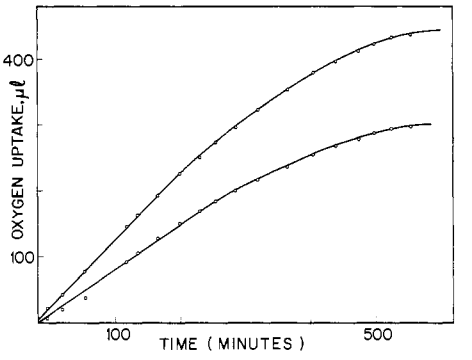


FIGURE 2: Oxygen uptake by the reduced pyridine coenzymes in the presence of 2.0 mM 4-amino-2,6-diiodophenol. Upper curve: 10.1 mM NADH; lower curve: 7.7 mM NADPH. Solvent: 0.2 M Tris buffer–CH_3OH (1:1, v/v), pH 6.8.

In agreement with this expectation, the amount of NAD formed was quantitative (Table I). Spectrophotometrically, the disappearance of the 340-mμ band is followed by an increasing of the peak at 260 mμ.

Autoxidation of the Model Coenzyme, 1-Benzyl-1,4-dihydronicotinamide. Manometrically, the behavior was very similar to that of the NADH system. However, DIPAP, as expected on the basis of the precedent work (Schreier and Cilento, 1969) does also catalyze solvent addition to the 5,6-double bond of the model coenzyme. The latter reaction could be properly followed spectrophotometrically, using evacuated Thunberg cells, as it leads to disappearance of the 355-mμ band and appearance of a stronger one at 290 mμ.

The stoichiometric O_2 uptake for water formation implies that also the 5,6-saturated derivatives or the intermediates leading to their formation are autoxidized in the presence of DIPAP. The most likely products to be formed are II, III, and IV as shown in Scheme I, in which HOR (R = H, CH_3) represents the solvent.

TABLE I: Stoichiometry of the 4-Amino-2,6-diiodophenol-Catalyzed Autoxidation of the Reduced Pyridine Coenzymes.[a] [4-Amino-2,6-diiodophenol] = 2.0 mM.

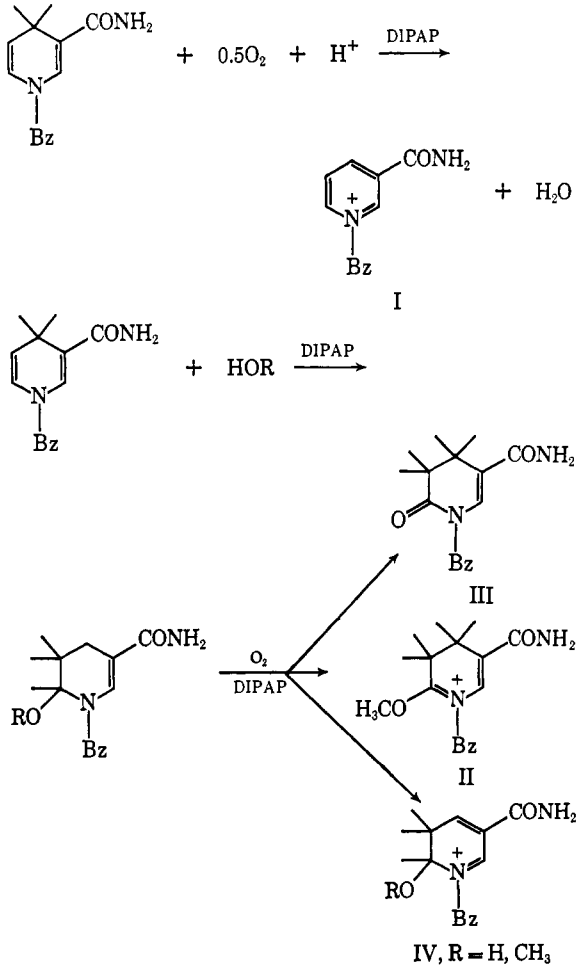
Coenzyme (mM)	pH	μl of O_2[c] Taken up	μl of O_2[c] Calcd[d]	[Oxidized Coenzyme] (mM)	% of Oxidn	O_2 Oxidative Coenzyme
NADH (10.0)	7.0			9.7	97	
NADH (10.1)	6.8	444	427	9.5	94	0.53
NADH (10.1)	6.9			8.8	87	
NADPH[b] (7.7)	6.8	300	325	6.3	80	0.56

[a] In 0.2 M Tris buffer–CH_3OH (1:1, v/v), except at pH 6.9, where Tris was substituted by phosphate. [b] Calculated from the absorbancy at 340 mμ. [c] 3.1 ml of solution. [d] For water formation.

Starch–iodide tests for H_2O_2 were slightly positive; hence if H_2O_2 was formed in substantial amounts, the observed total O_2 uptake indicates that H_2O_2 should either decompose or act as oxidant. No convincing evidence for either alternative could be obtained.

At pH values near 7.4, the yield of the pyridinium salt (I) as isolated with the Dowex resin was 62% in Tris and 42% in phosphate. No isolation was attempted at high pH values because of the well known instability of pyridinium cations in alkaline solutions.

SCHEME I



With the phosphate mixture after elution of I another cation comes out. This cation like I absorbs maximally at 265 mμ; yet the spectra are different (Figure 3). Presumably it has the structure II or IV (R = H, CH_3). The same or a similar cation is formed in much smaller amount in the Tris mixture. This new cation is not found when methanol is replaced by acetone in the solvent.

Spectrophotometrically the disappearance of the long-wavelength band of the model coenzyme is followed by increased absorption in the 260–300-mμ region. In Tris peaks at 268, 275, and 288 mμ can be recognized (Figure 4). After passing through the resin the solution shows only the 288-mμ peak; this absorption is presumably due to III.

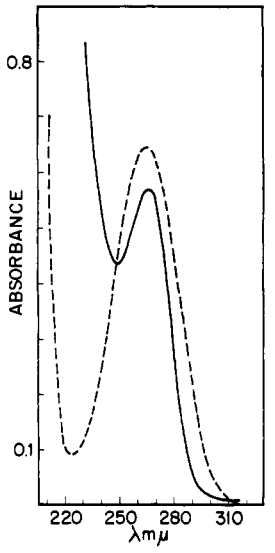


FIGURE 3: Qualitative comparison between the ultraviolet absorption spectrum of the first cation (——), that is 1-benzyl-3-carboxamidepyridinium chloride, and of the second cation (------) eluted from the resin.

In phosphate buffer the increased absorption in the ultraviolet is greatly dominated by the 288-mμ peak. After passing through the resin, the 288-mμ peak is twice stronger than that from the corresponding Tris buffer reaction.

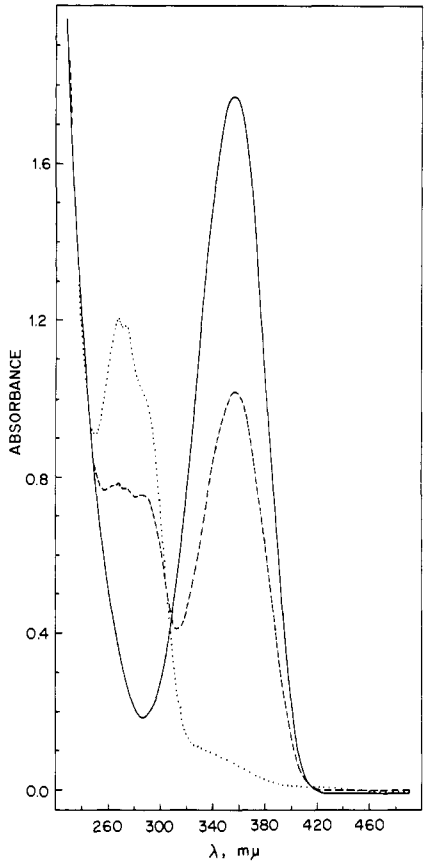
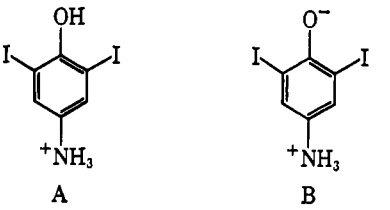


FIGURE 4: Spectrophotometric alterations following the 4-amino-2,6-diiodophenol- (1.5 mM) catalyzed oxidation of 15 mM 1-benzyl-1,4-dihydronicotinamide in 0.2 M Tris–CH_3OH (1:1, v/v) buffer, pH 6.8. The reaction was carried out in the Warburg apparatus using duplicate reaction mixtures; 0.4 ml of the reaction mixture was diluted to 25 ml with the solvent as soon as prepared (——), after 4 hr (------), and after 16 hr (.....); 1-cm optical path cells.

All the pertinent results of a typical experiment are summarized in Table II.

Effect of the pH. To ascertain what form of the catalyst is the active one, initial rates of O_2 uptake and of disappearance of the visible absorption of the model coenzyme have been measured at different pH values. The results are presented in Figure 5. From the decreased activity in going to higher pH values it is apparent that the main catalytical species is the positive anilonium ion (A). The flat shoulder at higher pH values may be ascribed to the zwitterion species (B) because of the expected ionization of the phenolic group. On a molar basis B should be much more active than A.



Stability of DIPAP. The species A is not expected to easily autoxidize; therefore DIPAP should not act cyclically through spontaneous autoxidation and regeneration by the dihydronicotinamide acting as reductant. To confirm this view, we followed the rate of autoxidation of DIPAP in concentrations quite higher than catalytical at neutral–acidic pH values and, as expected, found it spectrophotometrically and manometrically negligible. Moreover, oxidation of DIPAP would presumably generate some 2,6-diiodo-*p*-benzoquinone and this compound was found to be inactive at pH 6.0.

A cyclic oxidation–reduction role for DIPAP might contribute to the oxidation of the model dihydronicotinamide at alkaline pH values. However such a contribution is, at best, small, because the rate of O_2 uptake by the mixture is much higher than that by the catalyst alone. That even at alkaline pH values the catalytical effect of DIPAP does not depend upon the formation of another species is also indicated by the similar activity observed whether the catalyst had been preliminarly exposed to the alkalinity of the buffer or not.

TABLE II: Comparative Spectral Data for the 4-Amino-2,6-diiodophenol-Catalyzed Oxidation of 1-Benzyl-1,4-dihydronicotinamide in Methanolic Tris and Phosphate Buffers at pH 7.4.[a]

		Passage through Dowex 50X-4 Resin		
	Reaction Mixture at the End	Solution after Passage	First Cation (Pyridinium) and Yield (%)	Second Cation
Tris	270 (76.9) 275 (79.4) 283 (75.6)	288 (43.6)	265 (39.1), 62	265 (8.30)
Phosphate	288 (171.1)	290 (97.6)	265 (27.0), 42	265 (107.5)

[a] Position of the ultraviolet peaks in millimicrons and absorbancy; the latter is given in parentheses and is calculated for the original reaction mixture in 1-cm optical path cell. The concentration of the dihydronicotinamide was 15 mM, that of 4-amino-2,6-diiodophenol, 1.84 mM.

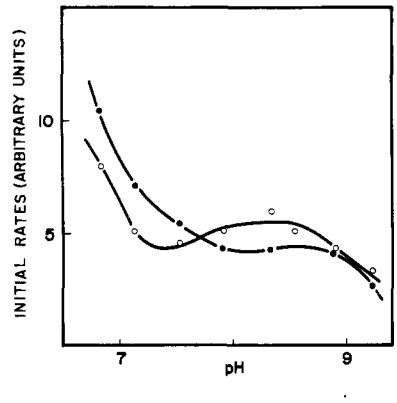


FIGURE 5: The effect of the pH upon the initial rate of 1-benzyl-1,4-dihydronicotinamide oxidation as determined manometrically (○—○—○—○) and spectrophotometrically (●—●—●—●). Solvent: 0.2 M Tris buffer–CH_3OH (1:1, v/v).

Kinetic Data. To obtain more information on the mechanism of catalysis, be it by species A or B, we have investigated the kinetic order in each participant, that is in the dihydronicotinamide, in oxygen and in DIPAP, at different pH values.

The study with the natural coenzyme was only carried out manometrically. The rate was found to be first order in NADH (Table III). Regarding the O_2 tension, a first-order dependence was observed (Table IV); the "spontaneous" oxidation showed a zero-order dependence indicating a substantially different mechanism of autoxidation.

The dependence of the initial rate upon the model coenzyme concentration may be appreciated from data in Table III. For the region of concentration investigated the order is close to one.

The effect of the oxygen tension upon the rate of autoxidation of the model coenzyme can be seen in Table IV. At pH 7.3 the rate of O_2 uptake is increased by a factor of two on passing from air to pure oxygen, instead of by a factor of about five as expected for a first-order dependence. Therefore one may suspect that in the concomitant process involving solvent addition and then oxidation, the latter step is not rate

TABLE III: Dependence of the Initial Rate of the 4-Amino-2,6-diiodophenol-Catalyzed Oxidation of Dihydronicotinamides upon the Concentration of the Dihydronicotinamide.[a]

pH	4-Amino-2,6-diiodophenol (mM)	NADH (mM)	Benzyldihydronicotinamide (mM)	Initial Rate (min)		Order
				μl of O_2[b]	$-\Delta A_{422}$[c]	
6.8	2.0	10.0		1.00		1.0
6.8	2.0	40.1		4.20		
6.8	1.9		5.0	0.40		
6.8	1.9		10.0	0.84		1.1
6.8	1.9		20.0	1.98		
6.8	1.9		40.0	4.24		
8.3	2.0		5.0	0.48		
8.3	2.0		10.0	0.78		
8.3	2.0		20.0	1.50		0.9
8.3	2.0		40.0	2.80		
8.3	3.0		10.0		0.0012	
8.3	3.0		20.0		0.0020	0.8
8.3	3.0		40.0		0.0034	

[a] In 0.2 M Tris buffer–CH_3OH(1:1, v/v). [b] Final volume, 3.1 ml. [c] 1-cm optical path cells.

TABLE IV: The Effect of Air and Pure Oxygen upon the 4-Amino-2,6-diiodophenol-Catalyzed Oxidation of Dihydronicotinamides.[a]

pH	4-Amino-2,6-diiodophenol (mM)	NADH (mM)	Benzyldihydronicotinamide (mM)	μl of O_2/min		$(-\Delta A/\text{min})$[b]		
				Air	Oxygen	Vacuum	Air	Oxygen
6.8	2.1	10.0		0.94 (0.76)[c]	3.30			
7.0	1.0		7.5			0.0016	0.0038	0.0068
7.3	1.0		5.1	0.32	0.66			
8.3	1.7		6.1	0.72	2.82			

[a] In 0.2 M Tris buffer–CH_3OH(1:1, v/v); final volume 3.1 ml. [b] At 412 mμ in 1-cm optical path cell. [c] Subtracting the rate of the uncatalyzed autoxidation.

determining. Even so, the increase in rate in going from air to oxygen is less than expected, because about 60% of the dihydronicotinamide undergoes oxidation to I. This is also apparent from spectrophotometric data: the relative initial rates of the catalyzed disappearance of the model compound *in vacuo*, air, and oxygen were approximately in the ratio 1:2.4:4.2 (model 7.5 mM; DIPAP 1.0 mM). By subtracting the rate in anaerobiosis, that is the rate of solvent addition, one obtains a factor of 2.3 for the increase in going from air to oxygen. It is clear that by both methods the increase is smaller than expected for a system in which about 60% of the model coenzyme molecules are directly oxidized by oxygen and 40% are first solvated in a rate-determining step and then oxidized.

Results more readily accounted for were obtained at pH 8.3. Manometrically the rate of O_2 uptake was increased by a factor of 4.0 on passing from air to pure oxygen in experiments in which the model compound was 6.1 mM and DIPAP 1.7 mM. Now, if α is the fraction of molecules which undergoes direct autoxidation, $1-\alpha$ will be the fraction which is first solvated and then oxidized. Hence

$$\frac{\text{rate}_{air}}{\text{rate}_{O_2}} = \frac{(\alpha) + (1 - \alpha)}{(5\alpha) + (1 - \alpha)} = \frac{1}{4}$$

whereby $\alpha = \frac{3}{4}$.

It appears therefore that under those conditions, 75% of the model coenzyme molecules are directly oxidized by oxygen and 25% are first solvated and then rapidly oxidized. In agreement with this, the spectral rate of the dihydronicotinamide disappearance increased about four times from vacuum to in air as expected from the ratio $1/(1 - \alpha)$.

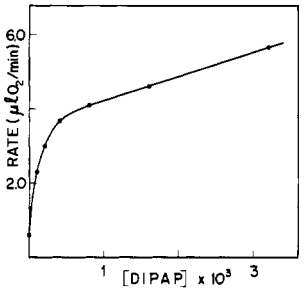


FIGURE 6: The effect of 4-amino-2,6-diiodophenol concentration upon the autoxidation of 40 mM 1-benzyl-1,4-dihydronicotinamide. Solvent: 0.2 M Tris buffer–CH_3OH (1:1, v/v), pH 6.8.

The effect of the catalyst concentration upon the rate was studied at pH 6.8 and 8.3, both manometrically and spectrophotometrically. Similar results were obtained at both pH values and by both techniques; a representative case is shown in Figure 6. It appears that two concomitant processes take place; one of them is extremely efficient and reaches saturation at very low concentration of the active forms of the catalyst.

Effect of Light. Essentially identical rates of O_2 uptake were obtained by studying the DIPAP-catalyzed autoxidation of the model coenzyme in the dark and in the light.

Effect of a Radical Scavenger. To test the effect of 9,10-dihydroanthracene, 0.2 M Tris–methanol mixture (1:2,v/v) was used as solvent. Even so the maximum concentration of the inhibitor attained was only 3×10^{-3} M. In an experiment in which the model dihydronicotinamide concentration was 15 mM, that of DIPAP 2 mM, and the pH of Tris 7.03, the inhibition was 15%.

Effect of DIPAP on the Autoxidation of Other Substrates. DIPAP failed to catalyze the autoxidation of *p*-hydroquinone (pH 5.5–8.8), of *p*-phenylenediamine (pH 7.0–8.5), and of benzenethiol (pH 7.6), compounds which autoxidize by one electron of H· atom transfer.

Structure and Catalysis. p-Aminophenol is much less active than DIPAP at neutral or acidic pH values. Not only the halogen atoms but also the *para* proton donor is important; in fact 3,5-diiodotyrosine does not catalyze the O_2 uptake.

Discussion

Nature of the Reaction. All the evidence clearly indicates that the reduced form of the pyridine coenzymes is directly oxidized by molecular O_2 in the presence of DIPAP.

A more complex scheme obtains for the model compound, because differently from the natural coenzymes, the model is also prone to solvent addition (Schreier and Cilento, 1969); as a result, a variety of products is formed.

At pH values near 7 one process is certainly solvent addition to the 5,6-double bond followed by autoxidation of the resulting products to compounds to which structures II, III, and IV (R = H, CH_3) have been tentatively assigned; it is however possible that oxidation takes place on intermediates leading to the 5,6 derivatives. Direct autoxidation of the model coenzyme does also occur but the dependence of the rate upon the O_2 pressure is lower than expected. At pH 8.3 the main process is the direct autoxidation.

The stability in aqueous solvents of a compound having a structure such as II is supported by the ionization of cotarnine (Ingold, 1953) and may be due to strong resonance.

Mechanism of Catalysis. We have seen that DIPAP does not act through cyclic autoxidation and reduction. Neither is its activity dependent upon the formation of another species; in this connection a further argument is the first-order dependency of the rate upon both the dihydronicotinamide concentration and the O_2 tension.

Since electron transfer occurs from a dihydropyridine to halocarbons (Kurz *et al.*, 1961; Kosower, 1965), one might consider the possibility that such a transfer occurs in our systems as a preliminary step, being then followed by the oxidation of the radicals formed. Such a mechanism can be ruled out on several grounds (kinetic order in the participants, inalterability of the

catalyst, inefficiency of light and of the radical scavenger).

Most certainly the function of DIPAP is simply to stabilize the transition state of the autoxidation, presumably through the oxygen molecule. Such a role for the anilonium cations A and B is not surprising because evidence has been presented (Cilento and Zinner, 1967b) that protonated *p*-phenylenediamine may play such a role. Moreover, experiments to be reported at a later date indicate that tetramethyl-*p*-phenylenediamine-H^+ (H^+ denotes the protonated form) is a good catalyst for the oxidation of NADH and of its models; however differently from DIPAP, tetramethyl-*p*-phenylenediamine-H^+ does also catalyze the autoxidation of one electron or hydrogen atom donors, such as of *p*-phenylenediamines themselves and of *p*-hydroquinone.

Earlier work (Cilento and Zinner, 1966, 1967a–c) suggests that this nonclassical oxygen activation should be a common phenomenon. The presumed reason is that entrance of one electron in O_2 favors entrance of a second electron, whereby O_2 should be a better electron acceptor in the transition state of autoxidation reactions than in the ground state; hence electron donors may speed up these reactions by reducing the free energy of activation through preferential stabilization of the transition state. Yet it appears that proton donation by the ammonium group of DIPAP to the oxygen molecules must also play a fundamental role in view of the decreased activity in going from pH values near 6 to pH values near 7.5 and in view of the catalytical inactivity of 3,5-diiodotyrosine. From the flat shoulder at higher pH values in the activity pH profile one may infer that the efficiency of DIPAP increases with the generation of the better complexing phenolate form (Cilento and Berenholc, 1965), but that a compromise exists with the protonation of the amino group. This compromise may not exist if a thyroid compound works in an enzymic system, the enzyme acting as proton donor.

Still another factor may be involved. Thus, the nonhalogenated *p*-hydroxyanilonium cation is, on a molar basis, much less active. In this connection we should consider the possibility that in the catalyzed autoxidation herein described an hydride ion is transferred to oxygen. This possibility is strongly supported by the failure of DIPAP to catalyze the autoxidation of one electron or hydrogen atom donors, either at neutral or alkaline pH values. Such an H^- ion transfer should be forbidden by the spin conservation rule because the O_2 molecule has a triplet ground state (Halpern and Orgel, 1960)

$$\underset{\text{singlet}}{X\text{-}H} + \underset{\text{triplet}}{O_2} \longrightarrow \underset{\text{singlet}}{X^+} + \underset{\text{singlet}}{HO_2^-}$$

Indeed the reverse process in which a peroxide ion transfers an H^- ion to a suitable acceptor leads to electronically excited, singlet oxygen (McKeown and Waters, 1966). Since the spin conservation rule breaks down in the presence of atoms of large atomic number (McClure, 1949; Kasha, 1952; McGlynn *et al.*, 1964), it may be that the heavy 2,6-diiodosubstituents "remove" to some extent the forbiddenness (Cilento and Berenholc, 1964, 1965; Cilento *et al.*, 1965). This would be, to the best of our knowledge, the first case of direct H^- ion transfer to oxygen and also the first case of a thermal reaction in which heavy atom perturbation is a contributing factor.

The catalytic efficiency of DIPAP is amazing; it is seen not only in solvent addition to the 5,6-double bond of 1,4-dihydronicotinamides and in the oxidative processes herein reported, but also in promoting the reduction of cytochrome *c* by NADH (I. Polacow and G. Cilento, in preparation).

Biological Oxidations. The reported autoxidation of the reduced pyridine coenzymes without electron carriers is novel and exciting; yet its biochemical significance is not obvious. However the possibility of transferring the elements of the hydride ion from a biochemical to O_2 has a great potential biological importance, especially if we consider that the catalyst is related to thyroid compounds.

Pending the results of experiments in course, it may well be that a real case is the oxidation of the reduced coenzymes which have added a reagent across the 5,6-double bond. This is an especially attractive possibility when we consider that in presence of phosphate, besides the pyridinium ion other compounds are generated in higher concentrations in our "*in vitro*" systems. Phosphate is known to catalyze the hydration of dihydronicotinamides (Alivisatos *et al.*, 1964, 1965; Anderson *et al.*, 1965) presumably by first adding to the 5,6-double bond. It is tempting to speculate that in the cell, in the presence of a suitable catalyst, perhaps a thyroid compound, the compound is oxidized and an energy-rich phosphate bond is generated as suggested by Barltrop *et al.* (1963).

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