Phosphate Catalysis in the Autoxidation of 1-*n*-Propyl-6-hydroxy-1,4,5,6-tetrahydronicotinamide. A Model for Adenosine Triphosphate Formation*

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ABSTRACT: *N,N,N',N'*-Tetramethyl-*p*-phenylenediamine, which is a catalyst for the autoxidation of 1,4-dihydronicotinamides, also catalyzes the autoxidation of 1-*n*-propyl-6-hydroxy-1,4,5,6-tetrahydronicotinamide provided phosphate or arsenate is present. High yields of the pyridinium cation are obtained. The reaction is slower in D_2O. The pyridinium cation appears to be formed from an oxidized phosphorylated intermediate. In pyridine as solvent, energy-rich bonds are generated as shown by the presence of pyrophosphate. The possibility arises that the formation of ATP at the level of the pyridine coenzymes is a result of the tendency to aromatize.

On studying the TMPD[1]-catalyzed autoxidation of 1,4-dihydronicotinamides (Bechara and Cilento, 1971) it was observed that, under circumstances in which 1-*n*-propyl-1,4-dihydronicotinamide had been converted into the 5,6-hydrated derivative, O_2 uptake still occurred albeit at a lower rate. This finding seemed especially interesting because the compound undergoing oxidation was the hydrated product itself and not the parent 1,4-dihydronicotinamide which is in equilibrium with it; furthermore this TMPD-catalyzed autoxidation of the hydrated product only occurred if phosphate was present.

Because of the potential importance of this result in connection with a model for the generation of ATP at the level of the pyridine coenzymes in the respiratory chain, an investigation has been carried out.

Materials

The C-4 monodeuterio analog of 1-*n*-propyl-1,4-dihydronicotinamide was prepared by reducing the 1-*n*-propyl-3-carboxamidopyridinium chloride with $Na_2S_2O_4$ in D_2O (Suelter and Metzler, 1960). All the other compounds were described in the preceding paper (Bechara and Cilento, 1971).

To prepare PHTN and other hydrated dihydronicotinamides a solution of the dihydronicotinamide in phosphate buffer was kept in anaerobiosis until the long-wavelength absorption dropped to a very low, constant value; phosphate acts as catalyst in this hydration (Stock *et al.*, 1961; Johnston *et al.*, 1963; Alivisatos *et al.*, 1965; Anderson *et al.*, 1965). The system was opened and the concentration of the hydrated product ascertained from the absorbance maximum in the 290-mμ region (ϵ 2 $\times$ 10^4). The hydration was slower in D_2O (Kim and Chaykin, 1968). In preparing solutions of PHTN in Tris buffer, TMPD too was initially present, as its monoprotonated form acts as an efficient catalyst for the hydration process.

Methods

The TMPD-catalyzed autoxidation of PHTN has been studied at 25° by following oxygen uptake in the Warburg manometer. Unless otherwise specified, the solvent was 0.2 M phosphate buffer (pH 6.88 $\pm$ 0.01), the TMPD concentration 1.0 $\times$ 10^{-3} M, and that of PHTN close to 1.5 $\times$ 10^{-2} M. For solvent isotope studies, the substrate and buffer solutions were freshly prepared in D_2O. Consideration was given to the fact that $pD = pH_{(measured)} + 0.4$ (Glasoe and Long, 1960; Srere *et al.*, 1961).

The reported pH values are as read on a Metrohm potentiometer. Spectra were taken either on a Cary 14 recording spectrophotometer or on a Beckman DB spectrophotometer.

The products of the reaction were separated with a Dowex 50W-X4 (dry mesh 200–400; 4% cross-linked) ion-exchange resin as described in the preceding paper (Bechara and Cilento, 1971), except that smaller fractions (1 ml) were initially eluted when the main aim was the identification of the pyridinium cation. The reason is that the latter may be contaminated by traces of another cation which is eluted later.

Paper chromatography was according to Bernhart and Chess (1959); the temperature was 22°.

Results

Part of a recently prepared, TMPD containing, PHTN solution in Tris was diluted with Tris, another part with phosphate buffer. Oxygen uptake by these solutions is shown in Figure 1. The phosphate containing solution absorbs much more oxygen; final consumption is somewhat substoichiometric for water formation. The spectrum of both final reaction mixtures is dominated by a band peaking in the 290–295-mμ region. With phosphate containing solutions the absorption due to the pyridinium cation can usually be observed also.

In the above experiment PHTN was prepared in Tris buffer; routinely however it was obtained in phosphate buffer.

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[1] The following abbreviations are used: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; PHTN, 1-*n*-propyl-6-hydroxy-1,4,5,6-tetrahydronicotinamide.

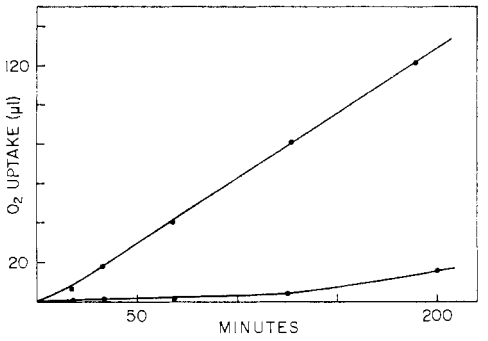


FIGURE 1: Oxygen uptake in the autoxidation of 15.1 mM PHTN catalyzed by 1.0 mM TMPD at pH 6.88. Lower curve, in 0.2 M Tris buffer; upper curve, in 0.1 M Tris–0.2 M phosphate buffer.

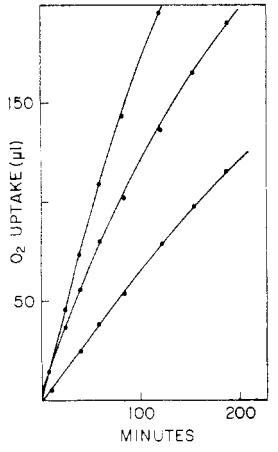
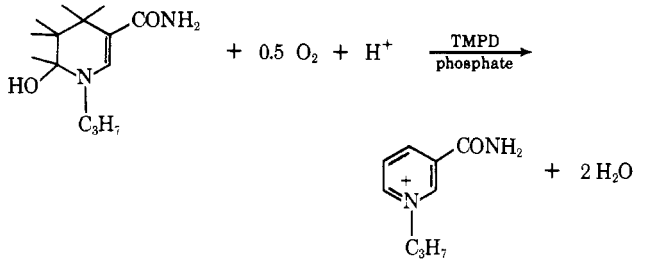


FIGURE 2: Oxygen uptake after addition of TMPD to a 16.0 mM solution of PHTN prepared in 0.2 M phosphate buffer (pH 6.87). The TMPD concentration was 2.0 mM (upper curve), 1.0 mM (middle), and 0.5 mM (lower). There was no absorption whatsoever in the absence of TMPD.

Under these conditions initial rates of O_2 uptake are considerably higher than that in Figure 1. A representative example showing also the effect of the TMPD concentration appears in Figure 2.

The pyridinium cation can be isolated from phosphate reaction mixtures with a Dowex cation-exchange resin (Figure 3) and easily identified (Figure 4). Yields are usually about 55%; they are considerably higher if calculated upon O_2 consumption as, during the long period of reaction, a fraction of PHTN undergoes a secondary modification reaction (Johnston *et al.*, 1963). In addition, the other cation which is eluted from the resin is formed in the preparation of PHTN and contributes to some extent to the absorption ascribed to PHTN.

The main overall reaction which takes place is



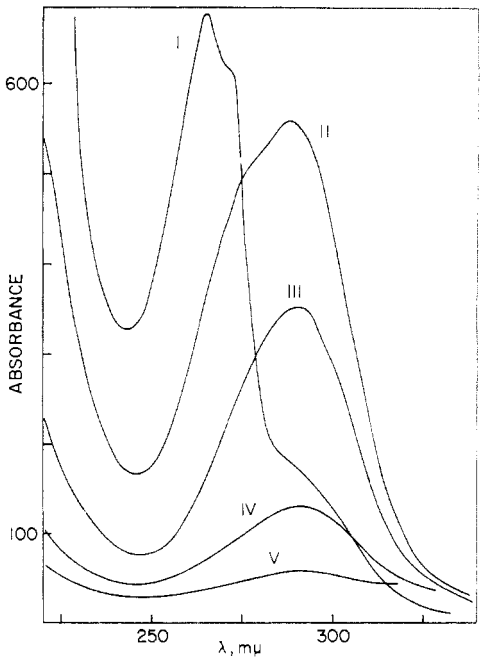
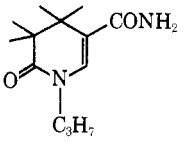


FIGURE 3: Separation (as described under Methods) of the cations present in the final reaction mixture in the autoxidation of 15 mM PHTN in 0.2 M phosphate buffer (pH 6.81), containing TMPD 1.0 mM. The curves represent the spectrum of the 5-ml fractions eluted with NH_4Cl. Numeration corresponds to the order of elution. Fraction I was diluted 1 to 4 and fraction II, 2 to 3. Fractions VI and VII did not absorb light. The cation eluted in fractions II to V does also form in the absence of O_2.

The spectrum of the material not retained by the column was similar to but not identical with that of PHTN. Partially oxidized mixtures afforded first unreacted PHTN and then the other similarly absorbing compound, which we tentatively suggest results from oxidation at C_6.



Effect of Varying the Concentration or the Nature of the Participants. Omission of TMPD or substitution of the latter by *p*-phenylenediamine totally abolished O_2 uptake. Addition of Mg^{2+} ion did not increase the rate.

Substituting phosphate by arsenate a 50% increase in rate was observed. Citrate, relative to Tris, was only slightly active.

Replacing the propyl group by benzyl in PHTN resulted in a much slower rate of O_2 uptake. Only the 1-benzyl-3-carboxamidopyridinium cation was eluted from the resin; it was easily identified by its ultraviolet spectrum. Hydrated NADH was not oxidized at all.

Table I shows the effect of increasing concentration of PHTN and of phosphate upon the initial rate of O_2 absorption. Figure 2 shows the effect of the TMPD concentration. A 150% increase in rate was observed on passing from air-saturated to pure O_2-saturated solutions.

Similar rates were observed at pH 6.8, 6.2, or 6.0 but a substantially lower one at pH 7.6. In methanolic phosphate buffer (1:1, v/v) the reaction was slower than in entirely aqueous buffer.

TABLE I: Dependence of the Initial Rate of the TMPD-Catalyzed Oxidation of 1-*n*-Propyl-6-hydroxytetrahydronicotinamide in Phosphate Buffer, pH 6.87, upon the Concentration of the Tetrahydronicotinamide and of Phosphate.[a]

Tetrahydronicotinamide (mM)	Phosphate (M)	μl of O_2 (min)[b]
17.0	0.20	1.04
34.0	0.20	1.32
48.0	0.20	1.51
17.0	0.20	1.04
17.0	0.40	1.21
17.0	0.66	1.36

[a] The concentration of TMPD was 1.0 mM. [b] Final volume, 3.1.

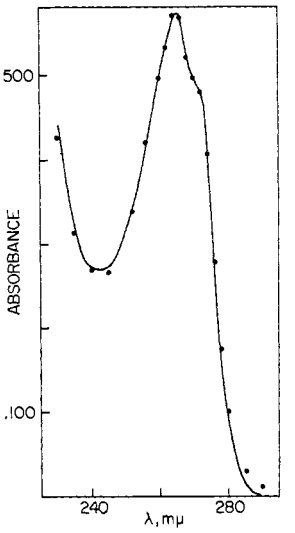
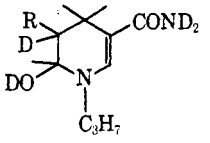


FIGURE 4: Matching of the spectrum of 1-*n*-propyl-3-carbonamidopyridinium chloride eluted from the resin (full curve) with that of an authentic sample (black circles).

Isotopic Substitutions. When PHTN was replaced by the C-4 monodeuterio analog, the rate was hardly affected.

In D_2O the rate of O_2 uptake by compound (R = H, D)



was smaller than that observed with PHTN in water and the pyridinium cation was formed in very low yields (<5%).

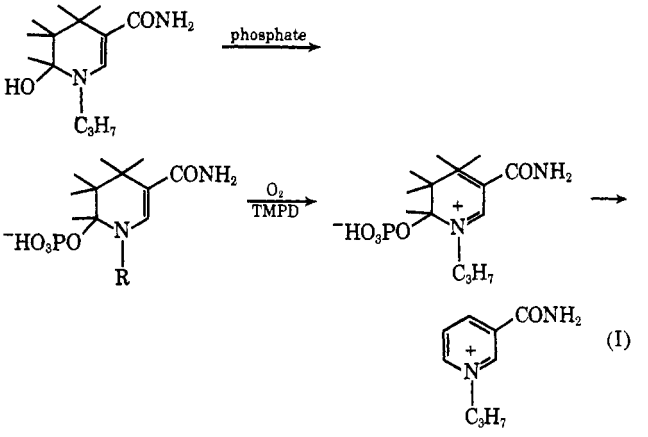
Formation of Pyrophosphate. Pyridine was used as solvent. Experiments were run under different conditions; thus the concentrations of phosphoric acid, dihydronicotinamide, and catalyst, the length of time during which oxygen was bubbled, and the temperature were varied. Positive results were always obtained. One of the most striking experiments is reported.

A warm solution 0.5 M in phosphoric acid (85–92%) was made 0.2 M in the dihydronicotinamide and after extensive saturation of the 5,6 double bond (as judged by the new absorption maximum around 290 mμ) was also made 0.02 M in TMPD. Oxygen was bubbled for 2 hr at 50. The pyridine was evaporated. Paper chromatography of the residue, dissolved in water, clearly showed pyrophosphate (R_F 0.55), behaving identically with an authentic sample (R_F 0.55). From another chromatogram the pyrophosphate was eluted, treated with pyrophosphate, and rechromatographed: only one spot was, as expected, observed.

Control experiments omitting the dihydronicotinamide were negative. Omitting the catalyst or by working *in vacuo* gave results which were negative or practically so. Actually a very faint positive result in the absence of catalyst is to be expected because of aerial oxidation (Barltrop *et al.*, 1963).

Discussion

The pyridinium cation is the main product of the PHTN oxidation. Although small amounts of the 1,4-dihydronicotinamide are in equilibrium with the 5,6-hydrated derivative, the pyridinium cation could not have directly been formed from the dihydronicotinamide as tentatively proposed by Johnston *et al.* (1963) when riboflavin acts as oxidant (Suelter, 1959). Thus the rate of the TMPD-catalyzed autoxidation of 1,4-dihydronicotinamides is first order in the concentration of the latter (Bechara and Cilento, 1971) and on the basis of the 1,4-dihydronicotinamide present no detectable autoxidation rate would be expected. Moreover, the pyridinium cation did not practically form in D_2O, whereas the TMPD-catalyzed autoxidation of 1,4-dihydronicotinamide is unaffected by substituting D_2O for H_2O (Bechara and Cilento, 1971). Therefore the pyridinium cation must have been formed secondarily from the oxidation product of PHTN. Considering that phosphate has never been found to influence the reactivity of oxygen (Cilento and Zinner, 1966, 1967a–c; Silva Araujo and Cilento, 1969) and that it is most likely incorporated in highly reactive intermediates we suggest eq I.



(I)

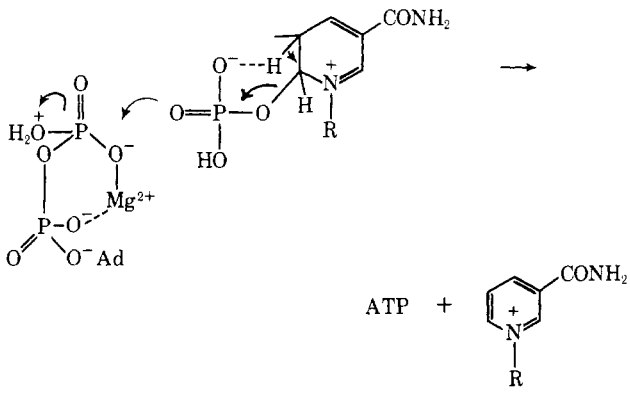
The fact that the rate is slower in D_2O accords with this scheme. Thus the oxidative phase will presumably be by the $HO_2\cdot$ chain mechanism and therefore unaffected by replacing H_2O by D_2O (Bechara and Cilento, 1971). On the other hand, formation of the phosphorylated intermediate, whether by addition of phosphate to the dihydronicotinamide in equilibrium with PHTN or to the aldehydic group resulting from ring-chain tautomerism in PHTN, should involve a

kinetically significant proton addition step and should therefore be slowed down in D_2O.

The very small formation of the pyridinium cation in D_2O is perhaps somehow connected with the much more difficult regeneration of the 5,6 double bond as saturation of this bond in 1,4-dihydronicotinamides is greatly slowed down in D_2O (Kim and Chaykin, 1968).

It is a result of the PHTN concentration-rate dependence (Table I) that there is practically no isotope effect when substituting C_4H by deuterium in PHTN. Probably the relatively small effect of PHTN and phosphate concentrations upon the rate of O_2 uptake may be due to the fact that in these experiments the initial rate of autoxidation of TMPD itself is relatively large.

The importance of the present work was to demonstrate that phosphate catalyzes the oxidation of a 6-hydroxy-1,4,5,6-tetrahydronicotinamide, that the pyridinium cation is the result of the oxidation, and that energy-rich bonds can be generated. Therefore the reaction is a model for the generation of ATP at the level of the pyridine coenzymes. The oxidation of the phosphorylated intermediate at C_6 would result in a scheme of the Barltrop type (Barltrop *et al.*, 1963). Oxidation at C_4 raises the interesting and more likely possibility that the formation of ATP may be connected with the tendency to aromatize, a process in which phosphate increases the acidity of the adjacent proton and also behaves as a good leaving group.



Among the 1,4-dihydronicotinamides generally employed as models of NADH and NADPH, the *N*-alkyl-substituted ones are usually more reactive. This view may now be extended to the 5,6-hydrated derivatives.

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