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Formation of Adenosine Triphosphate in the Oxidation of a Model for the Reduced Pyridine Nucleotides†

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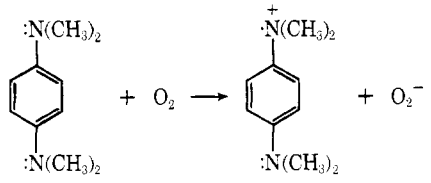
ABSTRACT: In the oxidation of 1-*n*-propyl-6-hydroxy-1,4,5,6-tetrahydronicotinamide to the 1-*n*-propyl-3-carboxamidopyridinium cation by the *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine–O_2 system in phosphate buffer, the Wurster's Blue radical is an active oxidizing species. FMN cannot replace this semiquinone. In pyridine–water (49:1, v/v) pyrophosphate is formed in yields up to 4% of the initial concentration of the dihydronicotinamide; if ADP is also present the yield of pyrophosphate is increased (up to 14%) and ATP is also formed (about 3%). In anhydrous pyridine, based upon the amount of reacted 1-*n*-propyl-1,4-dihydronicotinamide, a total yield of energy-rich bonds of about 24% could be observed. ATP may also be formed from AMP. The fact that energy-rich bonds may be generated from the oxidation of the 1,4-dihydronicotinamide itself and not necessarily from its hydrated form, reinforces the values of the model system. From the model system itself and from available information, a mechanism is tentatively suggested for the formation of ATP at the level of the pyridine coenzymes in mitochondria. The proposed mechanism is similar to that operating in the 3-phosphoglyceraldehyde dehydrogenase system.

Tetramethyl-*p*-phenylenediamine[1] catalyzes the autoxidation of 1,4-dihydronicotinamides (Bechara and Cilento, 1971a) and, provided phosphate is present, that of PHTN also (Bechara and Cilento, 1971b). In the latter case the reaction is of considerable interest as a model for ATP formation at the level of the pyridine coenzymes. Indeed the 1-*n*-propyl-3-carboxamidopyridinium cation is formed and in pyridine energy-rich bonds (pyrophosphate) are generated.

It was therefore important to verify if other oxidizing species, including flavins, could, like the TMPD–O_2 system, promote the oxidation at the C_4 (para) position of the hydroxytetrahydronicotinamide. Regarding the specificity of this oxidation it was of interest to know first if with the TMPD–O_2 system, the oxidizing species was the perhydroxyl radical (superoxide ion) or the Wurster's Blue semiquinone or both.



Next, for what concerns the generation of energy-rich bonds, the question arose if in the presence of ADP the reaction could lead to ATP. A further question was whether the generation of energy-rich bonds requires the hydroxytetrahydronicotinamide or whether the 1,4-dihydronicotinamide itself could be used for such a purpose. This paper deals with these further aspects of our model system and with possible biological implications.

Materials

TMPD·2HCl was purified as described in an earlier paper (Bechara and Cilento, 1971a). Wurster's Blue perchlorate was obtained by the procedure of Michaelis and Granick (1943). 1-*n*-Propyl-1,4-dihydronicotinamide, prepared according to Suelter and Metzler (1960), was recrystallized from water (mp 91–92°). NADH, FMN, and AMP were from Sigma Chemical Co. whereas NaATP was from Pabst Laboratories. Two different samples of Na_2ADP were used, one from Sigma Chemical Co., the other from Pabst Laboratories.

The tetra-*n*-butylammonium salts [$(Bu_4N)_3ADP$, $(Bu_4N)_2$-AMP, and $(Bu_4N)_2HPO_4$] were prepared by the method of Wieland and Bäuerlein (1967) as described by Lambeth and Lardy (1969). The solution of tetrabutylammonium hydroxide in benzene–methanol used in these preparations was in turn prepared from tetrabutylammonium iodide (BDH) and Ag_2O (Cundiff and Markunas, 1956). PHTN was formed as described under Methods. Superoxide dismutase was prepared according to McCord and Fridovich (1969).

Pyridine was carefully purified (Vogel, 1956). For reactions

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[1] Abbreviations used are: TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; PHTN, 1-*n*-propyl-6-hydroxy-1,4,5,6-tetrahydronicotinamide; Bu_4N, tetra-*n*-butylammonium.

in anhydrous media, pyridine was dried over KOH and the other reagents over P_2O_5.

Oxygen uptake was measured in Warburg. Spectra were taken either on a Zeiss DMR-21 or on a Cary 14 recording spectrophotometer. For ^{32}P measurements a Geiger-Muller tube (Nuclear-Chicago, Model 181 B) was empolyed. pH values were read on a Metrohm potentiometer.

Methods

Formation of Labile Phosphate. Most of the experiments concerning the generation of energy-rich bonds were carried out in pyridine containing 2% water but a few were carried out in anhydrous pyridine. The reason for selecting pyridine as solvent stems from the fact that it allows formation of pyrophosphate (Bechara and Cilento, 1971b) and dissolves the 1-*n*-propyl-1,4-dihydronicotinamide and the tetrabutylammonium salts of phosphoric acid, of AMP, and of ADP.

The initial concentration of the 1,4-dihydronicotinamide was 6.0×10^{-2} M. Phosphate (0.22 M) was added in the form of phosphoric acid to 98% pyridine and in the form of $(Bu_4N)_2HPO_4$ to anhydrous pyridine. However if in 98% pyridine a higher phosphate concentration was required, $(Bu_4N)_2HPO_4$ was added to the solution.

When the aim was to investigate the formation of ATP, 0.10–0.15 M $(Bu_4N)_3ADP$ was present in the reaction mixture. To investigate the possibility of ADP formation, the reaction was performed in the presence of 0.20 M $(Bu_4N)_2AMP$. In several cases a small amount of [^{32}P]phosphate was added to the reaction mixtures.

In the experiments in 98% pyridine, PHTN was always present either because we initially accelerated its formation from the dihydronicotinamide (by keeping the system at 50° during 2–3 hr under N_2) or because of the spontaneous formation from the dihydronicotinamide during the oxidative phase.

The oxidation was carried out in the presence of 1×10^{-2} M TMPD by bubbling oxygen for 2–3 hr through 2 ml of the reaction mixture at 48–50°. The solvent was removed *in vacuo* and the residue taken up in 2 ml of cold water. This solution was extracted five times with cold ether. In some instances the volume was made up to 3.0 ml.

Chromatographic Separation by Anion-Exchange Resin. The resin used was a Dowex 1-X2, 200–400 mesh in the chloride form. The position of the peaks corresponding to the elution of phosphate, pyrophosphate, AMP, ADP, and ATP was first ascertained with authentic samples and mixtures. This in turn permitted the determination of contaminants (such as ATP) and then their concentration in our samples of AMP and ADP. The procedure (Cohn, 1957) was as described below for the separation of products present in the reaction mixture.

The aqueous solution (0.5 ml), representing one-fourth or one-sixth of the final reaction mixture, was slowly passed through the column (0.8 × 6.0 cm). The column was thoroughly washed with water until no absorption in the ultraviolet region was detected. Elution of AMP, phosphate, and ADP was carried out with 250 ml of a solution 0.01 M in both HCl and KCl. To elute pyrophosphate and ATP, the concentration of components of the eluent solution was made 0.05 M and 75 ml was required. Operations were performed at 25°. Fractions of 5 ml were collected every 2–3 min.

Elution was followed by measurements at 260 mμ, where the adenine ring absorbs strongly, and when [^{32}P]phosphate was present in the initial reaction mixture also by measurements of radioactivity. Labile phosphate, that is phosphate resulting from hydrolysis of pyrophosphate and of ATP by 1.5 N H_2SO_4 at 92° for 30 min (Lambeth and Lardy, 1969), was determined according to Sumner (1944).

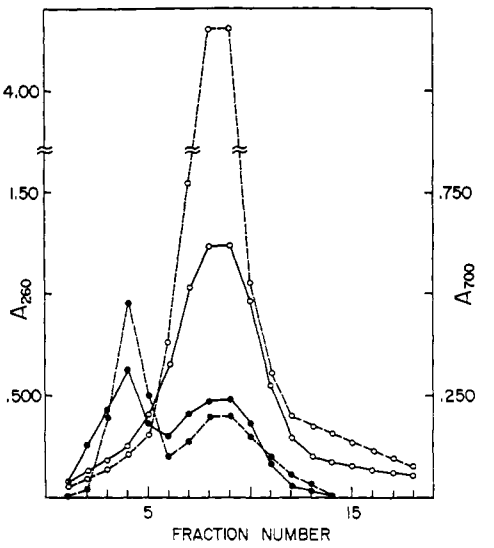


FIGURE 1: Representative example of the elution of pyrophosphate and ATP from the Dowex resin as described under Methods. The original reaction mixture in pyridine–water (49:1, v/v), containing 0.22 M phosphate and 0.15 M $(Bu_4N)_3ADP$, was 60 mM in 1-*n*-propyl-1,4-dihydronicotinamide and 10 mM in TMPD. Ordinates on the left represent the absorbance at 260 mμ (empty circles); ordinates on the right represent the absorbance at 700 mμ (solid circles) after acid hydrolysis and treatment by the Sumner method. The dotted curves were obtained with an authentic mixture containing 10 μmoles of pyrophosphate and 5 μmoles of ATP. It is to be pointed out that the development of the blue color for the authentic mixture was done under different conditions of hydrolysis (time and dilution).

As expected, the positions of the peaks corresponding to pyrophosphate and ATP were the same whether the absorbancy at 260 mμ, or the radioactivity or labile phosphate was measured. A representative example is presented in Figure 1.

Chromatographic Separation by Cation-Exchange Resin. While the separation of the 1-*n*-propyl-3-carboxamidopyridinium cation formed in aqueous mixtures presented no difficulty, a different situation obtains when pyridine is used as solvent, mainly because of contamination by the unsubstituted pyridinium cation.

The following procedure was adopted. One-fourth or one-sixth of the aqueous solution prepared from the final reaction mixture was brought to pH 8–9. The solution was passed through the Dowex 50W-X4, 200–400 mesh cation-exchange resin, the column thoroughly washed with water, and the cations eluted with a 0.1 or 1.0 M ammonium chloride solution (Bechara and Cilento, 1971a). The first cations to appear are the unsubstituted pyridinium cation and the 1-*n*-propyl-3-carboxamidopyridinium cation. The last one to appear was the 290-mμ-absorbing cation (Bechara and Cilento, 1971b).

The spectrum of the eluted fractions was run at acid and alkaline pHs. The difference spectrum was identical to the difference spectrum obtained with pyridine. This in turn permitted to calculate the concentration of pyridine in the eluted fractions. Therefore the spectrum of the unsubstituted pyridinium cation could be subtracted from the spectrum of the eluted fraction. The resulting spectrum was similar to that of the 1-*n*-propyl-3-carboxamidopyridinium cation except in the later fractions which also showed the presence of the 290-mμ

cation. Since the spectrum of this 290-mμ cation is known and since it only partially overlaps that of typical pyridinium derivatives, its contribution could also be subtracted. Often the spectrum of the 1-*n*-propyl-3-carboxamidopyridinium cation so determined was somewhat shifted to shorter wavelengths. That this is a result of the procedure could be ascertained by starting from reaction mixtures in which the dihydronicotinamide had been substituted by authentic 1-*n*-propyl-3-carboxamidopyridinium chloride.

The above procedure is admittedly lengthy and approximate. It was only applied to those eluted fractions which appeared to be rich in 1-*n*-propyl-3-carboxamidopyridinium chloride (strong absorbance in the 250- to 265-mμ region and small drop in absorbance with alkalinization).

Yields. The yields are calculated upon the initial concentration of the dihydronicotinamide. Since only a fraction of the dihydronicotinamide reacts in our systems (either directly or presumably also as PHTN) the true yields must be considerably higher. In presenting yields of ATP, the presence of the latter as a contaminant in ADP was taken in account.

Results

Oxidation by the TMPD–O_2 System in the Presence of Scavengers of the Superoxide Ion. The TMPD–O_2 system promotes the oxidation of PHTN either through the superoxide ion (chain mechanism) or through the Wurster's Blue semiquinone or through both. It was therefore of interest to verify if scavengers of the O_2^-(HO_2) radical, such as the enzyme superoxide dismutase (McCord and Fridovich, 1969) and catechol (Miller, 1970; Silva Araujo *et al.*, 1970), could inhibit the autoxidation of PHTN in water.

These scavengers were first tested upon the TMPD (1.0 mM)-promoted autoxidation of NADH (15 mM) and of 1-*n*-propyl-1,4-dihydronicotinamide (10 mM) in 0.2 M Tris buffer (pH 7.0). The enzyme (2.0×10^{-7} M) inhibited (23%) the initial rate of O_2 uptake by NADH and, at the level of 5×10^{-8} M, to an extent of 35% the rate of O_2 uptake by the model dihydronicotinamide. On the other hand, catechol (6.0×10^{-4} M) inhibited by 40% the rate of autoxidation of NADH. Yet these scavengers, even in high concentration (1×10^{-5} M superoxide dismutase and 1×10^{-3} M catechol), had no effect whatsoever upon the oxidation of PHTN by the TMPD–O_2 system in the presence of phosphate.

Wurster's Blue perchlorate was found to oxidize PHTN. Essentially the same O_2 uptake curve was obtained with either TMPD or Wurster's Blue perchlorate. EDTA (1×10^{-4} M) did not affect the results.

Oxidation by FMN. The autoxidation of 14 mM PHTN in the presence of 2 mM FMN was studied at pH 6.96 in 0.4 M Tris buffer and in a mixture of 0.2 M Tris plus 0.2 M phosphate buffers; the rates of O_2 uptake in microliters per hours were 18 and 27, respectively.

Several experiments were run in anaerobiosis, with PHTN (20 mM) and FMN (10–20 mM). The reactions were followed up by the increase in absorbance at 1000 mμ where the FMN·$FMNH_2$ charge-transfer complex absorbs strongly (Gibson *et al.*, 1962). In every case the increase in absorbance was much faster in Tris–phosphate than in Tris buffer. In Tris–phosphate a flocculent green precipitate was formed with time. In comparative experiments in 0.49 M buffers, as soon as turvation started, both reaction mixtures were analyzed for the 1-*n*-propyl-3-carboxamidopyridinium cation. Surprisingly the yield in Tris–phosphate (17%) was only slightly larger than that in Tris (14%).

Oxidation by KBr_3. The procedure was similar to that employed by Barltrop *et al.* (1963) when they attempted the formation of pyrophosphate from the oxidation of 1-methyl-1,4-dihydronicotinamide. We performed the reaction for 2 hr at 60°. The following concentrations were used: PHTN, 19 mM; KBr_3, 0.16 M; NaH_2PO_4, 0.16 M; and $MgCl_2$, 78 mM.

The 1-*n*-propyl-3-carboxamidopyridinium cation was formed in 17% yield, but no pyrophosphate could be detected. No products absorbing maximally near 290 mμ were found.

Formation of Pyrophosphate and ATP in the Oxidation by the TMPD–O_2 System. In aqueous solutions despite numerous attempts in no instance could any pyrophosphate or ATP be found. Negative results were also obtained in the presence of Ca^{2+}, Mg^{2+}, or Mn^{2+} ions. Neither using [^{32}P]phosphate, could any pyrophosphate be detected.

In pyridine–water (49:1, v/v) the reaction produces pyrophosphate as detected by paper chromatography (Bechara and Cilento, 1971b). Pyrophosphate has now been isolated by an anion-exchange resin. The yields as a function of the phosphate concentration were: 0.22 M, 2.0%; 0.32 M, 3.1%; 0.6 M, 3.7%. In three other experiments in which phosphate was 0.22 M and $(Bu_4N)_3$ADP in concentration 0.15 M was also present, the yield of pyrophosphate ranged from 5.3 to 6.1% and ATP was formed in 3.0–3.3% yields.

Since these results suggested that ADP increases the yield of pyrophosphate, two parallel experiments were run. In the first experiment the phosphate concentration was 0.62 M and $(Bu_4N)_3$ADP, 0.15 M, was also present. In the second, the ADP salt was omitted. In the first experiment the yields were 14.3% for pyrophosphate and 2.6% for ATP. In the second the yield of pyrophosphate was 4.0%. Control experiments in which only ADP and phosphate were present showed that the increased pyrophosphate formation was not due to a direct reaction between ADP and phosphate.

Other experiments were run to test the influence of 1×10^{-3} M $MgCl_2$ and of 2.0×10^{-3} M, 2,4-DNP upon the yield of energy-rich bonds but no effect could be noticed. In anhydrous pyridine containing 0.2 M $(Bu_4N)_2HPO_4$ and 0.1 M $(Bu_4N)_3$ADP the yields were 2.1% for pyrophosphate and 4.0% for ATP. However considering that only one-fourth of the 1,4-dihydronicotinamide reacted, the true yields are 8% and 16%; the yield of the 1-*n*-propyl-3-carboxamidopyridinium cation was approximately stoichiometric.

In another experiment in anhydrous pyridine, but with a lower $(Bu_4N)_2HPO_4$ concentration (0.1 M) and 0.2 M $(Bu_4N)_2$-AMP instead of ADP, the yield of pyrophosphate dropped to less than 1%, ADP was formed in 3.0% yield, and ATP in 1.5% yield. Since again only one-fourth of the dihydronicotinamide reacted, the true yield of ADP is 12%, that of ATP 6%, and therefore the total yield of energy-rich bonds no less than 24%.

Discussion

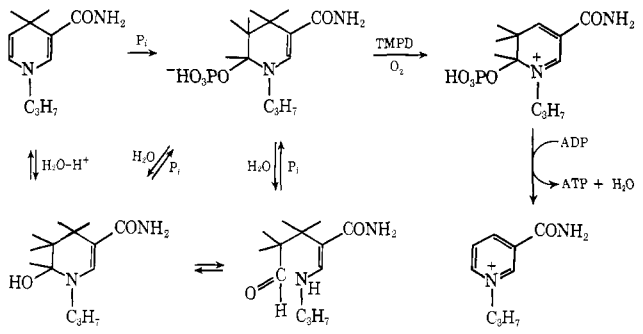
Specificity of the Oxidant Species. The fact that the same rate of oxidation of PHTH by the TMPD–O_2 system is observed in the presence of superoxide dismutase, that is under conditions in which the O_2^-–$HO_2\cdot$ species could not have existed, might suggest that the Wurster's Blue radical semiquinone is the primary oxidizing species

$$\text{TMPD} + O_2 \longrightarrow S^+ + O_2^-$$

$$\text{PHTN–P}_i + S^+ \longrightarrow \text{PHTN}\cdot\text{–P}_i + \text{TMPD} + H^+$$

$$\text{PHTN}\cdot\text{–P}_i + O_2 \longrightarrow P^+ + P_i + O_2^-$$

SCHEME I



In this scheme S^+ is the Wurster's Blue semiquinone PHTN–P_i is the hypothetical phosphorylated intermediate and P^+ is the 1-*n*-propyl-3-carboxamidopyridinium cation. Consistent with this scheme, Wurster's Blue perchlorate was found to oxidize PHTN. However the operation of the $HO_2\cdot$ chain mechanism—which is of primary importance in the case of 1,4-dihydronicotinamides (Bechara and Cilento, 1971a)—cannot be excluded as the nonobservance of inhibition in the presence of superoxide dismutase might also be due to inactivation of the enzyme by the system under investigation.

As when the TMPD–O_2 is the oxidizing system, the oxidation of PHTN with FMN goes faster in phosphate than in Tris buffer. Yet with FMN the faster reaction in phosphate might result from the oxidation of the 1-*n*-propyldihydronicotinamide (Johnston *et al.*, 1963), whose regeneration from PHTN should be catalyzed by phosphate. On the other hand, if phosphate leads to the formation of a more reactive intermediate, the greater reactivity of the latter toward FMN is not manifested at the C_4 positions as the yields of the 1-*n*-propyl-3-carboxamidopyridinium cation were very similar in Tris and Tris–phosphate buffers. It is pertinent to note that Suelter and Metzler (1960) observed that the oxidation of 1-*n*-propyl-1,4-dihydronicotinamide by riboflavin is faster in phosphate than in amine buffers, a fact which they tentatively ascribed to general acid catalysis by the $H_2PO_4^-$ anion.

When KBr_3 reacted with PHTN the yield of the pyridinium cation was only 17%. In conclusion our results indicate that the ability of the TMPD–O_2 system to promote the oxidation of PHTN to the 1-*n*-propyl-3-carboxamidopyridinium cation in phosphate buffer is not matched by any one of the other oxidizing species tested.

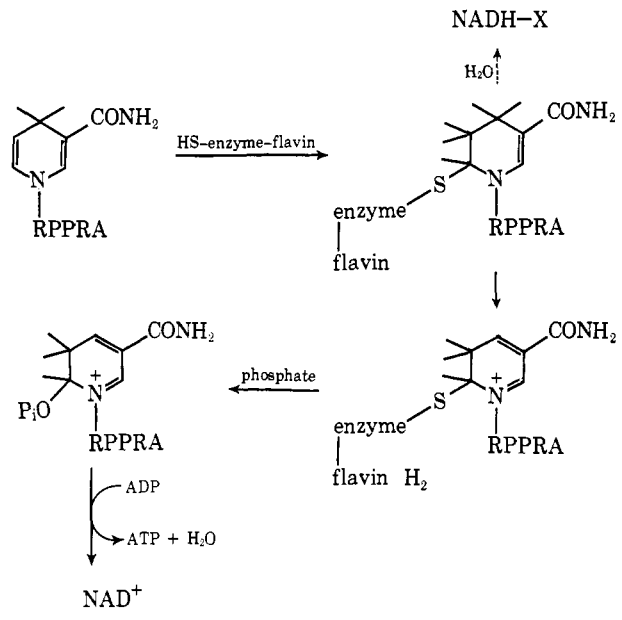
Generation of Energy-Rich Bonds. The present work clearly shows that energy-rich bonds are formed in the oxidation of 1-*n*-propyl-1,4-dihydronicotinamide in pyridine. ADP acts as phosphate acceptor not only because ATP is formed, but also because of pyrophosphate generation. In the latter case AMP is presumably also formed and phosphorylated again. However in the process despite the substitution of a energy-rich

$$ADP + X\sim P_i \longrightarrow AMP + P\sim P_i + X$$

bond (of ADP) by another (in pyrophosphate) and therefore no net gain, a high-potential phosphate ($X\sim P_i$) should be involved. Therefore here the formation of pyrophosphate also counts as energy-rich bond.

The explanation we have proposed for the generation of labile phosphate (Bechara and Cilento, 1971b) demands a close connection between formation of the pyridinium cation and the generation of the energy-rich bonds. Although there

SCHEME II



is little doubt that in our reaction mixture the 1-*n*-propyl-3-carboxamidopyridinium cation is formed, its identification and determination could not be very accurate. Even so it seems safe to infer that in our system for every few molecules of the 1,4-dihydronicotinamide oxidized one energy-rich bond is generated. This presumably occurs in Scheme I.

The alternative possibility, oxidation at the C_6 position of the phosphorylated intermediate seems to be excluded for the reaction studied by Barltrop *et al.* (1963) because we could not detect amide formation in their system. We hope to investigate our model reaction in other solvents which might allow a better determination of the 1-*n*-propyl-3-carboxamidopyridinium cation, although preliminary experiments indicate other difficulties. At any rate, as complemented by the present work, our model system is almost perfect: a 1,4-dihydronicotinamide is oxidized by a semiquinone to the pyridinium form with generation of ATP. In oxidative phosphorylation, NAD^+ is the primary product of NADH oxidation (Chaykin, 1967). Furthermore as radical reactions generally require a much lower activation free energy than nucleophilic attack, it is likely that they are important in oxidative phosphorylation (Cooper *et al.*, 1968).

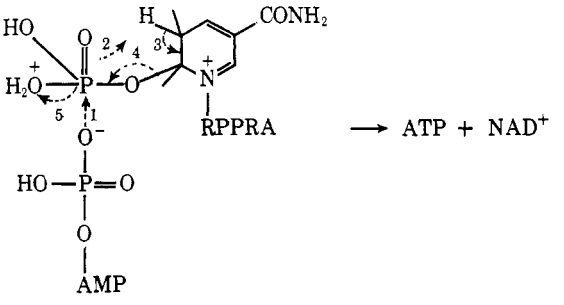
Biological Implications. If our mechanism of generation of energy-rich bonds is operative in mitochondria, the oxidizing species is a flavoprotein. When one considers that the specificity of flavin action is dependent upon the apoenzyme (Dixon, 1971) it is by no means disturbing that in our model system, FMN as opposed to the TMPD–O_2 system, did not lead to a substantial greater formation of the pyridinium cation from PHTN in phosphate buffer. Actually from the observation that the Wurster's Blue semiquinone seems to be the primary oxidizing species in the model system one might even infer that in mitochondria the semiquinone form of the flavin is involved in the oxidation. We tentatively suggest the following adaptation of our scheme to the situation in mitochondria (Scheme II).

This scheme is consistent with recent views on mitochondrial oxidation phosphorylation (Cross *et al.*, 1970; Sabadie-Pialoux and Gautheron, 1971). The scheme has obvious similarities with the generally accepted mechanism of ATP

formation by the glyceraldehyde-3-phosphate dehydrogenase system. The main difference is that in our scheme oxidation does not take place at the carbon to which sulfur is bound; this is as required by a prompt regeneration of the oxidized form of the coenzyme. This regeneration is known to be concomitant with the formation of ATP (Chaykin, 1967). On the other hand the mechanism proposed by Barltrop *et al.* (1963) requires two functionally different molecules of the pyridine coenzyme.

It is interesting to note that the enzyme 3-phosphoglyceraldehyde dehydrogenase may promote the formation of NADH-X (Rafter *et al.*, 1954; Hilvers *et al.*, 1966) presumably through a preliminary addition to the 5,6-double bond of the coenzyme by way of an $-SH$ group.

The last step of the above scheme deserves some comment. In the earlier paper (Bechara and Cilento, 1971b) we proposed that the phosphate linked to the pyridine ring would attack the terminal phosphate in ADP. Since isotopic data indicates that the terminal oxygen bridge of ATP formed in mitochondria comes from ADP (Boyer, 1957) a slight modification as shown below is necessary.



Formally there would not be enough energy for step 1 to be followed by step 5. Probably a pentacovalent intermediate is first formed in which pseudorotation occurs. This allows the 90° geometrical relationship between the entering ADP group and the leaving water molecule (McLick and Korman, 1971).

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