# Coenzyme $B_{12}$ Dependent Propanediol Dehydratase System. Nature of Cobalamin Binding and Some Properties of Apoenzyme–Coenzyme $B_{12}$ Analog Complexes†

Tetsuo Toraya, Masao Kondo, Yoshiharu Isemura, and Saburo Fukui*

ABSTRACT: New information regarding the binding of coenzyme $B_{12}$ analogs to propanediol dehydratase was obtained from a study of apoenzyme–coenzyme $B_{12}$ analog complexes which may be used as a model for the holoenzyme. Requirements for the binding of some coenzyme analogs, such as cyano- and methylcobalamin, to the apoenzyme were the same as those for coenzyme $B_{12}$ binding. A monovalent cation such as potassium ion was absolutely required; the substrate also facilitated the binding, but to a lesser degree. Cyanocobalamin was bound to the apoenzyme to approximately the same extent as coenzyme $B_{12}$. Treatment of the apoenzyme with a sulfhydryl inhibitor, *p*-chloromercuribenzoate, caused a remarkable inhibition of the binding of these cobalamins. The apoenzyme–cyanocobalamin and apoenzyme–methylcobalamin complexes, like the holoenzyme, resisted inactivation by *p*-chloromercuribenzoate. These results suggest that the binding of coenzyme $B_{12}$ to the apoenzyme is similar to the binding of these coenzyme analogs, and that mercurial-sensitive sulfhydryl group(s) are involved at the cobalamin binding site of the enzyme. On the other hand, excessive amounts of hydroxocobalamin were bound to the apoprotein, irrespective of *p*-chloromercuribenzoate treatment of the apoprotein and of the presence of inorganic monovalent cation. Although the apoenzyme–hydroxocobalamin complex is not resolvable upon Sephadex G-25 gel filtration, even in the absence of both monovalent cation and substrate in contrast to other apoenzyme–cobalamin complexes, it could be resolved by Sephadex G-25 gel filtration in the absence of both monovalent cation and substrate after treatment with urea–dithiothreitol or KCN. The enzyme-bound cyanocobalamin did not appreciably react with KCN to form dicyanocobalamin. The greater stabilities of apoenzyme–coenzyme $B_{12}$ analog complexes to heat and to change in pH as compared to the apoenzyme might suggest that the apoenzyme acquires much greater stability by forming the holoenzyme.

Coenzyme $B_{12}$[1] dependent propanediol dehydratase (DL-1,2-propanediol hydro-lyase, EC 4.2.1.28) from *Aerobacter aerogenes* catalyzes the conversion of L- or D-1,2-propanediol to propionaldehyde and of 1,2-ethanediol to acetaldehyde (Lee and Abeles, 1963). Some analogs of coenzyme $B_{12}$, such as cyano-, hydroxo-, and methylcobalamin, inhibit coenzyme $B_{12}$ requiring enzymes, such as propanediol dehydratase (Lee and Abeles, 1963), glycerol dehydratase (Pawelkiewicz and Schneider, 1967), and ethanolamine deaminase (Kaplan and Stadtman, 1968b; Babior, 1969), very strongly and almost irreversibly. It has been generally accepted that the powerful inhibition by these cobalamins is due to their tight binding to an apoenzyme, forming inactive, undissociable apoenzyme–coenzyme analog complexes. These complexes themselves are catalytically inactive, and no method for resolving the complexes into the native apoenzyme and cobalamin under mild conditions has hitherto been found. Therefore, the properties of apoenzyme–coenzyme $B_{12}$ analog complexes remain almost unknown in any coenzyme $B_{12}$ dependent enzymes, except for some properties of the glycerol dehydratase apoenzyme–hydroxocobalamin complex (Schneider *et al.*, 1970).

The knowledge of the structure and properties of holoenzyme would provide useful information on the mechanism of action of coenzyme $B_{12}$ dependent enzymes. However, it is difficult to investigate the above-mentioned enzymes on the basis of this idea, since the holoenzymes undergo rapid, irreversible inactivation in the absence of substrate under aerobic conditions (Lee and Abeles, 1963; Wagner *et al.*, 1966; Schneider and Pawelkiewicz, 1966; Kaplan and Stadtman, 1968a,b). Hence, a study of properties of apoenzyme–coenzyme $B_{12}$ analog complexes appears to be of much interest and significance as a model for the holoenzyme.

Our previous papers (Toraya *et al.*, 1970, 1971) established the role of monovalent cations in the binding of coenzyme $B_{12}$ or its analogs to the propanediol dehydratase apoenzyme and presented a method for resolving the apoenzyme–coenzyme $B_{12}$ and certain apoenzyme–coenzyme analog complexes except for the apoenzyme–hydroxocobalamin complex. The apoenzyme thus obtained was reconstitutable into the catalytically active holoenzyme. The present communication describes two methods for resolving the apoenzyme–hydroxocobalamin complex under mild conditions. These techniques were applied in this study to investigate the nature of the binding of coenzyme $B_{12}$ analogs to the apoenzyme of propanediol dehydratase and some properties of apoenzyme–coenzyme $B_{12}$ analog complexes.

## Materials and Methods

*Materials.* The crystalline coenzyme $B_{12}$ used in this study was the gift from Yamanouchi Pharmaceutical Co., Tokyo, Japan. Cyano- and hydroxocobalamin were obtained from Glaxo Lab, Greenford, U. K. Methylcobalamin was prepared

† From the Laboratory of Industrial Biochemistry, Department of Industrial Chemistry, Faculty of Engineering, Kyoto University, Kyoto, Japan. *Received December 22, 1971.* Paper II in this series. The first publication of this series is Toraya *et al.* (1971).

[1] Abbreviations used are: coenzyme $B_{12}$, α-(5,6-dimethylbenzimidazolyl)-*Co*-5′-deoxyadenosylcobamide or 5′-deoxyadenosylcobalamin; $CNB_{12}$, cyanocobalamin; $OHB_{12}$, hydroxocobalamin; $CH_3B_{12}$, methylcobalamin; *p*CMB, *p*-chloromercuribenzoate; *p*HMB, *p*-hydroxymercuribenzoate.

TABLE I: Effects of Potassium Ion and Substrate on Resolution of Apoenzyme–Cyanocobalamin Complex by Sephadex G-25 Gel Filtration.[a]

| Enzyme | Buffer[b] | Sp Act. (Units/mg) | Resolution (%) |
|---|---|---|---|
| Apoenzyme[c] | 0.05 M Tris·HCl | 0.37 | (100) |
| Apoenzyme·$CNB_{12}$ | 0.05 M Tris·HCl | 0.35 | 95 |
| | 0.05 M Tris·HCl–0.10 M 1,2-propanediol | 0.22 | 60 |
| | 0.05 M Tris·HCl–0.10 M KCl | 0.01 | 3 |
| | 0.05 M Tris·HCl–0.10 M KCl–0.10 M 1,2-propanediol | 0.00 | 0 |

[a] Mixtures (2.0 ml), containing 4.0 units of the apoenzyme–cyanocobalamin complex, were chromatographed on a column (1.6 × 20 cm) of Sephadex G-25 (fine) using the four different buffers indicated. The flow rate was about 6 drops per minute. The degree of the resolution was determined as described in the text. [b] All buffers, pH 8.0. [c] Apoenzyme was treated in a similar manner as control.

by the procedure of Smith and Mervyn (1963). All other chemicals were reagent grade commercial products and were used without further purification. Propanediol dehydratase apoenzyme was prepared from *Aerobacter aerogenes* (ATCC 8724) and assayed by the same procedure as described previously (Toraya *et al.*, 1971), which is similar to that of Lee and Abeles (1963).

*Formation of Apoenzyme–Coenzyme $B_{12}$ Analog Complexes.* Apopropanediol dehydratase was completely converted into apoenzyme–coenzyme $B_{12}$ analog complexes according to the procedure described before (Toraya *et al.*, 1971), except for the slight variation of the compositions of incubation mixtures.

*Resolution of Apoenzyme–Coenzyme $B_{12}$ Analog Complexes and Determination of Degree of Resolution.* After being treated as described below, the mixture containing the apoenzyme–cyanocobalamin or apoenzyme–methylcobalamin complex was subjected to gel filtration on Sephadex G-25 (fine) column (1.6 × 20 cm) using 0.05 M Tris·HCl buffer (pH 8.0) as an eluting agent. In the case of the apoenzyme–hydroxocobalamin complex, a mixture containing 4.0 units of the complex was incubated at 37° for 15 min either with 6 M urea, 20 mM dithiothreitol, and 0.10 M potassium phosphate buffer (pH 8.0) (urea–dithiothreitol method) or with $5 \times 10^{-3}$ M KCN (KCN method) in a final volume of 2.0 ml and was then subjected to gel filtration on Sephadex G-25 as described above. The conditions used for the urea–dithiothreitol treatment were identical with those employed by Taylor (1970) for resolving *Escherichia coli* B $N^5$-methyltetrahydrofolate–homocysteine holomethyltransferase. The degree of the resolution of a complex was determined by assaying the resolved apoenzyme activity in the complete system as described earlier (Toraya *et al.*, 1971). In the KCN method, the protein-containing fractions of gel filtration were incubated at 37° for 15 min with 0.10 M 1,2-propanediol prior to estimation of the degree of the resolution in order to obtain the maximum activity of the resolved apoenzyme. Protein was routinely determined by the procedure of Lowry *et al.* (1951). Crystalline bovine serum albumin was used as the standard.

*Binding of Coenzyme $B_{12}$ and Its Analogs to Native and pCMB-Treated Apoenzymes.* pCMB treatment of the apoenzyme was achieved by incubating the apoenzyme with $8.3 \times 10^{-4}$ M pCMB at 37° for 15 min. The native apoenzyme, 219 units, or the same amount of pCMB-treated apoenzyme was incubated at 37° for 15 min with 80 μM coenzyme $B_{12}$ or other cobalamin in the presence of 0.12 M KCl–0.40 M 1,2-propanediol–0.01 M potassium phosphate buffer (pH 8.0), in a total volume of 25 ml. This treatment led to complete complex formation. In order to remove the excess unbound cobalamin, the entire mixture was applied to a column (3.6 × 30 cm) of Sephadex G-25 (fine) equilibrated previously with 0.05 M potassium phosphate buffer (pH 8.0) containing both 0.10 M 1,2-propanediol and 0.05 M KCl, and was then eluted with the same buffer. Under the conditions employed for the gel filtration, neither apoenzyme–coenzyme $B_{12}$ nor apoenzyme–coenzyme analog complexes should be resolved at all (Toraya *et al.*, 1971). The bound cobamide was extracted from the protein-containing fraction by the method of Ertel *et al.* (1968). The amount of cobamide extracted as the dicyano derivative was determined by the microbiological and spectrophotometric assay methods. *E. coli* 215, a vitamin $B_{12}$ or methionine-requiring mutant strain isolated by Hayashi *et al.* (1955), was used as a test organism in the turbidimetric assay (Kato *et al.*, 1964). The spectrophotometric assay was based on the molar extinction coefficient at 367 nm for dicyanocobalamin, $\epsilon_{367}$ $30.4 \times 10^3$ $M^{-1}$ $cm^{-1}$ (Barker *et al.*, 1960).

## Results

*Factors Necessary for Binding of Coenzyme $B_{12}$ Analogs to Apopropanediol Dehydratase.* Requirements for the binding of coenzyme $B_{12}$ analogs to the apoenzyme were investigated by means of the gel filtration method. Table I summarizes the extent of the resolution of the apoenzyme–cyanocobalamin complex by the Sephadex G-25 gel filtration using four different buffers. No resolution occurred when the complex was subjected to gel filtration in the presence of potassium ion, irrespective of the presence of the substrate. In contrast, the complex was almost completely resolved upon gel filtration in the absence of both potassium ion and substrate, yielding the apoenzyme. On the other hand, a greater part of the complex was resolved when gel filtration was performed using Tris·HCl buffer containing the substrate only.

The apoenzyme–methylcobalamin complex could also be resolved upon gel filtration in the absence of both potassium ion and substrate (Toraya *et al.*, 1971). These results indicate that a monovalent cation such as potassium ion is the most essential factor for the binding of the apoenzyme with cyano- or methylcobalamin. The substrate also facilitated the binding and retarded the resolution, but to a lesser degree. This conclusion is consistent with the case of the binding of coenzyme $B_{12}$ to the apoenzyme (Toraya *et al.*, 1971).

*Resolution of Apoenzyme–Hydroxocobalamin Complex.* Of the coenzyme analogs tested, only hydroxocobalamin could be attached to the apoprotein without monovalent cation or substrate. The resulting apoenzyme–hydroxocobalamin complex was not resolvable upon the Sephadex G-25 gel filtration procedure (Toraya *et al.*, 1971). For the res-

TABLE II: Resolution of Apoenzyme–Hydroxocobalamin Complex by the Urea–Dithiothreitol Resolution Method.[a]

| Type of Treatment | Sp Act. (units/mg) | Recovery (%) | Resolution (%) |
|---|---|---|---|
| Native apoenzyme[b] | 0.51 | (100) | |
| Urea–dithiothreitol treated apoenzyme[c] | 0.14 | 27 | (100) |
| Urea–dithiothreitol treated apoenzyme·$OHB_{12}$ | 0.14 | 27 | 100 |

[a] Experimental procedure is described in the text. [b,c] Native and urea–dithiothreitol treated apoenzymes were treated in a similar manner as controls of the recovery and the resolution, respectively.

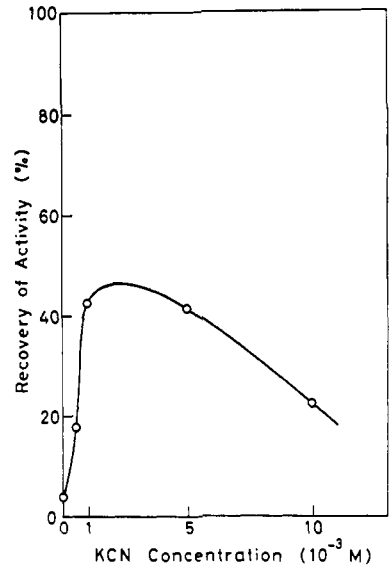



FIGURE 1: Effect of KCN concentration on the resolution of the apoenzyme–hydroxocobalamin complex. Mixtures (1.8 ml), containing 4.0 units of the apoenzyme–hydroxocobalamin complex, were treated at 37° for 30 min with the indicated concentrations of KCN in a final volume of 2.0 ml and then subjected to the Sephadex G-25 gel filtration and the determination of the degree of the resolution as described in the text. Apoenzyme incubated without KCN was treated in a similar manner as control (100%).

olution of this complex, the following two methods were found to be effective. Both of them involve the chemical conversion of the enzyme-bound hydroxocobalamin to other cobalamin derivatives which are resolvable upon gel filtration in the absence of both potassium ion and substrate.

A. UREA–DITHIOTHREITOL METHOD. The treatment of the apoenzyme–hydroxocobalamin complex with the urea–dithiothreitol resolving system was followed by the Sephadex G-25 gel filtration using Tris·HCl buffer. This resolving system can be expected to reduce the protein-bound hydroxocobalamin and to destroy the higher order structures of the enzyme. Table II indicates that the complex was completely resolved by this method. The recovery, however, of the apoenzyme was rather low (about 27%) because of the severity of the conditions employed.

B. KCN METHOD. The KCN treatment of the apoenzyme–hydroxocobalamin complex and the subsequent Sephadex G-25 gel filtration using Tris·HCl buffer were also effective for the resolution of the complex. The protein-bound hydroxocobalamin would be converted into the cyano derivative(s) of the cobalamin. The presence of the substrate in eluting buffer diminished the resolution by this method, in agreement with the case of the resolution of the apoenzyme–cyanocobalamin complex (Table I). Figure 1 shows the remarkable dependency of the recovery of the resolved apoenzyme on the concentration of KCN. A KCN concentration of $5 \times 10^{-3}$ M was chosen as the optimum. The activity of the resolved apoenzyme increased with the time of standing at 0°. Figure 2 shows the dependency of the activity of the resolved apoenzyme on the time of incubation at 37° with the substrate and/or potassium ion. The incubation of the resolved apoenzyme at 37° for 15 min with the substrate elicited the maximum activity, irrespective of the presence of potassium ion, while the incubation in the absence of substrate could not increase the activity. It seems likely that the substrate accelerates the renaturation of the resolved apoprotein into the native conformation. Figure 3 depicts the dependency of the degree of the resolution on the time of incubation with KCN and suggests that the conversion of bound hydroxocobalamin into other cobalamin derivative(s) was completed by the KCN treatment at 37° for 10–20 min. A 15-min incubation was carried out in the following experiments. Under the optimal conditions the KCN resolution procedure provided the high recovery (about 55–60%) of the active apoenzyme from the apoenzyme–hydroxocobalamin complex, as compared with the urea–dithiothreitol method, even though the resolution of the complex was still incomplete (about 75% resolution).

It appeared of interest to investigate what kind of cobalamin(s) was formed from the enzyme-bound hydroxocobalamin by the KCN treatment. As shown in Table V, excessive amounts of hydroxocobalamin were attached to the apoenzyme in such a manner that the cobalamin could not be removed by Sephadex G-25 gel filtration. From this observa-

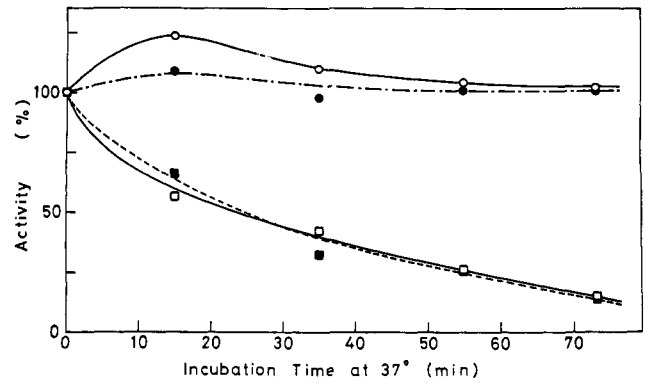



FIGURE 2: Effects of the substrate and potassium ion on the time course change of the activity of the apoenzyme resolved from the apoenzyme–hydroxocobalamin complex. A mixture (1.8 ml), containing 4.0 units of the apoenzyme–hydroxocobalamin complex, was treated at 37° for 30 min with $5 \times 10^{-3}$ M KCN in a final volume of 2.0 ml and then subjected to the Sephadex G-25 gel filtration as described in the text. After incubation of the protein-containing fractions at 37° for the indicated time with or without added substrate and/or KCl, the activity of the resolved apoenzyme was assayed as described in the text. Additions: (□-□) none; (■--■) 0.10 M KCl; (○-○) 0.10 M 1,2-propanediol; (●——●) 0.10 M 1,2-propanediol plus 0.10 M KCl.

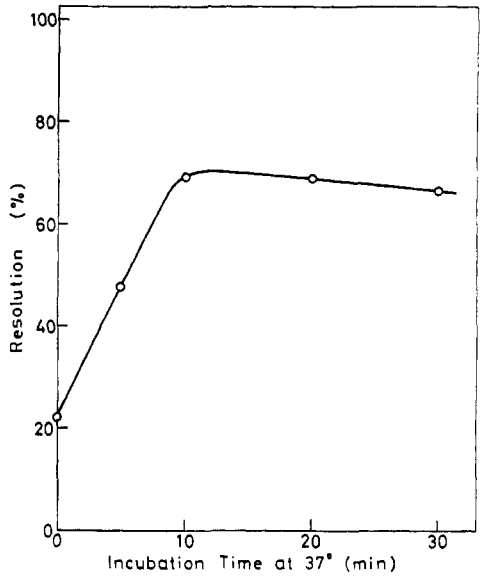



FIGURE 3: Effect of the time of incubation with KCN on the resolution of the apoenzyme-hydroxocobalamin complex. Mixtures (1.8 ml), containing 4.0 units of the apoenzyme-hydroxocobalamin complex were treated at 37° for the indicated time with $5 \times 10^{-3}$ M KCN in a final volume of 2.0 ml, and then subjected to the Sephadex G-25 gel filtration and the determination of the degree of the resolution as described in the text. Apoenzyme was treated in a similar manner as control (100%).

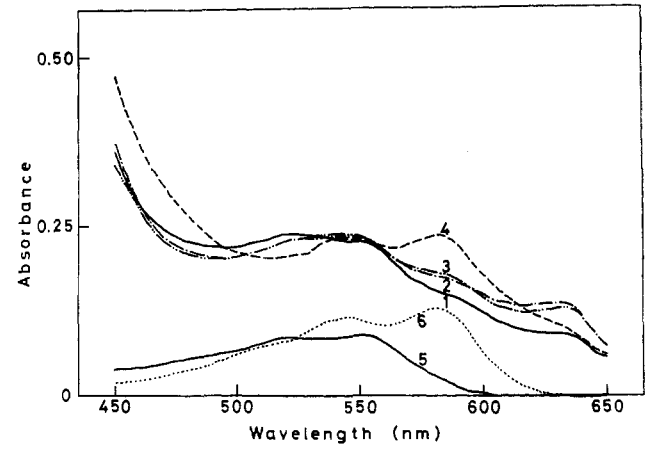



FIGURE 4: Comparison of the effects of KCN on the absorption spectra of the apoenzyme-cyanocobalamin complex and of free cyancobalamin. The apoenzyme-cyanocobalamin complex was partially purified by the procedure (T. Toraya and M. Kondo, unpublished results) based on its greater stability. KCN was added to 2.7 ml of 13.7 $\mu$M free cyanocobalamin and to a mixture (2.7 ml) containing about 67 units of the apoenzyme-cyanocobalamin complex and 0.023 M 1,2-propanediol-0.057 M KCl-0.0023 M potassium phosphate buffer (pH 8.0), final concentration being $5 \times 10^{-3}$ M; final volume, 3.0 ml. Absorption spectra are corrected for dilution. Curve 1, apoenzyme·$CNB_{12}$ before addition of KCN; curves 2–4, 2 min at room temperature, 10 min at 37°, and 5 min of boiling after addition of KCN to apoenzyme·$CNB_{12}$; curve 5, free $CNB_{12}$; curve 6, 12 min at room temperature after addition of KCN to free $CNB_{12}$.

tion, it can be presumed that more than one type of binding was involved in the attachment of this cobalamin to the apoenzyme, as has been described for other proteins (Bonnett, 1963). As discussed later, there would be only one catalytically active site per molecule of propanediol dehydratase. Therefore 1 equiv of hydroxocobalamin would be bound to the active site; additional binding would be of a nonspecific nature. The possibility must also be considered that a part of the hydroxocobalamin might be attached to contaminating proteins. Thus, it seems meaningless to study a spectral change caused by the KCN treatment of the apoprotein-hydroxocobalamin complex. It has been reasonably assumed that upon the KCN treatment of the apoenzyme-hydroxocobalamin complex, $CN^-$ reacts rapidly with the hydroxocobalamin which is bound to nonspecific binding sites in the apoprotein, converting the hydroxo compound to dicyanocobalamin, and that $CN^-$ displaces the upper ligand of the hydroxocobalamin which is bound to the active site of the enzyme. A possibility of further displacement by $CN^-$ of the lower ligand of the cobalamin bound to the active site to form dicyanocobalamin was examined as follows. The partially purified apoenzyme-cyanocobalamin complex was subjected to KCN treatment in the place of the apoprotein-hydroxocobalamin complex. As shown in Figure 4, there was no significant difference in the absorption spectrum between the KCN-treatment system (curves 2 and 3) and the untreated apoenzyme-cyanocobalamin complex (curve 1), although the spectrum of free cyanocobalamin (curve 5) was rapidly changed into the one of dicyanocobalamin (curve 6) under the conditions employed. The spectrum indicated that dicyanocobalamin was formed when the KCN-treated system was denatured by heating at 100° (curve 4). These observations suggest that the cyanocobalamin which is bound to the active site of the enzyme is less readily converted to dicyanocobalamin upon the KCN treatment. In other words, the active site bound cobalamin is not readily accessible to some reagents. Hence, upon the resolution of the apoprotein-hydroxocobalamin complex by the KCN method, it is suggested that the nonspecifically bound hydroxocobalamin was converted into dicyanocobalamin to be detached from the protein, and that the active site bound hydroxocobalamin was converted into cyanocobalamin to be resolved by the gel filtration in the absence of both potassium ion and substrate. The development of techniques for resolving apoenzyme-coenzyme $B_{12}$ analog complexes made it possible to measure the activity remaining after the appropriate treatment of the complexes.

*Thermal Stabilities of Apoenzyme and Apoenzyme-Coenzyme $B_{12}$ Analog Complexes.* In order to test the effectiveness of heat treatment for the purification of propanediol dehydratase as an inactive apoenzyme-coenzyme analog complex, the thermal stability of apoenzyme-coenzyme $B_{12}$ analog complexes was compared with that of the apoenzyme. As indicated in Figure 5A, the apoenzyme was heat labile, and about 90% of the enzyme activity was lost when heated at 50° for 10 min. In contrast, both apoenzyme-cyanocobalamin and apoenzyme-hydroxocobalamin complexes were quite heat stable and retained almost all of the activity even after heating at 70° for 10 min. Figure 5B shows the heat stability of the two apoenzyme-coenzyme analog complexes at 70°. Both complexes of the apoenzyme with cyano- and hydroxocobalamin could be heated at 70° for 20 min without loss of activity. Thus, heat treatment is clearly a very effective procedure for purifying the enzyme as an apoenzyme-coenzyme analog complex. This remarkable thermostability of apoenzyme-coenzyme $B_{12}$ analog complexes probably reflects their more rigid structures.

*pH-Dependent Stabilities of Apoenzyme and Apoenzyme-Coenzyme $B_{12}$ Analog Complexes.* As shown in Figure 6, the apoenzyme was stable at relatively high pH values, even at pH 10.5, while there was a remarkable loss of the activity

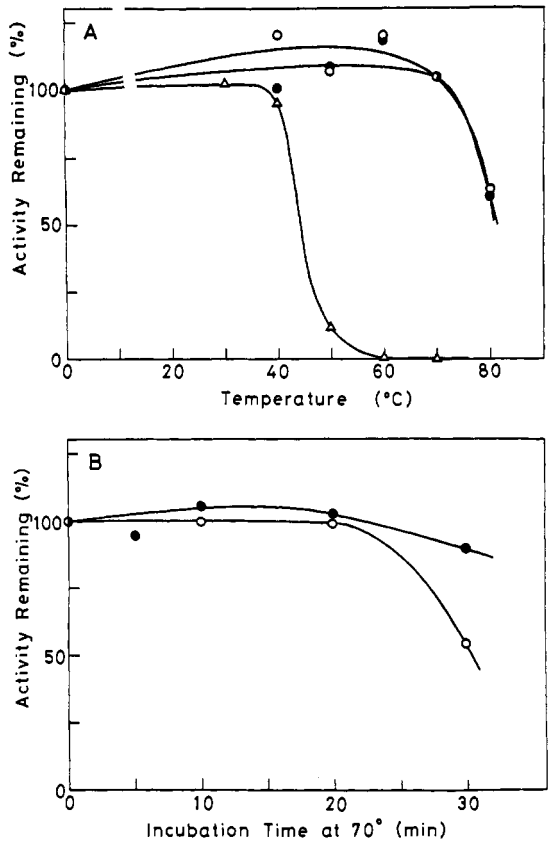



FIGURE 5: Thermal stabilities of the apoenzyme and apoenzyme–coenzyme $B_{12}$ analog complexes at different temperatures (A) and at 70° (B). Mixtures (3.0 ml) containing 10 units of the apoenzyme or each apoenzyme–coenzyme $B_{12}$ analog complex were heated for 10 min at the temperatures indicated (A) or at 70° for the indicated time (B). After removal of the denatured protein by centrifugation, the remaining activity of the apoenzyme was measured in the complete system after dilution of the supernatant. In the cases of apoenzyme–coenzyme analog complexes, 2 ml of the supernatant was subjected to the resolution steps followed by the determination of the activity of the resolved apoenzyme as described in the text. The KCN method was used for the resolution of the apoenzyme–hydroxocobalamin complex. (△-△) Apoenzyme; (○-○) apoenzyme·$CNB_{12}$; (●-●) apoenzyme·$OHB_{12}$.

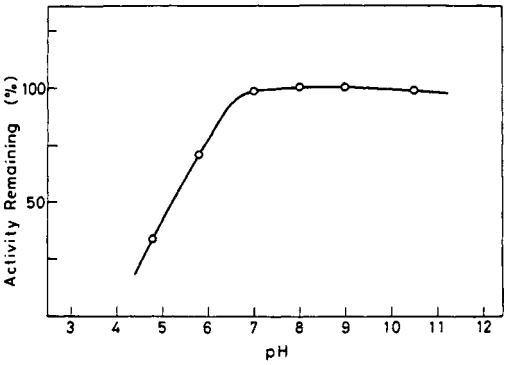



FIGURE 6: pH-dependent stability of the apoenzyme. Mixtures composed of 10 units of the apoenzyme and 0.30 M 1,2-propanediol–0.10 M KCl–0.030 M potassium phosphate buffer (pH 8.0) in a total volume of 10 ml were incubated at 37° for 10 min and then adjusted to the indicated pH with 0.1 M $H_3PO_4$ or with 10% KOH. After standing at 0° for 4 hr, the mixtures were adjusted to pH 8.0. The total activity remaining was measured in the complete system after dilution. The total activity of the apoenzyme which was allowed to stand at pH 8.0 for 4 hr at 0° was taken as control (100 %).

at pH lower than 7.0 when the apoenzyme was allowed to stand at 0° for 4 hr at different pH. The stability of apoenzyme–coenzyme $B_{12}$ analog complexes was compared with that of the apoenzyme at pH 6.0 where the apoenzyme is fairly unstable. As indicated in Table III, more than 40% of the activity of the apoenzyme was lost under the experimental conditions, whereas both apoenzyme–cyanocobalamin and apoenzyme–hydroxocobalamin complexes were stable at pH 6.0 for 4 hr, and there was very little loss of the activity under the same conditions.

*pCMB Susceptibility of Apoenzyme–Coenzyme $B_{12}$ Analog Complexes.* Although the propanediol dehydratase apoenzyme is a sulfhydryl protein and is readily inactivated by incubation with *p*HMB or *p*CMB, the holoenzyme once formed by incubating the apoenzyme with coenzyme $B_{12}$ in the presence of a certain monovalent cation is resistant to the inactivation by *p*HMB or *p*CMB (Lee and Abeles, 1963; Toraya *et al.*, 1971). In Table IV, the susceptibility of the complexes to *p*CMB was compared with that of the apoenzyme. As expected, apoenzyme–cyanocobalamin and apoenzyme–methylcobalamin complexes were not inactivated by *p*CMB, while the apoenzyme was almost completely inactivated under the same conditions. These results suggest that coenzyme analogs such as cyano- and methylcobalamin may cover the same sulfhydryl group(s) of the enzyme as the coenzyme does. On the other hand, it is intriguing that only the apoenzyme–hydroxocobalamin complex was fairly sensitive to *p*CMB. No clear-cut explanation for this observation can be offered at this time, but it seems likely that the attachment of excessive amounts of hydroxocobalamin to the apoprotein (Table V) resulted in a conformational change which rendered the essential sulfhydryl group(s) more sensitive to *p*CMB.

*Binding of Coenzyme $B_{12}$ and Its Analogs to Native and p-CMB-Treated Apoenzymes.* Gel filtration on Sephadex G-25 using potassium phosphate buffer containing both KCl and substrate was used to separate apoenzyme–cobalamin complexes from excess unbound cobalamin without any appreciable resolution (Toraya *et al.*, 1971) and was also used to

TABLE III: Comparison of pH-Dependent Stabilities of Apoenzyme and Apoenzyme–Coenzyme $B_{12}$ Analog Complexes at pH 6.0.[a]

| Enzyme | Specific Activity (units/mg) (Remaining Activity (%)) | |
|---|---|---|
| | pH 8.0[b] | pH 6.0 |
| Apoenzyme | 0.67 (100) | 0.39 (58) |
| Apoenzyme·$CNB_{12}$ | 0.46 (100) | 0.39 (85) |
| Apoenzyme·$OHB_{12}$ | 0.43 (100) | 0.39 (91) |

[a] Mixtures (5.0 ml), containing 15 units of the apoenzyme or each apoenzyme–coenzyme $B_{12}$ analog complex, were adjusted to pH 6.0 with 0.1 M $H_3PO_4$. After standing at 0° for 4 hr, the mixtures were adjusted to pH 8.0 with 10% KOH. Two milliliters of each mixture was then subjected to the resolution steps followed by the determination of the activity of the resolved apoenzyme as described in the text. The KCN method was used for the resolution of the apoenzyme–hydroxocobalamin complex. [b] The mixtures which were allowed to stand at pH 8.0 for 4 hr at 0° were treated in a similar manner as controls.

TABLE IV: Comparison of *p*CMB Susceptibilities of Apoenzyme and Apoenzyme–Coenzyme $B_{12}$ Analog Complexes.[a]

| Enzyme | Specific Activity (units/mg) (Remaining Activity (%)) | |
|---|---|---|
| | Untreated[b] | *p*CMB Treated |
| Apoenzyme | 0.59 (100) | 0.01 (2) |
| Apoenzyme·$CNB_{12}$ | 0.56 (100) | 0.48 (86) |
| Apoenzyme·$CH_3B_{12}$ | 0.28 (100) | 0.26 (93) |
| Apoenzyme·$OHB_{12}$ | 0.33 (100) | 0.08 (24) |

[a] Mixtures (1.8 ml), containing 7.0 units of the apoenzyme or each apoenzyme–coenzyme $B_{12}$ analog complex, were incubated at 37° for 6 min with $5 \times 10^{-5}$ M *p*CMB in a final volume of 2.0 ml and then subjected to the resolution steps followed by the determination of the activity of the resolved apoenzyme as described in the text. The KCN method was used for the resolution of the apoenzyme–hydroxocobalamin complex. [b] The mixtures incubated without *p*CMB were treated in a similar manner as controls.

estimate the cobalamin contents in these complexes. The amounts of coenzyme $B_{12}$ and its analogs which could be bound to the native and *p*CMB-treated apoenzymes were determined by the microbiological (*E. coli* 215) and spectrophotometric assay methods. The *p*CMB-treated apoenzyme did not show any appreciable activity. Treatment with mercaptoethanol or dithiothreitol reversed the inhibition caused by *p*CMB (T. Toraya, unpublished results). This observation undoubtedly established that *p*CMB reacts with the essential sulfhydryl group(s) of the apoenzyme. Since a homogeneous preparation of propanediol dehydratase apoprotein is not available at the present time, the results presented in Table V on the cobalamin binding are only qualitative. The data given in Table V show three important facts. Firstly, coenzyme $B_{12}$ and cyanocobalamin were bound to the native apoenzyme to nearly the same level. Secondly, when the native apoenzyme was previously treated with *p*CMB, the binding of coenzyme $B_{12}$ and cyanocobalamin was markedly inhibited. Mercurial reagents reacted only slightly with apoenzyme–coenzyme $B_{12}$ and apoenzyme–coenzyme $B_{12}$ analog complexes although they react very rapidly with the sulfhydryl group(s) of the apoenzyme. Hence, these lines of evidence strongly suggest that coenzyme $B_{12}$ analogs such as cyanocobalamin bind to the apoenzyme at the same site as does coenzyme $B_{12}$, and furthermore that the mercurial-sensitive sulfhydryl group(s) of the enzyme play an important role in the binding of coenzyme $B_{12}$ and its analogs. Thirdly, in contrast to cyanocobalamin, hydroxocobalamin was attached to the apoprotein in large amounts independent of the *p*CMB treatment of the apoprotein, indicating that excessive amounts of this cobalamin bound nonspecifically, in addition to at the active site, to the apoenzyme. This seems to be comparable to the peculiar behavior of hydroxocobalamin in the interaction with the apoprotein (Toraya *et al.*, 1971) and to the observation that only the apoenzyme–hydroxocobalamin complex was considerably susceptible to *p*CMB.

TABLE V: Binding of Coenzyme $B_{12}$ and Its Analogs to Native and *p*CMB-Treated Apoenzymes.[a]

| Cobalamin | Cobalamin Bound[b] (nmoles/100 units of Enzyme) | | | |
|---|---|---|---|---|
| | To Native Apoenzyme | | To *p*CMB-Treated Apoenzyme | |
| | Microbiological[c] | Spectrophotometric | Microbiological[c] | Spectrophotometric |
| Coenzyme $B_{12}$ | 9.2 | | 2.5 | |
| $CNB_{12}$ | 6.0 | | 1.2 | |
| $OHB_{12}$ | 168 | 202 | 102 | 123 |

[a] The experimental procedure is described in the text. [b] Samples incubated without any cobalamins exogenously added were treated in a similar manner as controls. [c] Values are averages of two independent bioassays.

## Discussion

A monovalent cation such as potassium ion was absolutely required for the binding of the apoenzyme with cyano- or methylcobalamin. The substrate also played an important part, but to a lesser degree, in the maintenance of the complex. These facts are consistent with the case for the binding of coenzyme $B_{12}$ to the apoenzyme (Toraya *et al.*, 1971). Cyanocobalamin and coenzyme $B_{12}$ were bound to the native apoenzyme to approximately the same extent. The treatment of the apoenzyme with a sulfhydryl inhibitor, *p*CMB, resulted in a remarkable inhibition of the binding of cyanocobalamin as well as of coenzyme $B_{12}$ to the apoenzyme. Despite the fact that the apoenzyme is highly susceptible to mercurial reagents, complexes of the apoenzyme with certain coenzyme analogs, like apoenzyme–coenzyme $B_{12}$ complex (holoenzyme), were almost insusceptible to *p*CMB. From these results, it is concluded that coenzyme analogs such as cyanocobalamin bind to the apoenzyme at the active site in a manner similar to that of the binding of coenzyme $B_{12}$, and that the mercurial-sensitive sulfhydryl group(s) of the enzyme are required for the binding. It has been observed that the inhibition of *Lactobacillus* 208-A glycerol dehydratase as well as of a mammalian methylmalonyl-CoA mutase by mercurial compounds can be relieved by coenzyme $B_{12}$ (Smiley and Sobolov, 1962; Cannata *et al.*, 1965). In contrast to our observation, coenzyme $B_{12}$ can be bound by the *p*HMB-blocked apoenzyme of the latter in a manner that protects the cofactor from photoinactivation (Cannata *et al.*, 1965). Switzer and Barker (1967) have reported that the sulfhydryl protein component of the glutamate mutase system increases the affinity of the cobamide binding protein moiety for coenzyme $B_{12}$. Recently, Essenberg *et al.* (1971) have estimated from the amount of enzyme activated by 1 $\mu$g of coenzyme $B_{12}$ that the equivalent weight of propanediol dehydratase is $2.6 \times 10^5$ g/mole using the value of 60 units/mg for the specific activity of their most highly purified enzyme preparation. According to their value of the specific activity, it can be calculated from the data shown in Table V that 1 mole of coenzyme $B_{12}$ or 1 mole of cyanocobalamin was bound per $1.81 \times 10^5$ g and $2.78 \times 10^5$ g of the propanediol dehydratase protein, respectively, in reasonable agreement with the value of Essenberg *et al.* (1971). From the value of 2.2–2.4 $\times 10^5$ for the molecular weight of this enzyme (Essenberg *et al.*, 1971), it might be suggested that one molecule of propanediol dehydratase

possesses one active site where one molecule of coenzyme $B_{12}$ or its analogs can be bound.

On the other hand, excessive amounts of hydroxocobalamin were bound to the apoenzyme in such a manner that the cobalamin could not be removed by Sephadex G-25 gel filtration, irrespective of the *p*CMB treatment of the apoprotein and of the absence of monovalent cation. Whether the binding of hydroxocobalamin to the active site of the enzyme, like those of other cobalamins, requires a certain monovalent cation and sulfhydryl group(s) of the apoenzyme is not yet clear. The nature, however, of the nonspecific, excessive binding of hydroxocobalamin to the apoenzyme may be analogous to the type of the nonspecific, excessive attachment of this cobalamin to apoethanolamine deaminase (Kaplan and Stadtman, 1968b), *E. coli* B $N^5$-methyltetrahydrofolate–homocysteine apomethyltransferase (Taylor, 1970), and bovine serum albumin (Taylor and Hanna, 1970). Stable complex formation between hydroxocobalamin and bovine serum albumin does not require any sulfhydryl groups, being interpreted as due to the substitution of the imidazole side chains for $H_2O$ at the sixth coordinating position of the cobalt accompanied by the locking of the cobalamin into the protein by the interaction between other nearby amino acid side chains and the corrin ring (Taylor and Hanna, 1970).

The apopropanediol dehydratase–hydroxocobalamin complex could be resolved by both the urea–dithiothreitol and KCN methods, both of which are based on the conversion of the enzyme-bound hydroxocobalamin into other cobalamin derivatives resolvable upon Sephadex G-25 gel filtration in the absence of both potassium ion and substrate. The spectroscopic result showed that cyanocobalamin bound to the active site of the enzyme can not be converted to dicyanocobalamin under the conditions employed for the resolution of the apoenzyme–hydroxocobalamin complex by the KCN method. Hence, it is strongly suggested that by the KCN method the nonspecifically attached hydroxocobalamin was converted into dicyanocobalamin to be detached from the protein, and that the active site bound hydroxocobalamin was converted to cyanocobalamin to be resolved by the gel filtration in the absence of both potassium ion and substrate. The slow reaction of the ethanolamine deaminase bound cobamide with KCN has similarly been observed (Kaplan and Stadtman, 1968b). Taylor and Hanna (1970) have reported that all of the hydroxocobalamin bound to the bovine serum albumin is readily removed by conversion into dicyanocobalamin with KCN. An acid–ammonium sulfate treatment, which is effective for partial removal of ethanolamine deaminase bound hydroxocobalamin (Kaplan and Stadtman, 1968b), led to a marked decrease in the propanediol dehydratase activity. In the case of *E. coli* B $N^5$-methyltetrahydrofolate–homocysteine methyltransferase, the initial holoenzyme and complexes of the apoenzyme with propyl- and methylcobalamin have been resolved by urea–dithiothreitol treatment (Taylor, 1970). Unlike glycerol dehydratase (Schneider *et al.*, 1970), the replacement of propanediol dehydratase bound hydroxocobalamin with coenzyme $B_{12}$ in the presence of $Mg^{2+}$ and $SO_3^{2-}$ was unsuccessful.

Through the present methods for resolving inactive apoenzyme–coenzyme $B_{12}$ analog complexes of propanediol dehydratase, it was possible to investigate the properties of the complexes. As described above, the mode of the binding of coenzyme $B_{12}$ to the apoenzyme is similar to that of the binding of the cobamide inhibitors, especially cyano- and methylcobalamin. This indicates that these apoenzyme–coenzyme $B_{12}$ analog complexes are a suitable model for the holoenzyme. Apoenzyme–coenzyme $B_{12}$ analog complexes showed the greater stabilities to heat and to change in pH, probably resulting from their more rigid conformation induced by the cobalamin binding. This may also be the case for the holoenzyme. The greater stability of the apoprotein–hydroxocobalamin complex of glycerol dehydratase as compared to the apoenzyme has been reported by Schneider *et al.* (1970). The apoenzyme of coenzyme $B_{12}$ dependent enzymes might acquire much greater stability by forming a complex with coenzyme $B_{12}$ analogs, in general. Further study on the properties of apoenzyme–coenzyme $B_{12}$ analog complexes, a stable model for the holoenzyme, could be expected to provide valuable information regarding the holoenzyme. Whether such an approach to the problem is applicable to other coenzyme $B_{12}$ enzymes or not cannot be determined until a method for resolving inactive apoprotein–coenzyme $B_{12}$ analog complexes under mild conditions has been found. The methods described here might be applicable for some enzymes.

## Acknowledgment

The authors are grateful to Assistant Professor Shoichi Shimizu for helpful discussions and suggestions during this work. Thanks are also due to Mr. Yukio Ohya for the microbiological assay of cobamide using *E. coli* 215.

## References

Babior, B. M. (1969), *J. Biol. Chem. 244*, 2917.

Barker, H. A., Smyth, R. D., Weissbach, H., Munch-Petersen, A., Toohey, J. I., Ladd, J. N., Volcani, B. E., and Wilson, R. M. (1960), *J. Biol. Chem. 235*, 181.

Bonnett, R. (1963), *Chem. Rev. 63*, 573.

Cannata, J. J. B., Focesi, A., Jr., Mazumder, R., Warner, R. C., and Ochoa, S. (1965), *J. Biol. Chem. 240*, 3249.

Ertel, R., Brot, N., Taylor, R., and Weissbach, H. (1968), *Arch. Biochem. Biophys. 126*, 353.

Essenberg, M. K., Frey, P. A., and Abeles, R. H. (1971), *J. Amer. Chem. Soc. 93*, 1242.

Hayashi, R., Nakayama, H., and Ikeda, H. (1955), *Nippon Saikingaku Zasshi 10*, 749.

Kaplan, B. H., and Stadtman, E. R. (1968a), *J. Biol. Chem. 243*, 1787.

Kaplan, B. H., and Stadtman, E. R. (1968b), *J. Biol. Chem. 243*, 1794.

Kato, T., Shimizu, S., and Fukui, S. (1964), *J. Vitaminol. (Kyoto) 10*, 89.

Lee, H. A., Jr., and Abeles, R. H. (1963), *J. Biol. Chem. 238*, 2367.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Pawelkiewicz, J., and Schneider, Z. (1967), *Bull. Acad. Polon. Sci. 15*, 65.

Schneider, Z., Larsen, E. G., Jacobson, G., Johnson, B. C., and Pawelkiewicz, J. (1970), *J. Biol. Chem. 245*, 3383.

Schneider, Z., and Pawelkiewicz, J. (1966), *Acta Biochim. Polon. 13*, 311.

Smiley, K. L., and Sobolov, M. (1962), *Arch. Biochem. Biophys. 97*, 538.

Smith, E. L., and Mervyn, L. (1963), *Biochem. J. 86*, 2p.

Switzer, R. L., and Barker, H. A. (1967), *J. Biol. Chem. 242*, 2658.

Taylor, R. T. (1970), *Arch. Biochem. Biophys. 137*, 529.

Taylor, R. T., and Hanna, M. L. (1970), *Arch. Biochem. Biophys. 141*, 247.

Toraya, T., Sugimoto, Y., Tamao, Y., Shimizu, S., and Fukui, S. (1970), *Biochem. Biophys. Res. Commun. 41*, 1314.

Toraya, T., Sugimoto, Y., Tamao, Y., Shimizu, S., and Fukui, S. (1971), *Biochemistry 10*, 3475.

Wagner, O. W., Lee, H. A., Jr., Frey, P. A., and Abeles, H. A. (1966), *J. Biol. Chem. 241*, 1751.

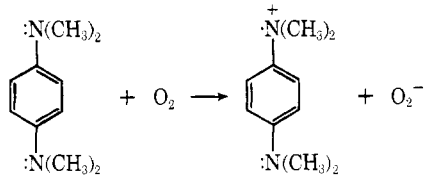
# Formation of Adenosine Triphosphate in the Oxidation of a Model for the Reduced Pyridine Nucleotides†

E. J. H. Bechara‡ and G. Cilento*

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

† From the Department of Biochemistry, Instituto de Quimica da Universidade de São Paulo, São Paulo, Brazil. *Received February 14, 1972.* This investigation was greatly aided by grants from the Fundação de Amparo a Pesquisa do Estado de São Paulo (BIOQ-FAPESP programme). The paper is part of the doctoral dissertation to be submitted by E. J. H. B. to the Universidade de São Paulo. This is Paper XIII in the series of the Pyridine Coenzymes. Paper XII is Casadei de Baptista and Cilento (1971). Papers X and XI are Bechara and Cilento (1971a,b); Paper IX is Polacow and Cilento (1970), whereas paper VIII is Silva Araujo and Cilento (1969).

‡ Predoctoral fellow of the Fundação de Amparo a Pesquisa do Estado de São Paulo.

[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.