Propanediol Dehydratase System. Role of Monovalent Cations in Binding of Vitamin B_{12} Coenzyme or Its Analogs to Apoenzyme*

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ABSTRACT: Holopropanediol dehydratase (EC 4.2.1.28) was resolved completely into the apoenzyme and corrinoid(s) upon gel filtration on a column of Sephadex G-25 in the absence of potassium ion. The apoprotein obtained by this resolution procedure could be reconstituted into the catalytically active holoenzyme by the incubation at 37° with added B_{12} coenzyme in the presence of potassium ion. The recovery of dehydratase activity was nearly quantitative. The major corrinoid recovered was identified as 5′-deoxyadenosylcobalamin. Of inactive complexes between the apoenzyme and irreversible cobamide inhibitors of propanediol dehydratase, cyanocobalamin- and methylcobalamin–apoenzyme complexes were also mostly resolved upon the gel filtration in the absence of both potassium ion and the substrate, yielding the apoenzyme which was reconstitutable into the active holoenzyme. Hydroxocobalamin–apoenzyme complex, however, was hardly resolvable under the same conditions. In the presence of both potassium ion and the substrate, gel filtration of the holoenzyme or coenzyme analog–apoenzyme complexes resulted in negligible resolution. When gel filtration of the holoenzyme was carried out in the presence of potassium ion only, the holoenzyme was found to be resolved according to the kinetics of first-order reaction. These experiments indicate that potassium ion plays an essential role in the binding of the apoenzyme with coenzyme B_{12} or its analogs except for hydroxocobalamin. Only hydroxocobalamin could bind to the apoenzyme even in the absence of both potassium ion and the substrate. Besides potassium ion, certain monovalent cations, such as ammonium, rubidium, methylammonium, cesium, and sodium ions, were effective as the cofactor in activation of the propanediol dehydratase system. These monovalent cations, like potassium ion, were found to be able to function as a factor essential both for the holoenzyme formation and for the maintenance of the binding of coenzyme B_{12} to the apoenzyme. A close relationship was observed between their effectivenesses and their ionic radii.

It has been generally considered that vitamin B_{12} coenzyme or its analogs bind to the apoprotein of propanediol dehydratase (DL-1,2-propanediol hydro-lyase, EC 4.2.1.28) or to those of many other B_{12} coenzyme-dependent enzymes almost irreversibly and the resolution of B_{12} coenzyme–apoenzyme complex (holoenzyme) or coenzyme analog–apoenzyme complex into the active apoprotein and corresponding cobalamin is usually accompanied by a significant loss of the enzyme activity. Charcoal treatment or exhaustive dialysis was found not to be effective. An acid ammonium sulfate resolution procedure, which was effectively used for mammalian methylmalonyl-CoA mutase (Lengyel *et al.*, 1960) and ethanolamine deaminase (Kaplan and Stadtman, 1968b), led to a marked decrease in the propanediol dehydratase activity (T. Toraya, S. Shimizu, and S. Fukui, 1969, unpublished results).

During the course of an investigation on factors necessary for binding of B_{12} coenzyme or its analogs to the apoprotein of propanediol dehydratase, our attention was drawn to the function of a monovalent cation which was required as a cofactor by this enzyme. Although it has previously been reported that propanediol dehydratase and many other coenzyme B_{12} dependent enzymes show an absolute requirement for a monovalent cation, such as potassium ion, for their catalytic activities (Smiley and Sobolov, 1962; Lee and Abeles, 1963; Pawelkiewicz and Zagalak, 1965; Kaplan and Stadtman, 1968a; Stadtman and Renz, 1968; Jacobsen and Huennekens, 1969; Morley and Stadtman, 1970), little attention has been paid to the role of monovalent cations in B_{12} enzyme systems except that, in glycerol dehydratase system, the presence of potassium or ammonium ion has been reported to be one of the factors necessary for association of the enzyme components (Schneider *et al.*, 1966). Thus, the greater part of the role of monovalent cations in B_{12} enzyme systems remains to be elucidated.

This report describes both a study of the effects of potassium ion and the substrate on the binding of B_{12} coenzyme or its analogs to apopropanediol dehydratase, and a method for resolving the B_{12} coenzyme- or coenzyme analog–apoenzyme complex. Incubation of the resolved apoenzyme with added B_{12} coenzyme in the presence of potassium ion resulted in the complete reconstitution of the catalytically active holoenzyme. The effects of other monovalent cations both on the enzymatic activity and on the binding of coenzyme B_{12} to the apoprotein of propanediol dehydratase are also reported here.

Materials and Methods

Chemicals. 5,6-Dimethylbenzimidazolylcobamide coenzyme was the generous gift of Yamanouchi Pharmaceutical Co., Tokyo. Cyanocobalamin and hydroxocobalamin were purchased from Glaxo Lab., Greenford, U. K. Methylcobalamin was prepared from cyanocobalamin by the procedure of Smith and Mervyn (1963). The purity of these cobalamins was established by paper electrophoresis (Armitage *et al.*, 1953). Determinations of the cobalamin concentrations were based on the molar extinction coefficient at 367 mμ for dicyanocobalamin ϵ_{367} 30.4×10^3 M^{-1} cm^{-1} (Barker *et al.*, 1960). The cobalamins were converted into dicyanocobalamin by photolysis in the

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presence of KCN, and the absorbance at 367 mμ was measured. All other materials were obtained from commercial sources and were used without further purification.

Enzyme Preparation. Apopropanediol dehydratase was obtained from *Aerobacter aerogenes* (ATCC 8724) by a procedure similar to that reported by Lee and Abeles (1963). Fraction E-3 was dialyzed against 0.01 M K_2HPO_4 and used.

K^+-Free Apoenzyme. When potassium ion-free apoenzyme was required in certain experiments, apopropanediol dehydratase, obtained as described above, was applied to a column (1.6 × 20 cm) of Sephadex G-25 (fine) previously equilibrated with 0.05 M Tris·HCl buffer (pH 8.0). The column was eluted with the same buffer. Enzyme thus prepared did not show any observable activity in the absence of added monovalent cation such as potassium ion, and was assumed to be potassium ion free although metal analysis was not made.

Enzyme Assay. Propanediol dehydratase activity was assayed essentially as described by Lee and Abeles (1963). Reaction mixtures which contained enzyme, 15 μM 5′-deoxyadenosylcobalamin, 10 mM 1,2-propanediol, and potassium ion or other monovalent cation, in a total volume of 1.0 ml, were incubated at 37° for 10 min. Enzyme reaction was then stopped by the addition of 0.5 ml of 10% (w/v) trichloroacetic acid and the amount of propionaldehyde formed was determined colorimetrically by conversion to its 2,4-dinitrophenylhydrazone (Lee and Abeles, 1963). The amount of enzyme that catalyzes the formation of 1 μmole of propionaldehyde per minute under the assay conditions is defined as 1 unit (Lee and Abeles, 1963).

Maintenance of Binding of Cobalamin to Apoenzyme. In order to investigate the effects of monovalent cations and the substrate on the maintenance of the binding of B_{12} coenzyme or its analogs to the apoprotein of propanediol dehydratase, apoenzyme–cobalamin complexes formed previously were subjected to gel filtration on Sephadex G-25 using various buffers, containing the above-mentioned factors or not, and the degree of the resolution was determined. The compositions of the buffers used are described in Table I and other corresponding tables.

Formation and gel filtration of apoenzyme–cobalamin complexes were carried out as follows. Apopropanediol dehydratase could be completely converted into B_{12} coenzyme– or its analog–apoenzyme complexes by incubating at 37° for 10 min the reaction mixtures composed of 2.0 units of the apoenzyme, 61 μM 5′-deoxyadenosylcobalamin or other cobalamin, 0.10 M KCl, 1.0 M 1,2-propanediol, and 0.0125 M potassium phosphate buffer (pH 8.0) in a total volume of 2.0 ml (apoenzyme–cobalamin complex formation step). Substrate was added in order to promote the complex formation and protect the complex formed, especially in the case of the holoenzyme formation, against the inactivation by oxygen. After incubation, the reaction mixture was then applied to a column (1.6 × 20 cm) of Sephadex G-25 (fine) previously equilibrated with an appropriate buffer and eluted with the same buffer (gel filtration step). Since the holopropanediol dehydratase is gradually inactivated by oxygen in the absence of the substrate (Wagner *et al.*, 1966), small fractions (2–4 ml) were collected into tubes which contained 0.2 mmole of 1,2-propanediol to protect the holoenzyme against the inactivation. Gel filtration was performed in dim light at 0–4°, and the degree of the resolution of B_{12} coenzyme–apoenzyme or coenzyme analog–apoenzyme complexes was determined with protein-containing fractions as described in the following sections.

Determination of Degree of Resolution of Holoenzyme. The degree of resolution of the B_{12} coenzyme–apoenzyme complex was determined by duplicate assays of propanediol dehydratase activity in each protein-containing fraction in the presence and absence of exogenously added B_{12} coenzyme. The activity measured in the complete system containing 10 mM 1,2-propanediol, 15 μM 5′-deoxyadenosylcobalamin, 0.05 M KCl, 0.025 M potassium phosphate buffer (pH 8.0), and 0.2 ml of each fraction in a total volume of 1.0 ml was interpreted as a measure of the total amount of active protein or total enzyme activity (apoenzyme plus remaining holoenzyme), whereas the activity measured in the reaction system in which 5′-deoxyadenosylcobalamin was omitted from the complete system was attributed to the holoenzyme remaining after gel filtration. The degree of the resolution could therefore be calculated in terms of percentage according to the following formula.

Resolution (%) = 100(1 − holoenzyme activity/total enzyme activity), where (holoenzyme activity/total enzyme activity) × 100 represents the percentage of the remaining holoenzyme. The value obtained from the fraction which contained the largest amount of protein of all the fractions was employed to minimize the error of the measurement.

Determination of Degree of Resolution of Coenzyme Analog–Apoenzyme Complexes. In the cases of gel filtration of coenzyme analog–apoenzyme complexes, the following procedure was employed for the estimation of the degree of resolution of coenzyme analog–apoenzyme complexes. This method was based on the fact that these inactive coenzyme analog–apoenzyme complexes can not be converted into the active coenzyme–apoenzyme complex (holoenzyme) by the replacement of each of these coenzyme analogs with exogenously added B_{12} coenzyme. After gel filtration, dehydratase specific activity (sp $act._{resol}$) of each protein-containing fraction was measured in the complete system described above. Sp $act._{resol}$ represents the amount of the reconstitutable apoenzyme which was produced by the resolution of inactive coenzyme analog–apoenzyme complexes.

To estimate the amount of the apoprotein which was converted into the coenzyme analog–apoenzyme complexes, the apoenzyme was treated in a similar manner in the absence of any coenzyme analogs and its specific activity (sp $act._{control}$) was measured in the complete system. The degree of the resolution would be expressed as follows. Resolution (%) = (sp $act._{resol}$/sp $act._{control}$) × 100.

Determination of Eluted Substances. Protein was routinely determined by the procedure of Lowry *et al.* (1951). Crystalline bovine serum albumin was used as standard. The amount of propionaldehyde formed by preincubation was measured colorimetrically by conversion to its 2,4-dinitrophenylhydrazone. Cobalamins were assayed spectrophotometrically as described above. Determination of the amount of potassium ion was based on its activating effect on propanediol dehydratase reaction.

Identification of Corrinoids Released by Gel Filtration. Corrinoids derived from the coenzyme part of the holopropanediol dehydratase resolved by gel filtration in the absence of potassium ion were identified by their absorption spectra and coenzyme activity in the propanediol dehydratase system.

Complex Formation between Hydroxocobalamin and Apoenzyme in the Absence of Potassium Ion and Substrate. A reaction mixture, containing 4.0 units of potassium ion free apoenzyme, 100 μM hydroxocobalamin, and 0.03 M Tris·HCl buffer (pH 8.0) in a total volume of 2.0 ml, was incubated at 37° for 10 min and then placed on a column (1.6 × 20 cm) of Sephadex G-25 (fine) previously equilibrated with 0.05 M Tris·

HCl buffer (pH 8.0). The column was developed with the same buffer. Specific activities (sp act.$_{remain}$) of the protein-containing fractions were determined in the complete system described above and compared with that (sp act.$_{control}$) obtained from the control experiment in which apoenzyme was treated in a similar manner without addition of hydroxocobalamin. The extent of apoenzyme–hydroxocobalamin complex formation would therefore be expressed by the value obtained by substracting the ratio of these specific activities from 100. Complex formation (%) = 100(1 − sp act.$_{remain}$/sp act.$_{control}$).

Cofactor Activity of Monovalent Cations. In the experiments concerning the investigation of monovalent cation effects, monovalent cations were added as their chlorides without pH adjustment except when necessary, since the propanediol dehydratase has the same activity in the pH range from 6.0 to 10.0 (Lee and Abeles, 1963). In these experiments, potassium ion free apoenzyme, prepared as described above, was used, and 0.05 M Tris·HCl buffer (pH 8.0) was employed instead of 0.05 M potassium phosphate buffer (pH 8.0).

The activating effects of varying concentrations of monovalent cation on the propanediol dehydratase system were examined in the absence of potassium ion in order to estimate their effectivenesses as the cofactor (cofactor activity). Enzyme reaction was carried out at 37° for 10 min by incubating the reaction mixture composed of 0.12 unit of potassium ion free apoenzyme, 15 μM 5′-deoxyadenosylcobalamin, 10 mM 1,2-propanediol, 0.035 M Tris·HCl buffer (pH 8.0), and varied concentrations of each monovalent cation, in a total volume of 1.0 ml, and the amount of propionaldehyde formed was determined in the usual way. K_m and V_{max} values for each monovalent cation which was effective as the cofactor (active) were determined by the method of Lineweaver and Burk (1934).

Inhibition by Inactive Monovalent Cations. Inhibition of propanediol dehydratase reaction by monovalent cations which were ineffective as the cofactor (inactive) was studied with respect to potassium ion. Enzyme reaction was carried out at 37° for 10 min by incubating the reaction mixture containing 0.10 unit of potassium ion free apoenzyme, 15 μM 5′-deoxyadenosylcobalamin, 10 mM 1,2-propanediol, 0.03 M Tris·HCl buffer (pH 8.0), varied concentrations of potassium ion with or without added inactive monovalent cation, in a total volume of 1.0 ml. The product was assayed in the usual way. Types of inhibition by these inactive monovalent cations with respect to potassium ion and their K_i values were determined by double reciprocal plots.

Effects of Monovalent Cations on Holoenzyme Resolution. Effect of each monovalent cation on the resolution of the holopropanediol dehydratase by gel filtration was examined as described above. Buffers used for gel filtration as eluting agent were 0.05 M Tris·HCl buffer (pH 7.0) containing each cation at the concentration of 50 times K_m value for activating cations or 10 times K_i value for inhibiting cations with or without 0.10 M substrate.

O_2 Inactivation of Enzyme. Effects of 5′-deoxyadenosylcobalamin and monovalent cations on inactivation of propanediol dehydratase by oxygen were studied as follows. Potassium ion free apoenzyme, 0.10 unit, was preincubated at 37° for 10 min with 18.8 μM 5′-deoxyadenosylcobalamin, 0.031 M Tris·HCl buffer (pH 8.0), and varied concentrations of each monovalent cation in a total volume of 0.8 ml in the absence of the substrate. Then 0.20 M substrate and 0.10 M KCl were added to this reaction mixture in a final volume of 1.0 ml, and enzyme activity remaining was determined by the incubation of the mixture at 37° for 10 min. Similar experiments in which each cation was added at a fixed concentration of 10 times K_m value for activating cations or 5 times K_i value for inhibiting cations with varying preincubation time were also performed.

Alleviation of pCMB-Susceptibility of Enzyme. Effects of 5′-deoxyadenosylcobalamin and monovalent cations on alleviation of sensitivity of propanediol dehydratase to *p*-chloromercuribenzoate was studied by experiments I, II, and III. In these experiments, tubes preincubated in a similar manner without added *p*CMB[1] were offered as controls (100%).

EXPERIMENT I. The reaction mixture used contained 0.10 unit of potassium ion free apoenzyme, 0.033 M Tris·HCl buffer (pH 8.0), and each monovalent cation at a concentration of 13.3 times K_m value in a total volume of 0.6 ml. After incubation at 37° for 4 min, the mixture was incubated again at 37° for a further 4 min with added 1.43×10^{-5} M *p*CMB in a total volume of 0.7 ml. Then 10 mM 1,2-propanediol, 15 μM 5′-deoxyadenosylcobalamin, and 0.10 M KCl were added to the mixture in a final volume of 1.0 ml, and enzyme activity remaining was determined by the incubation of the reaction mixture at 37° for 10 min.

EXPERIMENT II. The reaction mixture contained 0.10 unit of potassium ion free apoenzyme, 0.029 M Tris·HCl buffer (pH 8.0), 21.4 μM 5′-deoxyadenosylcobalamin, and each monovalent cation at a concentration of 11.4 times K_m value in a total volume of 0.7 ml. After incubated at 37° for 4 min, the mixture was incubated again at 37° for a further 4 min with added 1.25×10^{-5} M *p*CMB in a total volume of 0.8 ml. Then 10 mM 1,2-propanediol and 0.10 M KCl were added to the mixture in a final volume of 1.0 ml, and the enzyme activity remaining was determined by the incubation of the reaction mixture at 37° for 10 min.

EXPERIMENT III. The reaction mixture contained 0.050 unit of potassium ion free apoenzyme, 0.025 M Tris·HCl buffer (pH 8.0), 18.8 μM 5′-deoxyadenosylcobalamin, 12.5 mM 1,2-propanediol, and each monovalent cation at a concentration of 10 times K_m value in a total volume of 0.8 ml. After incubated at 37° for 4 min, the reaction mixture was incubated again at 37° for a further 4 min with added 1.11×10^{-5} M *p*CMB in a total volume of 0.9 ml. Then 0.10 M KCl was added to the mixture in a final volume of 1.0 ml, and the enzyme activity remaining was determined by the incubation of the reaction mixture at 37° for 10 min.

Results

Resolution of Holopropanediol Dehydratase. Figure 1 shows the result of determination of substances eluted by gel filtration on a Sephadex G-25 column using 0.05 M Tris·HCl buffer (pH 8.0) as an eluting agent. The elution pattern indicates that macromolecules such as enzyme protein were completely separated from small molecules such as 5′-deoxyadenosylcobalamin, potassium ion, and propionaldehyde, and that the holopropanediol dehydratase was resolved completely to liberate the apoenzyme under these conditions. The apoenzyme thus obtained was reconstituted into the catalytically active holoenzyme upon the incubation with added 5′-deoxyadenosylcobalamin in the presence of potassium ion and the substrate.

[1] Abbreviations used are: *p*CMB, *p*-chloromercuribenzoate; *p*HMB, *p*-hydroxymercuribenzoate; DBCC, α-(5,6-dimethylbenzimidazolyl)-cobamide coenzyme; OHB_{12}, hydroxocobalamin; CNB_{12}, cyanocobalamin; CH_3B_{12}, methylcobalamin.

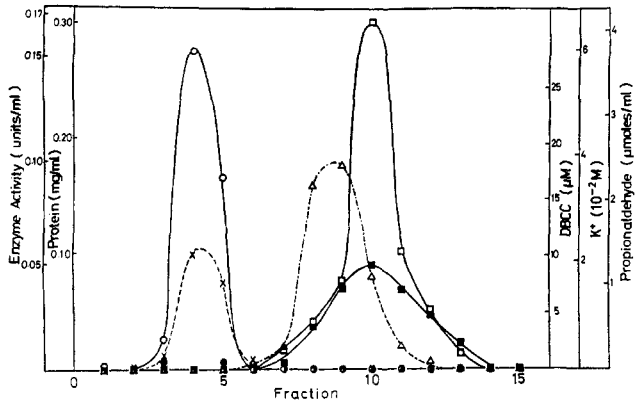


FIGURE 1: Elution patterns of enzyme protein and certain small molecules from Sephadex G-25 column using Tris·HCl buffer. Holopropanediol dehydratase was formed, chromatographed on a column (1.6 × 20 cm) of Sephadex G-25 (fine) using 0.05 M Tris·HCl buffer (pH 8.0), and the following substances eluted were determined as described in the text. (○—○) Total enzyme activity; (●—●) holoenzyme activity; (×- - -×) protein; (△-·-△) propionaldehyde; (■—■) 5′-deoxyadenosylcobalamin; (□—□) potassium ion.

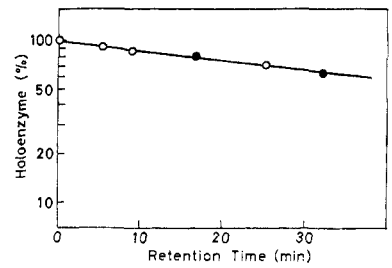


FIGURE 2: Kinetics of dissociation of holopropanediol dehydratase during gel filtration using potassium phosphate buffer. The holoenzyme was formed by the incubation of the following mixture. Apoenzyme, 4.0 units; 103 μM 5′-deoxyadenosylcobalamin; 0.10 M KCl; 0.30 M 1,2-propanediol; 0.025 M potassium phosphate buffer (pH 8.0). Total volume, 2.0 ml. The incubated mixture, 1.5 ml, was applied to a column, 1.6 × 10 cm (○) or 1.6 × 20 cm (●), of Sephadex G-25 (fine) previously equilibrated with 0.05 M potassium phosphate buffer (pH 8.0) containing 0.05 M KCl, and eluted with the same buffer over a period of indicated retention time. The amount of the holoenzyme in the protein-containing fraction was determined as described in the text.

Factors Necessary for Binding of Coenzyme B_{12} to Apoenzyme. Table I shows the behaviors of holopropanediol dehydratase in the gel filtration experiments which were performed using six different buffers in order to investigate the effects of potassium ion and the substrate on the maintenance of the binding of coenzyme B_{12} to the apoprotein. No resolution of the holoenzyme was observed when the holoenzyme coexisted with both potassium ion and the substrate. On the contrary, almost all of the coenzyme–apoenzyme complex was resolved when 0.05 M Tris·HCl buffer (pH 8.0) containing no potassium ion was used in the gel filtration, whether or not it contained the substrate. From this fact, it would be concluded that potassium ion plays the most essential role in the binding of the apopropanediol dehydratase with coenzyme B_{12}. On the other hand, a part of the holoenzyme was resolved when gel filtration was carried out using either 0.05 M potassium phosphate or 0.05 M Tris·HCl buffer (pH 8.0) containing potassium ion only and no substrate. These results suggest that the substrate also serves for the binding of the coenzyme B_{12} to the apoenzyme. To study the resolution in the presence of potassium ion alone kinetically, the holoenzyme was subjected to the gel filtration with varying retention time on the gel column. In this case, the resolution occurred as a function of the retention time and was independent of the bed volume of gel column so far tested. Figure 2 shows that an approximately linear relationship exists between the retention time on the gel column and the logarithms of the amount of holoenzyme remaining, indicating that the resolution of the holoenzyme proceeds according to the kinetics of first-order reaction when the holoenzyme coexisted with potassium ion only ($k = 1.4 \times 10^{-2}$ min^{-1} at 0°).

TABLE I: Effects of Potassium Ion and Substrate on Resolution of Holopropanediol Dehydratase by Gel Filtration.[a]

Expt	Buffer[b]	Specific Activity (units/mg) −DBCC[c]	Specific Activity (units/mg) +DBCC[c]	Resolution (%)
I	0.05 M Potassium phosphate–0.05 M KCl–0.10 M 1,2-propanediol	0.50	0.51	2
	0.05 M Potassium phosphate–0.05 M KCl	0.32	0.52	38
	0.05 M Tris·HCl–0.10 M 1,2-propanediol	0.00	0.52	100
	0.05 M Tris·HCl	0.00	0.43	100
II	0.05 M Tris·HCl–0.10 M KCl–0.10 M 1,2-propanediol	0.34[d]	0.35[d]	2
	0.05 M Tris·HCl–0.10 M KCl	0.18[d]	0.29[d]	38

[a] Experimental procedure is described in the text. [b] All buffers, pH 8.0. [c] Assays were carried out with (+), or without (−), exogenously added DBCC. [d] Value of activity (units/ml fraction).

Fate of Coenzyme Part after Resolution of Holoenzyme. The fate of the coenzyme moiety of holopropanediol dehydratase was investigated after the holoenzyme was resolved by the gel filtration in the absence of potassium ion. The holoenzyme, formed from 118 units of the apoenzyme in the usual way, was separated from unbound coenzyme B_{12} by gel filtration on a column (3.6 × 25 cm) of Sephadex G-25 (fine) using 0.05 M potassium phosphate buffer (pH 8.0) containing both 0.10 M 1,2-propanediol and 0.05 M KCl as an eluent. The second gel filtration of the holoenzyme thus obtained (93 units), performed with 0.05 M Tris·HCl buffer (pH 8.0) containing 0.10 M substrate as an eluting solution, resulted in complete resolution of the holoenzyme. Detection of the corrinoids in the effluents were tried by visible absorption spectra and coenzyme activity after the effluents were concentrated into small volumes. The corrinoid-containing fractions were eluted after the elution of protein, and gave the visible spectrum similar to that of 5′-deoxyadenosylcobalamin. The absorption spectrum was changed into hydroxocobalamin-like one by illumination, and further into the one similar to that of dicyanocobalamin by KCN treatment. Although observable

TABLE II: Resolution of Coenzyme Analog–Apoenzyme Complexes by Gel Filtration.[a]

Complex	0.05 M Tris·HCl–0.10 M KCl–0.10 M 1,2-Propanediol (pH 8.0)		0.05 M Tris·HCl (pH 8.0)	
	Specific Activity (units/mg)	Resolution (%)	Specific Activity (units/mg)	Resolution (%)
Apoenzyme[b]	0.46	(100)	0.50	(100)
OHB_{12}·apoenzyme	0.00	0	0.02	4
CNB_{12}·apoenzyme	0.02	4	0.44	88
CH_3B_{12}·apoenzyme	0.00	0	0.25	50

[a] Experimental procedure is described in the text. [b] Apoenzyme was treated in a similar manner as control.

coenzyme activity failed to be detected in our previous small-scale experiment (Toraya *et al.*, 1970), the corrinoid-containing fraction obtained here by treatment of a larger amount of the holoenzyme showed the coenzyme activity. These results seem to indicate that most of the coenzyme B_{12} part of the holoenzyme was released unmodified during the resolution procedure and mostly recovered as intact 5′-deoxyadenosylcobalamin under the conditions employed.

Resolution of B_{12} Coenzyme Analog–Apoenzyme Complex. It is well known that coenzyme analogs such as hydroxo- and cyanocobalamin bind very tightly to the apoprotein of propanediol dehydratase to act as an irreversible inhibitor (Lee and Abeles, 1963). It appeared of much interest to investigate whether or not the binding of these coenzyme analogs to the apoenzyme is dependent on the existence of potassium ion and/or the substrate. Since these inactive coenzyme analog–apoenzyme complexes could not be converted to the active holoenzyme by the replacement of each coenzyme analog with added B_{12} coenzyme, the specific activity in Table II represents the amount of the active apoenzyme produced by the resolution of inactive coenzyme analog–apoenzyme complex. As shown in the first column of Table II, all the coenzyme analog–apoenzyme complexes tested were not resolved when eluted from a column of Sephadex G-25 with 0.05 M Tris·HCl buffer (pH 8.0) containing both potassium ion and the substrate. On the other hand, when gel filtration of these complexes was performed in the absence of both potassium ion and the substrate, cyano- and methylcobalamin were released from the corresponding coenzyme analog–apoenzyme complex, yielding the active apoenzyme. These results seem to indicate that the binding of the apoenzyme with cyano- or methylcobalamin also depends on potassium ion and the substrate. In the case of hydroxocobalamin–apoenzyme complex, however, almost no resolution was observed under the same conditions.

Potassium Ion Independent Binding of Hydroxocobalamin to Apoenzyme. A markedly peculiar property of hydroxocobalamin in the interaction with the apoprotein was confirmed by the experiment in which the complex formation between hydroxocobalamin and the apoenzyme was studied in the absence of potassium ion and the substrate. After potassium ion free apoenzyme was incubated with hydroxocobalamin at 37° for 10 min in the absence of both potassium ion and the substrate, gel filtration of this mixture was performed using 0.05 M Tris·HCl buffer (pH 8.0) and the specific activities of protein-containing fractions were determined. As shown in Table III, most of the enzyme was converted into the inactive complex with hydroxocobalamin. This result indicates that hydroxocobalamin could bind to the apopropanediol dehydratase irrespective of the presence of potassium ion or the substrate.

TABLE III: Binding of Hydroxocobalamin to Apopropanediol Dehydratase in the Absence of Potassium Ion and Substrate.[a]

Incubation Mixture	Specific Activity (units/mg)	Complex Formation (%)
Apoenzyme[b]	0.45	(0)
Apoenzyme + OHB_{12}	0.04	91

[a] Experimental procedure is described in the text. [b] Apoenzyme was treated in a similar manner as control.

Effects of Monovalent Cations on Propanediol Dehydratase Activity. Table IV shows the V_{max} and K_m values for the monovalent cations which were effective as the cofactor and could replace potassium ion in the propanediol dehydratase system. Ammonium ion could fully replace potassium ion, and its K_m value was somewhat smaller than that of potassium ion. Rubidium and methylammonium ions were approximately as effective as potassium ion as the cofactor, but their K_m values were several times as large as that of potassium ion. Cesium and sodium ions were able to replace potassium ion partially, but their K_m values were fairly larger than that of potassium ion.

Of the monovalent cations tested, the following cations did not show any cofactor activity at a concentration of 10^{-5} to 10^{-1} M: lithium ion; dimethylammonium ion; trimethylammonium ion; ethylammonium ion; diethylammonium ion; and triethylammonium ion.

Inhibition by Inactive Monovalent Cations. As illustrated in Figure 3, both dimethylammonium and ethylammonium ions

TABLE IV: Effects of Monovalent Cations on Propanediol Dehydratase Activity.[a]

Monovalent Cation	V_{max} (μmoles/min)	Relative Cofactor Activity (%)[b]	K_m (M)
None	0.00	0	
Na	6.58×10^{-2}	57	1.92×10^{-2}
K	1.15×10^{-1}	(100)	6.67×10^{-4}
NH_4	1.22×10^{-1}	106	1.18×10^{-4}
Rb	1.08×10^{-1}	96	1.82×10^{-3}
CH_3NH_3	1.06×10^{-1}	92	8.34×10^{-3}
Cs	5.06×10^{-2}	44	5.00×10^{-3}

[a] Experimental procedure is described in the text. [b] Expressed in relation to the activity with K^+ taken as 100.

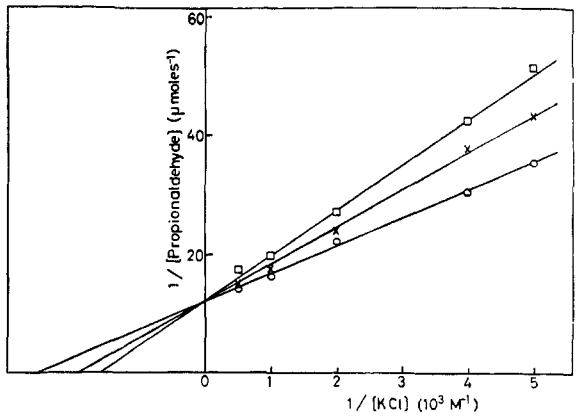


FIGURE 3: The Lineweaver–Burk plot for potassium ion in the presence and absence of either dimethylammonium or ethylammonium ion. Additions: (○—○) none; (□—□) 0.05 M $(CH_3)_2NH_2Cl$; (×—×) 0.05 M $C_2H_5NH_3Cl$. Incubation mixtures and conditions are given in the text.

behaved as weak competitive inhibitors with respect to potassium ion. Their K_i values were calculated from the double reciprocal plots to be 0.075 M and 0.143 M, respectively. Lithium ion showed a mixed type of inhibition, as indicated in Figure 4. The actions of other inactive cations were not further studied.

Effects of Monovalent Cations on the Maintenance of B_{12} Coenzyme–Apoenzyme Complex. To clarify the relationship between activating effects of monovalent cations on the propanediol dehydratase and their actions to maintain the binding of coenzyme B_{12} to the apoenzyme, abilities of monovalent cations to support the coenzyme–apoenzyme complex were evaluated from their effects on the resolution of the holoenzyme by gel filtration method. As shown in Table V, sodium or cesium ion which showed a low cofactor activity could hardly support the binding of coenzyme to the apoenzyme. Irrespective of the coexistence of the substrate, almost complete resolution of the holoenzyme was observed in the presence of these cations, although no significant resolution occurred when the holoenzyme coexisted with both the substrate and potassium or ammonium ion which possesses the highest cofactor activity. In the case of rubidium or methylammonium ion, the holoenzyme was partially resolved in the presence or

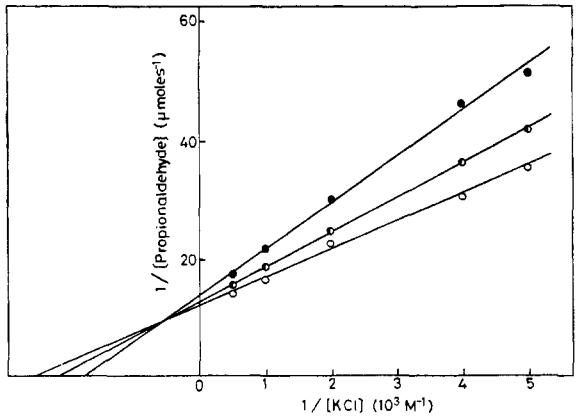


FIGURE 4: The Lineweaver–Burk plot for potassium ion in the presence and absence of lithium ion. Additions: (○—○) none; (◐—◐) 0.05 M LiCl; (●—●) 0.10 M LiCl. Incubation mixtures and conditions are given in the text.

TABLE V: Effects of Monovalent Cations on Resolution of Holopropanediol Dehydratase by Gel Filtration.[a]

Monovalent Cation	Resolution (%)	
	+ Substrate[b]	− Substrate[b]
Activator[c]		
Na	93	100
K	4	38
NH_4	3	28
Rb	16	56
CH_3NH_3	23	58
Cs	96	100
Inhibitor[d]		
Li	100	100
$(CH_3)_2NH_2$	100	100
$C_2H_5NH_3$	100	100

[a] Experimental procedure is described in the text. [b] Buffers containing 0.10 M 1,2-propanediol (+), or not (−), in addition to each monovalent cation were used for gel filtration. [c] At a concentration of 50 times K_m value. [d] At a concentration of 10 times K_i value.

absence of the substrate. In common the extents of resolution in the absence of the substrate were found to be greater than those in the presence of the substrate, since the substrate, although less effective than certain monovalent cations, functions also as one of the factors necessary for the binding of coenzyme B_{12} to the apoenzyme as described above. The actions of monovalent cations, in any event, were well in agreement in the presence and absence of the substrate, and their abilities to maintain the binding of coenzyme to the apoprotein were shown to be closely related to their cofactor activities in this system.

On the other hand, the holoenzyme was completely resolved in the cases of lithium, dimethylammonium, and ethylammonium ions independently of the coexistence of the substrate. These data indicate that inactive monovalent cations, which acted as an inhibitor in the enzymatic reaction, could not maintain the coenzyme B_{12}–apoprotein complex or could do only weakly. The interaction of these inactive monovalent cations with the dehydratase might cause some unfavorable conformational changes of its polypeptide chain(s) or dissociation into subunits, resulting in the conversion of the enzyme protein into the inactive form to which B_{12} coenzyme could not bind.

Effects of Monovalent Cations on Formation of B_{12} Coenzyme–Apoenzyme Complex. In order to investigate capacities of monovalent cations to form the coenzyme–apoenzyme complex, the amount of the holoenzyme formed by incubating the apoenzyme with B_{12} coenzyme in the presence of each monovalent cation was determined by the following two methods.

A. INACTIVATION OF DEHYDRATASE BY OXYGEN. Holopropanediol dehydratase is irreversibly inactivated by being incubated in the absence of the substrate under aerobic conditions (Wagner *et al.*, 1966), although the apoenzyme is not inactivated under the same conditions. Accordingly, the extent of the inactivation by oxygen in the absence of the substrate would reflect the amount of the holoenzyme formed from the apoenzyme and 5′-deoxyadenosylcobalamin under

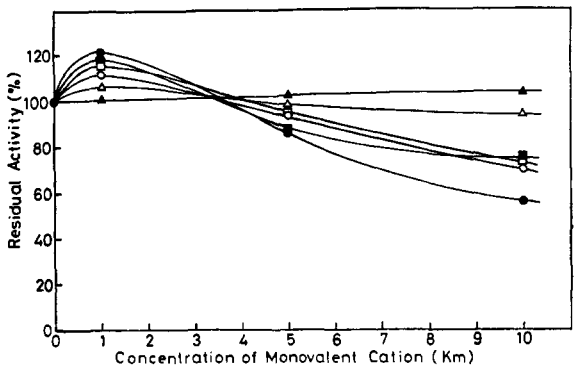


FIGURE 5: Effects of the existence of both 5′-deoxyadenosylcobalamin and varying concentration of monovalent cations on inactivation of propanediol dehydratase by oxygen. Experimental procedure is described in the text. (○—○) KCl; (●—●) NH_4Cl; (□—□) RbCl; (■—■) CH_3NH_3Cl; (△—△) CsCl; (▲—▲) NaCl. The activity remaining after the same treatment without addition of monovalent cation was served as the control (100%).

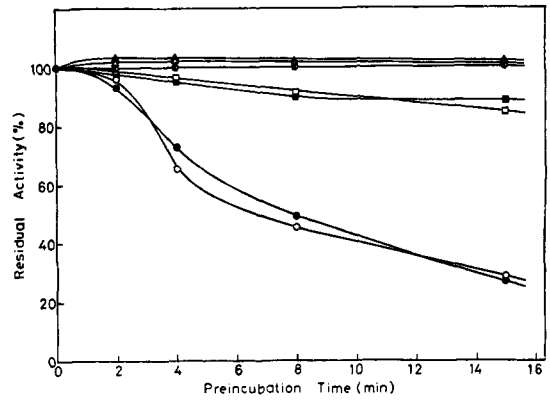


FIGURE 6: Comparison of inactivation rates of propanediol dehydratase in the presence of inhibiting monovalent cations with and without addition of 5′-deoxyadenosylcobalamin. Experimental procedure is described in the text. (⊗—⊗) No monovalent cation plus 5′-deoxyadenosylcobalamin; (○—○) LiCl; (●—●) LiCl plus 5′-deoxyadenosylcobalamin; (□—□) $(CH_3)_2NH_2Cl$; (■—■) $(CH_3)_2NH_2Cl$ plus 5′-deoxyadenosylcobalamin; (△—△) $C_2H_5NH_3Cl$; (▲—▲) $C_2H_5NH_3Cl$ plus 5′-deoxyadenosylcobalamin. Activities of the apoenzyme incubated for indicated periods without addition of both 5′-deoxyadenosylcobalamin and monovalent cation were used as controls (100%).

the influences of cofactors. Based on the fact, it would be possible to estimate the abilities of monovalent cations to form the coenzyme–apoenzyme complex. Figure 5 shows the enzyme activities remaining after the incubation of the apoenzyme with 5′-deoxyadenosylcobalamin at 37° for 10 min in the presence of varied concentrations of each monovalent cation without added substrate (in the presence of air). As expected, the inactivation of dehydratase was dependent on the existence of both 5′-deoxyadenosylcobalamin and monovalent cation, in agreement with Wagner *et al.* (1966), and the cations which showed a higher cofactor activity had a greater effect on the inactivation. That is, a close relationship was observed between the extent of inactivation of the enzyme and the cofactor activity of each monovalent cation coexisting. Similar results were obtained in the experiment in which the concentration of each monovalent cation was fixed and incubation time at 37° was varied. Since the enzyme was not inactivated under these conditions when coenzyme was not added, the inactivation could be undoubtedly attributed to inactivation by oxygen of the holoenzyme formed. As shown in Figure 5, the activities remaining after the incubation were slightly higher than that of the control (no monovalent cation added) when each monovalent cation was added at a concentration near to its K_m value. This observation might be explained by assuming that the effects of monovalent cations on the formation of the holoenzyme and on the inactivation by oxygen of the holoenzyme formed were inferior to the protecting effects of the monovalent cations on the apoenzyme at their relatively low concentrations.

Figure 6 shows the results of similar experiments in which the inactive monovalent cations were used at a concentration of 5 times K_i value and the incubation time at 37° was varied. The activity of the apoenzyme incubated for indicated period in the absence of both 5′-deoxyadenosylcobalamin and monovalent cation was used as a control. Since almost the same enzyme activities remained after the incubation in the presence of these inactive monovalent cations irrespective of the coexistence of coenzyme B_{12}, it would be concluded that the active holoenzyme was not formed in these cases. In other words, dimethylammonium, ethylammonium, or lithium ion seems to have little or no ability to bind coenzyme B_{12} to the apoprotein, in agreement with the fact that these three monovalent cations were ineffective as the cofactor and could not activate the propanediol dehydratase system. Remarkable inactivation of the enzyme in the presence of lithium ion would be ascribed to denaturating effect of a high concentration of LiCl on the apoprotein.

B. ALLEVIATION OF *p*CMB-SUSCEPTIBILITY OF DEHYDRATASE. Even though apopropanediol dehydratase is completely inactivated when incubated with *p*-hydroxymercuribenzoate (*p*HMB), the holoenzyme once formed by preincubation of the apoenzyme with 5′-deoxyadenosylcobalamin resists the inactivation by the *p*HMB (Lee and Abeles, 1963). According to this fact, *p*CMB insusceptibility acquired by the preincubation of the apoenzyme with coenzyme B_{12} under different conditions would reflect the amount of the holoenzyme formed. Hence, the abilities of monovalent cations to form the coenzyme–apoenzyme complex would be reasonably estimated by the enzyme activities remaining after *p*CMB treatment of the preincubated mixtures consisting of the apoenzyme, 5′-deoxyadenosylcobalamin, and each monovalent cation. Table VI shows the effects of three types of treatment on the sensitivity of the enzyme to *p*CMB. As shown in experiment I, the apoenzyme was inactivated almost completely by *p*CMB treatment even after preincubated at 37° with any monovalent cation in the absence of coenzyme B_{12}. Propanediol dehydratase, however, acquired the insensitivity to *p*CMB when preincubated at 37° with certain monovalent cations in the presence of both 5′-deoxyadenosylcobalamin and the substrate or 5′-deoxyadenosylcobalamin only, as shown in experiments II and III. Especially, monovalent cations which showed a higher cofactor activity, such as potassium or ammonium ion, brought about a higher insusceptibility to *p*CMB, and less active monovalent cations such as cesium or sodium ion were found to have almost no effect. These results also show that the abilities of monovalent cations to form the binding of coenzyme B_{12} to the apoprotein were closely related to their effectivenesses as the cofactor in the propanediol dehydratase system, since *p*CMB-insusceptibility acquired was undoubtedly dependent on the existence of both coenzyme B_{12} and monovalent cation, indicating that it represents the amount of the holoenzyme produced.

TABLE VI: Effects of 5′-Deoxyadenosylcobalamin and Monovalent Cations on *p*CMB Susceptibility of Propanediol Dehydratase.[a]

Expt	Type of Treatment	Activity Remaining (%)[b]						
		None	K	NH_4	Rb	CH_3NH_3	Cs	Na
I	Apoenzyme	0.0	0.8	0.4	0.0	0.0	0.8	0.0
II	Apoenzyme + DBCC	36.6	89.5	83.1	77.5	58.7	42.0	1.6
III	Apoenzyme + DBCC + 1,2-propanediol	67.5	101.2	99.0	69.3	52.0	38.8	6.9

[a] Experimental procedure is described in the text. [b] Tubes treated in a similar manner without added *p*CMB are served as controls.

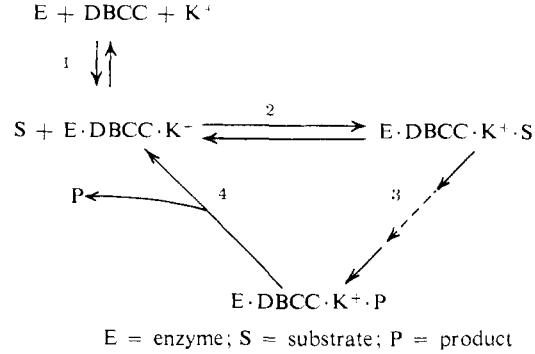
The dehydratase became insensitive to *p*CMB to a certain extent when preincubated at 37° with both 5′-deoxyadenosylcobalamin and the substrate or 5′-deoxyadenosylcobalamin alone in the absence of added monovalent cation. These findings may suggest that there occurred some weak interaction between the apoenzyme and coenzyme B_{12} even in 0.05 M Tris·HCl buffer (pH 8.0) containing no monovalent cation added, resulting in partial blocking of *p*CMB-sensitive SH groups of the enzyme. This interaction seems to be not so strong as to form the active holoenzyme and to be not so stable as to resist the resolution by gel filtration. The observation that dehydratase was extraordinary sensitive to *p*CMB after any type of treatment in the presence of sodium ion might suggest that a conformational change of the native enzyme was caused by high concentrations of NaCl, which rendered the SH group(s) essential for the catalytic activity more susceptible to the attack by sulfhydryl inhibitors such as *p*CMB.

Discussion

While it has previously been shown that propanediol dehydratase requires potassium ion or other monovalent cation in addition to coenzyme B_{12} and the substrate as a factor essential for the catalytic activity (Lee and Abeles, 1963), the function of the monovalent cations remains obscure. The results presented in this paper would indicate that monovalent cation such as potassium ion participates in the binding of coenzyme B_{12} to the apoprotein of propanediol dehydratase in a wide sense including a conformational change of enzyme protein or association of subunits. This function must be the most important one of roles played by potassium ion as the cofactor in this system.

Roles of potassium ion and the substrate are shown in Scheme I. The experimental data reported here could reasonably be explained by assuming that reactions 1 and 2 are reversible and reactions 3 and 4 are irreversible in this scheme. When the holoenzyme was submitted to gel filtration in the presence of sufficient amounts of both potassium ion and the substrate, the equilibrium of 2 lies so far to the right to form E·DBCC·K^+·S and enzymatic reaction proceeds normally such as 2 → 3 → 4 → 2 → →, resulting in no resolution of the holoenzyme. When the holoenzyme coexisted with potassium ion only, the absence of 5′-deoxyadenosylcobalamin shifts the equilibrium (2) to E·DBCC·K^+ and further displaces the equilibrium (1) slowly to the direction of resolution of E·DBCC·K^+ into the active apoenzyme and 5′-deoxyadenosylcobalamin, since the concentration of 5′-deoxyadenosylcobalamin is kept very low in protein zone during gel filtration and recombination between the apoenzyme and 5′-deoxyadenosylcobalamin released hardly occurs at 0–4°. The fact that in this case the resolution of the holoenzyme proceeded according to the kinetics of first-order reaction would support this hypothesis. When the holoenzyme was submitted to gel filtration in the absence of potassium ion, the absence of potassium ion leads to rapid displacement of the equilibrium (1) to resolve the holoenzyme completely into the active apoenzyme and 5′-deoxyadenosylcobalamin.

SCHEME I



E = enzyme; S = substrate; P = product

Of inactive complexes between the apoenzyme and irreversible cobamide inhibitors of propanediol dehydratase, cyanocobalamin- and methylcobalamin-apoenzyme complexes were also for the most part resolved upon gel filtration in the absence of both potassium ion and the substrate. Since the conditions of gel filtration, such as retention time, were almost identical, it seems probable that the difference in the extent of resolution between cyano- and methylcobalamin reflects the relative rates of the dissociation of the two coenzyme analog–apoenzyme complexes. It is of much interest that hydroxocobalamin–apoprotein complex, however, was not resolved by gel filtration under the same conditions. In contrast with 5′-deoxyadenosyl-, cyano-, and methylcobalamin, hydroxocobalamin could bind to the apoenzyme even in the absence of both potassium ion and the substrate. These markedly peculiar behaviors of hydroxocobalamin in the interaction with the apoprotein might be owing to its upper ligand easily exchangeable. It seems likely that the hydroxyl group of hydroxocobalamin interacts with a certain functional group of the protein, or, moreover, is displaced by the protein as the sixth ligand on the cobalt. The unique properties of the binding of hydroxocobalamin to apopropanediol dehydratase would correspond to the observation that excessive amounts of hydroxocobalamin were bound nonspecifically to apoethanolamine deaminase (Kaplan and Stadtman, 1968b) and *Escherichia coli* B N^5-

methyltetrahydrofolate–homocysteine apomethyltransferase (Taylor, 1970). Stable complex formation between hydroxocobalamin and bovine serum albumin has been reported by Taylor and Hanna (1970) to be due to the substitution of the imidazole side chains for H_2O at the sixth coordinating position of the cobalt.

On the other hand, it has been reported by Hill *et al.* (1970) that in the binding of corrinoids to proteins and polypeptides the formation of hydrogen bonds is far more important than coordination by the metal.

Recently, it has been reported by Schneider *et al.* (1970) that the inactive hydroxocobalamin–apoenzyme complex of glycerol dehydratase can be partially converted to the active holoenzyme by replacement of hydroxocobalamin with coenzyme B_{12} in the presence of Mg^{2+} and SO_3^{2-}.

Besides potassium ion, certain monovalent cations were shown to be effective as the cofactor in activation of the propanediol dehydratase system. In Figure 7, the relative cofactor activity of monovalent cation is plotted against its ionic radius described by Goldschmidt (1926). According to the values of Pauling (1927), a similar relationship can be obtained. There seems to be an optimal size of monovalent cation for this enzyme, since the highly active monovalent cations have ionic radii near to that of potassium ion in common. It has been reported that thallium ion shows a cofactor activity comparable to potassium ion (Lee and Abeles, 1963; Manners *et al.*, 1970) and K_m value for it is rather smaller than that of potassium or ammonium ion (Manners *et al.*, 1970) in the propanediol dehydratase system. This is easily acceptable because the ionic radius of thallium ion is 1.44 Å according to the value described by Pauling (1927) or 1.49 Å by Goldschmidt (1926).

Abilities of monovalent cations to maintain the binding of coenzyme B_{12} to the apoenzyme were estimated by the experiment of the resolution of the holoenzyme by gel filtration method, and abilities of monovalent cations to form the binding of coenzyme to the apoprotein were estimated by two experiments, that is, O_2 inactivation and alleviation of *p*CMB susceptibility. Comparatively good agreement is found between these two abilities of each monovalent cation, both of which are closely related to the effectiveness of the cation as the cofactor in this system. According to these results, it would be considered that the major role of potassium ion or other monovalent cations as the cofactor in the propanediol dehydratase system must be to participate in the binding of coenzyme B_{12} to the apoenzyme.

The mechanism whereby monovalent cation is involved in the binding of coenzyme B_{12} to the apoprotein is not yet clear, and further experimentation is necessary to explain fully this interesting observation. The findings that there is an optimal size of monovalent cation for this system might suggest that the monovalent cation occupies appropriate position(s) of the apoprotein and causes conformational changes of polypeptide chains or association of subunits of the enzyme by the electrostatic interaction through its positive charge, resulting in generation of the binding site(s) of B_{12} coenzyme. As a result of the binding of the coenzyme B_{12} at the normal binding site(s) thus formed, cobalt–carbon bond of coenzyme would be activated by the apoenzyme to exhibit the enzymatic activity. That might be a reason why the monovalent cation is absolutely required as the cofactor for exhibition of the enzymatic activity in the propanediol dehydratase system.

Besides propanediol dehydratase, many other coenzyme B_{12} dependent enzymes have been reported to require an appropriate monovalent cation such as potassium ion as a cofactor (Smiley and Sobolov, 1962; Lee and Abeles, 1963; Pawelkiewicz and Zagalak, 1965; Kaplan and Stadtman, 1968a; Stadtman and Renz, 1968; Jacobsen and Huennekens, 1969; Morley and Stadtman, 1970) even though their specificities for monovalent cations are slightly different from propanediol dehydratase. From the fact that the binding of B_{12} coenzyme to the apoprotein of these enzymes is found to be acid labile (Lengyel *et al.*, 1960; Wagner *et al.*, 1966; Kaplan and Stadtman, 1968b), it appears possible that coenzyme B_{12} is noncovalently bound to the apoprotein of these B_{12} enzymes. The conclusion obtained here in the propanediol dehydratase might essentially be applicable to the interaction between B_{12} coenzyme and the apoprotein of other B_{12} coenzyme dependent enzymes, in general.

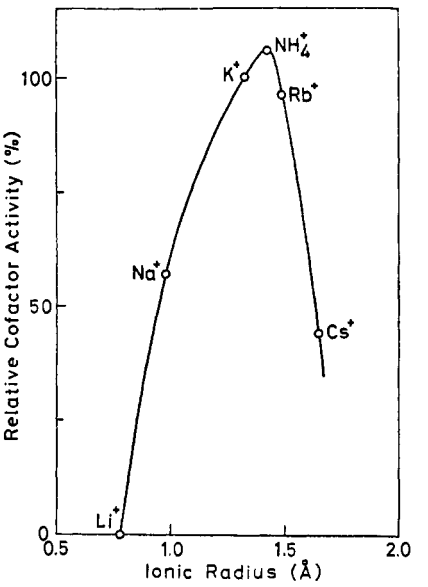


FIGURE 7: Relationship between ionic radii and relative cofactor activities of monovalent cations in the propanediol dehydratase system. Ionic radii are according to the values described by Goldschmidt (1926).

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