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Disproportionation of Vitamin B_{12r} under Various Mild Conditions*

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ABSTRACT: When vitamin B_{12r} dissolved in 1 M NaCl aqueous solution was allowed to stand in the presence of methyl iodide under anaerobic conditions in the dark overnight at room temperature, B_{12r} was converted into an almost equimolar mixture of methylcobalamin and aquocobalamin (vitamin B_{12a}). Under the experimental conditions mentioned above, the change did not occur in the absence of the salt. Other electrolytes, such as KCl, Na_2SO_4, $NaNO_3$, and $MgCl_2$, showed a similar effect. In the case of KCN or Na_2SO_3, cyanocobalamin or cobalamin sulfonate was formed instead of B_{12a}, respectively. Other alkylating agents, such as dimethyl sulfate, also yielded methylcobalamin. An equimolar mixture of B_{12r} and cobinamide$_r$, more easily reducible than B_{12r}, gave methylcobalamin and methylcobinamide in the yields corresponding to 16 and 80% of the initial amounts of B_{12r} and cobinamide$_r$, respectively. A comparative study on the effects of NaCl, NaI, and CsCl showed that the order of their effectiveness accorded with Hofmeister's lyotropic series. When a very high concentration of B_{12r} solution, where a portion of solid B_{12r} remained undissolved, was allowed to react with methyl iodide for 2 days in nitrogen atmosphere, methylcobalamin was obtained in about 20% yield without the help of electrolytes. In the visible absorption spectrum of B_{12r} in 1 M NaCl solution, no change was observed which demonstrated the occurrence of B_{12s} and B_{12a}. A comparative study on the alkylating actions of various monohaloacetates with different reactivity toward B_{12r} in 1 M NaCl revealed that the reactivity of alkylating agent had very marked influence on the reaction. These results would be explained as follows: a very small portion of B_{12r} disproportionates to B_{12s} and B_{12a}; B_{12s} thus formed is not only a very small amount but is also short lived; the formation of alkylcobalamin depends on whether an alkylating agent can trap this short-lived B_{12s} or not. When these reactions of B_{12r} were carried out in hydrogen atmosphere, the yield of alkylcobalamin was higher than that in nitrogen atmosphere.

The important role of reduced forms of vitamin B_{12} in biological systems has been increasingly suggested. Of the two reduced forms of the vitamin, B_{12s}, a two-electron reduction product, is highly reactive and yields alkylcobalamin by reacting with alkyl halide. This fact has led to the supposition that B_{12s} is an active species in several biochemical reactions, such as methane formation (Blaylock and Stadtman, 1964; Wood and Wolfe, 1966), methionine biosynthesis (Weissbach *et al.*, 1965), and conversion of cobalamin into its coenzyme form (Vitols *et al.*, 1964; Weissbach *et al.*, 1966). However, the appearance of B_{12s} has not been detected spectrophotometrically during the enzyme reactions. On the other hand, the absorption spectrum of B_{12r}, a one-elec-

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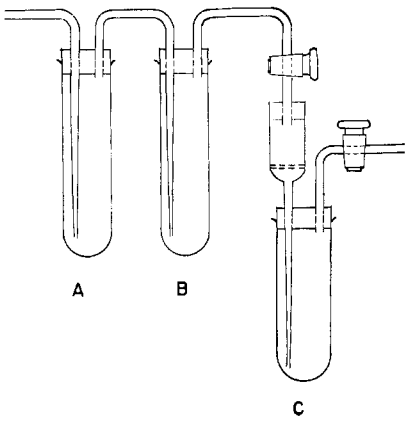

FIGURE 1: Apparatus used for preparation of B_{12r} by catalytic reduction of B_{12a} and subsequent reaction of B_{12r} with alkylating agents. The procedure is described in the text.

tron reduction product, or of something having analogous spectroscopic property, has been often observed during the course of some enzymatic reactions, such as biosynthesis of methionine (Taylor and Weissbach, 1967) and of 5′-deoxyadenosylcobalamin (Weissbach *et al.*, 1966).

A question also remains unsolved in the reduction of aquocobalamin (vitamin B_{12a}) with thiol compounds. It has been observed that the reaction product showed a B_{12r}-like absorption spectrum and an electron spin resonance signal like B_{12r} (Hill *et al.*, 1962, 1965). Nevertheless the product had a reactivity with alkyl halide yielding alkylcobalamin in a similar way to B_{12s} (Dolphin and Johnson, 1965).

This paper deals with the reaction of B_{12r} with appropriate alkylating agents *via* a disproportionation of B_{12r} to B_{12s} and B_{12a} under mild conditions. The results obtained would provide some information on the phenomena mentioned above.

Experimental Section

Materials. Cyanocobalamin was purchased from Roussel-Uclaf Co., France. Aquocobalamin, cyanodehydrocobalamin, and cyanocobinamide were prepared from cyanocobalamin according to the methods of previous workers (Bernhauer and Wagner, 1963; Bonnett *et al.*, 1957; Friedrich and Bernhauer, 1956). Alkylcobalamin, alkyldehydrocobalamin, and alkylcobinamide were prepared by the method of Smith and Mervyn (1963) from their corresponding cyano forms. Methyl iodide, *n*-propyl bromide, sodium monochloroacetate, and inorganic reagents were obtained from commercial sources. Sodium monobromoacetate and sodium monoiodoacetate were prepared from corresponding free acids.

Nitrogen used in this study was purified by passing through six wash bottles, each containing 100 ml of Fieser's solution (an alkaline sodium hydrosulfite solution containing sodium β-anthraquinone sulfonate) (Fieser, 1955). In an atmosphere of the nitrogen thus purified a solution of methylcobalamin (2.1×10^{-5} M) was irradiated with a 100-W tungsten lamp at a distance of 12 cm for 3 hr. A considerable amount of B_{12a} (*ca.* 35% of the initial amount of methylcobalamin) was detected by paper electrophoresis in the irradiated mixture. Dolphin *et al.* (1964) have observed that methylcobalamin was stable in the absence of oxygen (10^{-6} mm) in light of moderate intensity. On the other hand, Pratt (1964) has demonstrated that a trace of oxygen ($\leq 1.3 \times 10^{-7}$ M) markedly accelerated the rate of photolysis of methylcobalamin (5.1×10^{-5} M) in nitrogen atmosphere. These facts indicate that nitrogen used in our experiments still contained a trace of oxygen. By brisk bubbling of the nitrogen for 10 min through B_{12s} solution, B_{12s} oxidized to B_{12r}. But, when an aqueous solution (1×10^{-5} M) of B_{12r} was allowed to stand for 24 hr under a positive nitrogen pressure, no detectable spectral change, showing the formation of B_{12a}, was observed.

Preparation of Vitamin B_{12r} and Its Application to Reactions. METHOD A. B_{12r} was prepared by the catalytic hydrogenation of B_{12a} (Diehl and Murie, 1952). After mixing with an aqueous solution of an electrolyte and removal of hydrogen by bubbling nitrogen through the solution, the catalyst, $PtO_2 \cdot 2H_2O$, was filtered and the filtrate was led to a reaction vessel. In a representative experiment using a reaction apparatus as shown in Figure 1, hydrogen was bubbled for 20 min through 8 ml of aqueous solution of B_{12a} (4.3×10^{-4} M) containing 1 mg of $PtO_2 \cdot 2H_2O$ in vessel A and 8 ml of 2 M NaCl solution in vessel B. B_{12a} was almost completely converted into B_{12r} within 10 min. Nitrogen was then bubbled for 10 min and the B_{12r} solution was transferred into vessel B. After further 10-min bubbling of nitrogen, during which the catalyst was precipitated, the solution in vessel B was transferred into the reaction vessel C through a glass filter with pore diameter of 100–200 mμ.

METHOD B. B_{12r} was prepared by anaerobic photolysis of *n*-propylcobalamin and was applied to reactions with a similar apparatus to that used in method A except that the reaction vessel C was not furnished with the glass filter. In a typical experiment 5 ml of aqueous solution of *n*-propylcobalamin in vessel A and 5 ml of 2 M NaCl solution in vessel B were deoxygenated by 20-min bubbling of nitrogen. Then *n*-propylcobalamin solution was exposed to a 300-W tungsten lamp at a distance of 10 cm under vigorous bubbling of nitrogen. Photolysis was almost complete in 5 min.

In order to remove B_{12s} which would be formed (Yamada *et al.*, 1966b), nitrogen containing a trace of oxygen was briskly bubbled through the solution for further 10 min. By this treatment B_{12s} reoxidized to more stable B_{12r}. Then the B_{12r} solution was transferred into vessel B and next the mixed solution was poured into the reaction vessel C.

METHOD C. Solid B_{12r} prepared as previously described (Yamada *et al.*, 1966c) was used. (1) The apparatus used was similar to that of method A except that vessel A was omitted. Solid B_{12r} was placed on the glass filter (pore diameter, 20–30 μ) attached to the reaction vessel C and water or an aqueous solution of electrolyte was placed in vessel B. Nitrogen was made to pass through the filter *via* the solution in vessel B for 20 min. Then the solution was transferred into the vessel C

through the filter. By the treatment solid B_{12r} placed on the filter was dissolved. (2) When a large amount of B_{12r} was used, a two-necked flask was employed as a reaction vessel, and a glass filter (pore diameter, 20–30 μ) was attached to one neck. The solid B_{12r} was placed inside of the filter, and the flask was inclined so that the powder of B_{12r} might not drop into water in the bulb of the flask. Nitrogen, flushed into the flask through the other neck, was bubbled through the water and passed out through the filter for 20 min. Then the flask was made to stand vertically and was shaken to transfer the powder of B_{12r} into the water. Cobinamide$_r$ and dehydrovitamin B_{12r} were prepared and applied to subsequent reactions according to method B or C.

An appropriate alkylating agent was placed and deoxygenated in the reaction vessel (vessel C in method A, B, and C-1) prior to the addition of B_{12r} solution. In the case of methyl iodide, 0.5 ml in the reaction vessel was mildly bubbled with cooled nitrogen for 2–3 min before the addition of B_{12r} under cooling in ice water. In a particular case (see method C-2), methyl iodide was deoxygenated in another vessel and poured into the reaction vessel. In the cases of sodium monohaloacetates, these were dissolved in water and deoxygenated by 20-min bubbling of nitrogen before the addition of B_{12} solution.

Separation of Reaction Products. Paper electrophoresis was performed using the apparatus, type II-C of Toyo Roshi Co., and Toyo filter paper no. 50 in 0.5 M acetic acid (pH 2.7) and in 0.1 N phosphate buffer (pH 7.0), respectively. Paper chromatography was carried out using Toyo filter paper no. 50 by an ascending method. Solvent systems used were as follows: (1) water-saturated *sec*-butyl alcohol, (2) water-saturated *sec*-butyl alcohol–acetic acid (100:1, v/v), (3) *sec*-butyl alcohol–water–aqueous ammonia (28%) (100:36:14, v/v), (4) 0.1% aqueous KCN-saturated *sec*-butyl alcohol, and (5) *sec*-butyl alcohol–0.2 N aqueous KCN–aqueous ammonia (1000:10:2, v/v).

When the reaction mixture contained only cobalamins, products were well separated by paper electrophoresis in 0.5 M acetic acid and were extracted with water from the paper. Then they were determined, after converted into dicyanocobalamin. Recoveries of methylcobalamin and B_{12a} in these procedures were 87.0 ± 0.62 (standard deviation) and 88.4 ± 1.58% (standard deviation), respectively. In a case where the reaction mixture contained methylcobalamin, aquocobalamin (B_{12a}), methylcobinamide, and aquocobinamide, products were separated by a two-dimensional method which consists of a successive use of paper electrophoresis and paper chromatography. On paper electrophoresis in 0.5 M acetic acid the products were separated into three spots, the first, somewhat tailing, was that of aquocobinamide, the second was an overlapped spot of aquocobalamin and methylcobinamide, and the last was that of methylcobalamin. After the electrophoresis, the paper was exposed to light in order to convert the methylcorrinoids into aquocorrinoids. The paper chromatography was carried out at right angles to the paper electrophoresis with the solvent system 4 containing cyanide. The four products were separated in the forms of cyanocobalamin and dicyanocobonamide. Their recoveries were 86.5 ± 0.82 and 87.2 ± 0.76%, respectively.

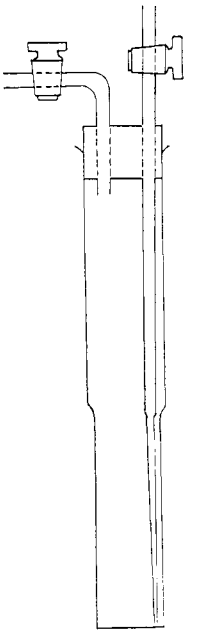

FIGURE 2: Modified quartz cell for absorption spectrum measurement under anaerobic conditions.

Absorption Spectra Measurement. A Hitachi EPS-II self-recording spectrophotometer was used. The measurement under anaerobic conditions was performed using a modified quartz cell fitted with a capillary in a manner as shown in Figure 2.

Results

Reaction of Vitamin B_{12r} with Methyl Iodide in 1 M NaCl. It has been generally accepted that B_{12r} is not reactive with methyl iodide. Under our experimental conditions, there was in fact no reaction observed when methyl iodide was added to a dilute aqueous solution of B_{12r}. In the concomitant presence of an electrolyte, *e.g.*, NaCl, Na_2SO_4, $NaNO_3$, NaOH, KCl, KCN, or $MgCl_2$, however, a considerable amount of methylcobalamin was found to be produced. An isolated product other than methylcobalamin was cyanocobalamin in the presence of KCN, cobalamin sulfonate in the presence of Na_2SO_3, and B_{12a} in the presence of the other electrolytes mentioned above. For subsequent experiments we mostly used NaCl, a typical, simple, and neutral electrolyte.

When 1 M NaCl solution containing B_{12r} prepared by method A and methyl iodide was vigorously shaken and was allowed to stand in the dark overnight under nitrogen, the brown color of B_{12r} turned to red, which was attributed to methylcobalamin and B_{12a}. After desalting with phenol extraction, methylcobalamin and B_{12a} were separated by paper electrophoresis in 0.5 M acetic acid (pH 2.7). Methylcobalamin was identified with the authentic sample by paper chromatography using the solvent systems 1–3. As shown in Table I the yield of methylcobalamin was slightly less than 50%. When the

TABLE I: Yield of Products from the Reaction of Vitamin B_{12r} with Methyl Iodide in 1 M NaCl.

Expt	Product (%) Methylcobalamin	Aquocobalamin
1[a]	46	54
2[a]	44	56
3[b] (without NaCl)	0	100[c]

[a] In expt 1 and 2, 16 ml of 2.1×10^{-4} M and 16 ml of 3.2×10^{-4} M B_{12r} prepared by method A were, respectively, allowed to react with methyl iodide. [b] B_{12r} prepared by method C was used in comparable amounts with that in expt 1 and 2. [c] Oxidation product of remaining B_{12r} by exposure to air.

TABLE II: Yield of Products of All the Corrinoids from the Reaction of an Equimolar Mixture of Vitamin B_{12r} and Cobinamide$_r$ with Methyl Iodide in 1 M NaCl.[a]

	Methylcorrinoid (%)	Aquocorrinoid (%)
Cobalamin	8	42
Cobinamide	40	10
Total	48	52

[a] Reaction mixture (12 ml) contained 2.1×10^{-4} M B_{12r} and 2.1×10^{-4} M cobinamide$_r$ prepared by method B.

reaction of B_{12r} with methyl iodide was attempted in the absence of NaCl, there was no formation of methylcobalamin as mentioned above, and only B_{12a} was obtained, which was considered to be derived from B_{12r} unreacted, by exposure to air. These results suggest that the yield of methylcobalamin would be 50% in ideal conditions in the presence of NaCl, and the slightly lower yield is owing to the difficulty of complete removal of oxygen in methyl iodide and nitrogen used.

Therefore, a following reaction route was supposed. Two molecules of B_{12r} disproportionated into one molecule of B_{12s} and one molecule of B_{12a}, the former of which produced methylcobalamin by reacting with methyl iodide. Dimethyl sulfate also served as alkylating agent, but the activity was markedly lower than that of methyl iodide under our experimental conditions.

Reaction of a Mixture of Vitamin B_{12r} and Cobinamide$_r$ with Methyl Iodide in 1 M NaCl. If B_{12r} disproportionated as described above, the presence of another corrinoid$_r$, which is more or less easily reducible than B_{12r}, would affect the disproportionation process and consequently the ratio of methylcobalamin to B_{12a} produced by the reaction with methyl iodide would not become 1:1.

In order to investigate this point, an equimolar mixture of B_{12r} and cobinamide$_r$, which were prepared by method B, was allowed to react with methyl iodide in 1 M NaCl. After standing overnight under slightly positive nitrogen pressure, products were separated by a successive use of paper electrophoresis in 0.5 M acetic acid and paper chromatography in the solvent system 4. As shown in Table II nearly 50% of all the corrinoids was methylated. However, the yield of methylcobalamin was 8% of all the corrinoids, namely 16% of the initial amount of B_{12r}. On the other hand, methylcobinamide was obtained in the yield of 40% of all the corrinoids, corresponding to 80% of cobinamide$_r$ used.

In this reaction system, it appears probable that some of the following routes would take place as a primary reaction: (1) disproportionation of B_{12r}, (2) disproportionation of cobinamide$_r$, and (3) crossed disproportionation between B_{12r} and cobinamide$_r$. The result in Table II strongly suggests that cobinamide$_r$, which would be more easily reducible than B_{12r}, was preferentially converted into cobinamide$_s$ by the crossed disproportionation and reacted with methyl iodide. The presumption that cobinamide$_r$ was more easily reduced than B_{12r} was supported by the following observation; the conversion rate of aquocobinamide into cobinamide$_s$ was higher than that of B_{12a} to B_{12s} in reduction with $NaBH_4$. The presumption was also supported by polarographic investigation (unpublished result).

Absorption Spectrum of Vitamin B_{12r} in 1 M NaCl. As shown in Figure 3, the visible absorption spectrum of B_{12r} in 1 M NaCl, was identical with that in water. There was nothing found that demonstrated the occurrence of B_{12s} or B_{12a}. This suggests that a very small portion of B_{12r}, if any, disproportionated in 1 M NaCl under the conditions.

Reaction of Vitamin B_{12r} with Monohaloacetates in 1 M NaCl. To obtain information on the property of B_{12s} formed by the disproportionation of B_{12r}, alkyl halides of different reactivity were allowed to react with B_{12r} in 1 M NaCl. For this purpose sodium monochloroacetate, sodium monobromoacetate, and sodium monoiodoacetate were used as alkylating agent and the degree of formation of carboxymethylcobalamin was compared.

In nitrogen atmosphere a mixture of B_{12r} prepared by method C and monohaloacetate in 1 M NaCl was allowed to stand overnight in the dark. This reaction gave by-products which were more acidic than carboxymethylcobalamin. When the reaction time was limited (335 min) for the comparison of the reactivities among the monohaloacetates, only one by-product was obtained in addition to carboxymethylcobalamin. A similarly behaving compound was obtained from carboxymethylcobalamin and monoiodoacetate irrespective of the presence of oxygen and NaCl. B_{12a}, cyanocobalamin, and methylcobalamin did not give such a compound in the presence of monoiodoacetate. Therefore the by-product is regarded as a secondary reaction product formed from carboxymethylcobalamin.

As given in Table III, when monoiodoacetate was

TABLE III: Yield of Products from the Reaction of Vitamin B_{12r} with Monohaloacetates in 1 M NaCl.[a]

Monohaloacetate Used	Product (%)		
	Aquoco-balamin	Carboxy-methyl-cobalamin	By-Product
ICH_2COONa	64	28	8
$BrCH_2COONa$	89	8	3
$ClCH_2COONa$	100	0	0

[a] Reaction mixture (10 ml) contained 2.7×10^{-4} M B_{12r} prepared by method C and 5×10^{-2} M monohaloacetate. Reaction was stopped before it was complete (335 min).

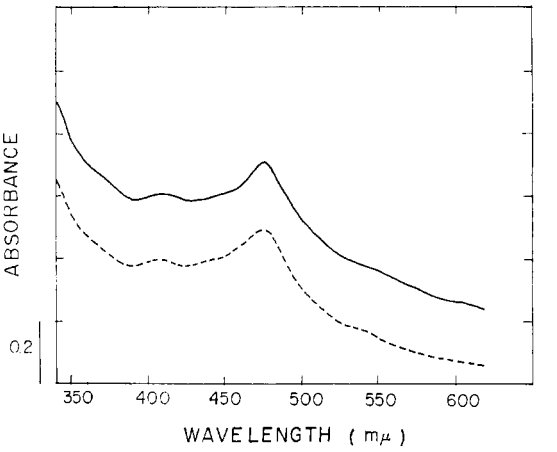

FIGURE 3: Absorption spectra of vitamin B_{12r} (5.1×10^{-5} M) in water and 1 M NaCl, respectively. (———) Vitamin B_{12r} in water and (- - - -) vitamin B_{12r} in 1 M NaCl.

used the yield of carboxymethylcobalamin was 28% and that of the single by-product was 8%. This means that carboxymethylation of B_{12r} occurred in the yield of 36%. Similarly, when monobromoacetate was used, 8% plus 3%, that is, 11% was carboxymethylated, while carboxymethylated products were negligible when monochloroacetate was used. Evidently the reactivity of alkylating agent had very marked influence on the reaction. These results would be explained as follows. B_{12s} formed from B_{12r} in 1 M NaCl is not only in a small amount, but also short lived, and the occurence of the alkylation reaction depends on whether an alkylating agent can trap this short-lived B_{12s} or not. Thus B_{12r} is considered to be indeed in an equilibrium with B_{12s} and B_{12a} and this equilibrium probably lies far to the direction of B_{12r}.

Comparison of the Effect of NaCl, NaI, and CsCl on the Reaction of Vitamin B_{12r} with Methyl Iodide. In order to investigate the role of electrolytes on the reaction of B_{12r} with methyl iodide, the effects of NaCl, NaI, and CsCl were compared. In 1 M solution of these salts, B_{12r} was allowed to react with methyl iodide in a limited time (140 min). As shown in Table IV, NaCl was most effective and yielded 19% of methylcobalamin, while the yield of methylcobalamin was markedly low, only 6 or 7%, respectively, when NaI or CsCl was employed. This order of the effectiveness of the salts was in accord with the Hofmeister's lyotropic series. Therefore it may be probable that the electrolytes remove water molecules around B_{12r} molecules and render them easier to interact with each other. This is in accord with an observation that solid B_{12r} is much less easily dissolved in 1 M NaCl than in water.

TABLE IV: Comparison of Effect of NaCl, NaI, and CsCl on the Reaction of Vitamin B_{12r} with Methyl Iodide.[a]

Electrolyte Added	Product (%)	
	Methylco-balamin	Aquoco-balamin
NaCl	19	81
NaI	6	94
CsCl	7	93

[a] Reaction mixture (10 ml) contained 1 M electrolyte and 1.4×10^{-4} M B_{12r}. Reaction was stopped before it was complete (140 min).

Reaction of Vitamin B_{12r} with Methyl Iodide in the Absence of Electrolytes. If the role of electrolytes is to stimulate the interaction of B_{12r} molecules as described above, it is possible that the disproportionation would occur in a very high concentration of B_{12r} solution without electrolytes. To test this presumption, 11 μmoles of solid B_{12r} prepared by method C was mixed with 0.1 ml of water, where a portion of solid B_{12r} remained undissolved. After adding methyl iodide the mixture was kept for 2 days in nitrogen atmosphere. Then methylcobalamin was obtained in the yield of 23%. Thus the disproportionation was proved to occur without the help of electrolytes under such conditions.

Effect of Hydrogen on the Reaction of Vitamin B_{12r} with Methyl Iodide. The reaction of B_{12r} with methyl iodide was compared in nitrogen and hydrogen atmosphere to test the possibility that molecular hydrogen serves as reducing agent to B_{12r}. When B_{12r} prepared by method C was allowed to react with methyl iodide overnight in 1 M NaCl, methylcobalamin was obtained in the yield of 43% under nitrogen, while 59% under hydrogen (Table V). When the reaction of B_{12r} or dehydrovitamin B_{12r} was performed in 0.1 N NaOH, the yield of the methylation product was also higher in hydrogen than in nitrogen. These results indicate that molecular hydrogen can act as a reducing agent to corrinoid$_r$. Tackett *et al.* (1963) have demonstrated the reduction of H_2O by B_{12s}, which is possibly a reverse of the reaction described here.

It was surprising that the reaction in 0.1 N NaOH gave methylcobalamin in the yield of more than 50% even in nitrogen atmosphere. Heating of cyanocobalamin or aquocobalamin in 0.1 N NaOH is known to lead to the dehydrogenation on the ring B of corrin nu-

TABLE V: Effect of Hydrogen on the Reaction of Vitamin B_{12r} and Dehydrovitamin B_{12r} with Methyl Iodide.[a]

Expt	Substance	Atmosphere	Electrolyte Added	Product (%)	
				Methyl-corrinoid	Aquo-corrinoid
1	B_{12r}	N_2	1 M NaCl	43	57
2	B_{12r}	H_2	1 M NaCl	59	41
3	B_{12r}	N_2	0.1 N NaOH	61	39
4	B_{12r}	H_2	0.1 N NaOH	83	17
5	Dehydro-B_{12r}	N_2	0.1 N NaOH	52	48
6	Dehydro-B_{12r}	H_2	0.1 N NaOH	58	42
7	Dehydro-B_{12r}	N_2	0.1 N NaOH	56	44

[a] Volume of reaction mixture and concentration of B_{12r} or dehydrovitamin B_{12r} was as follows: expt 1 and 2, 7 ml and 2.0×10^{-4} M; expt 3, 8.6 ml and 4.0×10^{-4} M; expt 4, 6.3 ml and 6.2×10^{-4} M; expt 5 and 6, 7 ml and 2.2×10^{-4} M; expt 7, 20 ml and 6.5×10^{-5} M; Dehydrovitamin B_{12r} used in expt 7 was prepared by method B and the others were prepared by method C.

cleus with simultaneous reduction of the cobalt atom. To test whether such a reaction occurred or not, methylcobalamin and B_{12a} produced from B_{12r} were converted into their dicyano forms and examined by visible absorption spectrum and by paper chromatography in solvent system 5 (Yamada *et al.*, 1966a). There was no dehydrocobalamin detected. Reaction of dehydrovitamin B_{12r}, which was already dehydrogenated on ring B, with methyl iodide in 0.1 N NaOH also gave its methylation product in the yield over 50%. Therefore another additional reduction mechanism should be sought in this case.

Discussion

Although a trace oxygen contaminating in our reaction systems might have some influences on the reaction, such as its equilibrium, reaction rate, or yields of alkylated products, the results obtained above strongly suggest the mechanism of the change of B_{12r} to B_{12a} and alkylcobalamin in eq 1.

$$\underset{B_{12r}}{2Co^{II}} \rightleftharpoons \underset{B_{12s}}{Co^{I}} + \underset{B_{12a}}{Co^{III}} \qquad (1)$$

$$\downarrow \text{alkylating agent}$$

$$\text{alkyl } B_{12}$$

In aqueous solutions B_{12r} disproportionates to B_{12s} and B_{12a} until an equilibrium is attained. This equilibrium lies so far to the direction of B_{12r} that B_{12s} or B_{12a} cannot be detected spectrophotometrically. An electrolyte added to this system promotes the disproportionation. When alkylating agents trap B_{12s} as alkylcobalamin to take it out of the equilibrium system, the equilibrium moves toward the right in eq 1 and finally an equimolar mixture of alkylcobalamin and B_{12a} is produced. Although it is difficult to define a conclusive mechanism of the action of electrolytes, a possible explanation might be offered by assuming that the electrolytes facilitate the interaction of B_{12r} molecules by removing the water molecules surrounding them. This view would be supported by the facts that the order of the effectiveness of the electrolytes, so far tested, was in accord with the lyotropic series and a very high concentration of B_{12r} solution gave alkylcobalamin even in the absence of electrolytes.

Schrauzer and Windgassen (1966) reported the occurrence of characteristic absorption peaks of B_{12s} when they added KOH at 1 N concentration to a methanolic solution of B_{12r}. This fact indicates that under specially vigorous conditions a sufficient amount of B_{12s} is formed as detected spectrophotometrically although the possibility of dehydrogenation on ring B or action of other reduction mechanisms cannot be ruled out. These phenomena show that B_{12s} produced by the same mechanisms can be spectrophotometrically detected in one case where vigorous condition is utilized, and cannot be detected in another case where the reaction was mildly carried out in neutral aqueous solution analogous to biochemical reactions.

For example, a reduction product of cyanocobalamin with ferredoxine can serve as substrate in the enzymatic synthesis of 5′-deoxyadenosylcobalamin and can also be methylated with methyl iodide, although it has an identical absorption spectrum with that of B_{12r} (Weissbach *et al.*, 1966). Considering the results of Vitols *et al.* (1964) that B_{12s} produced by $NaBH_4$ reduction of B_{12a} serves as substrate in the deoxyadenosylating enzyme system, a possible explanation for the phenomena mentioned above would involve a transient formation of B_{12s}. Similarly, in the terminal reaction of methionine biosynthesis, the spectrum of B_{12s} has not been observed in spite of the property of the B_{12} enzyme to be propylated with propyl iodide. In this case the spectrum of the enzyme is similar to that of B_{12r} (Brot and Weissbach, 1965), although the absence of electron spin resonance signal leaves a problem to be solved.

Reduction of cobalamins with several thiols gives a

compound having a similar absorption spectrum to that of B_{12r} (Hill *et al.*, 1962) as well as a characteristic electron spin resonance spectrum of B_{12r} (Hill *et al.*, 1965). This compound reacts with methyl iodide and 5′-iododeoxyadenosine to give methylcobalamin and 5′-deoxyadenosylcobalamin (Murakami *et al.*, 1966), respectively, while it hardly reacts with 2′,3′-isopropylidene-5′-tosyladenosine (Dolphin and Johnson, 1965; Morley and Blakley, 1967). These facts can be also understood as that the reactant is a small amount of B_{12s} which has only a short life. The difference of the behaviors between 5′-iododeoxyadenosine and 2′,3′-isopropylidene-5′-tosyladenosine is explained by the marked dependence of transient B_{12s} on the reactivity of alkylating agent, as is shown when B_{12r} was allowed to react with different monohaloacetates. When 2′,3′-isopropylidene-5′-tosyladenosine is employed, the rapid reaction will not be seen unless a considerable amount of B_{12s} is maintained with a strong reducing agent. An analogous interpretation may be offered for our findings that a B_{12r}-like compound formed by anaerobic heating of cyanocobalamin in 0.1 N NaOH reacts with methyl iodide or *n*-butyl bromide (Yamada *et al.*, 1964, 1966a).

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