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Studies on Cobaloxime Compounds. III. Effects of Various Cobaloximes as Vitamin B_{12} Models and of Substrate Analogs on Diol Dehydratase System

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Various types of cobaloximes with the general formula $[CoX(DH)_2B]$ or $[RCo(DH)_2B]$ (X: Cl, CN. R: alkyl groups such as methyl, ethyl, *n*-propyl, or *i*-propyl. DH: dimethylglyoximato monoanion. B: bases such as water, pyridine, nicotinamide, *γ*-picoline, *p*-toluidine, or imidazole) and some amide compounds were examined for the inhibitory effect or coenzyme activity in propanediol dehydratase of *Aerobacter aerogenes*. None of them act as coenzyme nor inhibitor, except for cyanoaquocobaloxime, which shows only a weak inhibitory effect that has been proved to be non-competitive. Water-soluble polymeric cobaloximes with the general formula [Co(OH) $(DH)_2$(Copoly–AM-VPy)] (Copoly–AM-VPy: a low molecular weight copolymer of acrylamide and 4-vinylpyridine) were also prepared and examined in the enzymatic system whose inhibitory effects were very small. Other cobaloximes with the general formula $[RCo(DH)_2(py)]$ (R: *β*-hydroxy-*n*-propyl, *β*-hydroxy-*i*-propyl, or *β*,*γ*-dihydroxy-*n*-propyl. py: pyridine) which were three of the possible intermediate models in propanediol dehydratase system, also showed little effect on the enzymatic reaction. Substrate anologs such as 2-bromo-1-propanol, 1-bromo-2-propanol, and glycerol *α*-monochlorohydrin, which are considered to interact with a nucleophilic Co(I) species, presumably a component of the active site in the holoenzyme, were found to serve as substrate.

Although many works have been done on vitamin B_{12} compounds, there are not many on the active sites in holoenzyme in which coenzyme B_{12} and substrate are assumed to be bound to apoenzyme. In the case of L-methylmalonyl-CoA mutase from sheep liver, it was assumed that holoenzyme formation involves at least a two-point attachment between the coenzyme and apoenzyme and that sulfhydryl groups are one binding site of the protein.[1] In ribonucleotide reductase from *L. leichmannii*, 5′-deoxyadenosylcobalamin with a slight alteration of *e*-propionamide group to *e*-propionic acid on the corrin ring showed neither activity nor inhibitory effect.[2] In propanediol dehydratase system, the analogs with two or three propionic acid side-chains on the corrin ring did not serve as the coenzyme, while all the three isomers of one propionic acid side-chain (at *b*, *d*, or *e* position on the corrin ring) were effective.[3]

We considered that for the appearance of the coenzyme activity in enzymatic reactions, there might be some amide groups in the neighborhood of the Co atom, since these groups in cobalamin might take part in the reactions *in vivo*, probably as binding sites to apoenzyme.[4] We have therefore studied the effect of cobaloximes having a ligand with amide groups. The resemblance of cobaloxime compounds to cobalamin compounds in chemical behavior has also been described elsewhere.[5–8]

There are a few examples dealing with the cobaloximes as the vitamin B_{12} model in biological systems. Some cobaloximes were examined in blockade of vitamin B_{12}-binding sites in gastric juice, serum, and saliva,[9] and some methylcobaloximes were demonstrated to be able to make methane in enzymatic reaction containing the extracts from *Methanobacillus omelianskii*.[10]

We have also studied the effect of various cobaloximes on propanediol dehydratase system. Among them, hydroxypropyl derivatives might be considered as model compounds for the possible intermediates in the enzymatic reaction. At present the following hypothesis seems to be acceptable: It is necessary for the appearance of coenzyme activity that Co–5′-deoxyadenosyl

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5) G. N. Schrauzer and J. Kohnle, *Chem. Ber.*, **97**, 3056 (1964).
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8) L-P. Lee and G. N. Schrauzer, *ibid.*, **90**, 5274 (1968).
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bond in DBCC bound to apoenzyme is heterolytically cleaved, producing enzyme-bound Co(I) species. Thus, the electrophilic substrate analogs such as propylene bromohydrin are considered to have the ability to interact with the Co(I) species and serve as substrate. The results are given in the following.

Experimental

Materials. Preparative methods of various cobaloximes were reported.[11]

Imidazole-4,5-dicarboxyamide was prepared according to the method described by Baxter and Spring.[12]

Found: C, 38.47; H, 3.98; N, 36.44%. Calcd for $C_5H_6N_4O_2$: C, 38.96; H, 3.92; N, 36.35%.

2-Bromo-1-propanol was synthesized as follows: $LiAlH_4$ 5.32 g was refluxed for 3 hr in 300 m*l* of dry ether. α-Bromopropionyl bromide 43.2 g was added slowly to the mixture at 0°C for an hour followed by stirring for 2 hr. After adding 15 m*l* of H_2O and 130 m*l* of 3N H_2SO_4 to the mixture, the upper ether layer was separated. The remaining water layer was extracted with isopropyl ether, and the extract was combined with the ether layer. After drying the ether solution with Na_2SO_4, the product was fractionally distilled under a reduced pressure (bp 71°C/33 mmHg). The yield was 17.6 g (64%). The structure was confirmed by NMR and IR spectra.

Found: C, 26.85; H, 5.23; Br, 57.77%. Calcd for C_3H_7OBr: C, 25.93; H, 5.08; Br, 57.49%.

Propylene bromohydrin (commercial E. P. reagent) was a mixture of 1-bromo-2-propanol (79%) and 2-bromo-1-propanol (21%). Its composition was determined by NMR and gas chromatography, and used without further purification, because it was impossible to fractionate it by the usual fractional distillation.

5,6-Dimethylbenzimidazolylcobamide coenzyme (DBCC) was supplied by Prof. S. Fukui and Eisai Co., and propanediol dehydratase (EC. 4.2.1.28) was generously given by Prof. S. Fukui.

All other reagents were of commercial G. R. grade.

Methods. *General Method for the Enzymatic Reaction*: The enzymatic reaction was carried out in the dark. DBCC and cobaloxime or amide were mixed prior to the addition of the apoenzyme solution. A typical method is as follows: 0.1 m*l* of 0.5M KCl, and 0.2 m*l* of cobaloxime or amide solution were pipetted into an assay tube. 0.1 m*l* of DBCC solution was then added in the dark and after being cooled in an ice bath, 0.5 m*l* of cold apoenzyme solution in potassium phosphate buffer (pH 8.0) was added. The reaction was conducted for 5, and 10 min at 37°C in the dark. The mixture was then rapidly cooled in an ice bath and 0.5 m*l* of 10% trichloroacetic acid was immediately added to the mixure to stop the reaction.

In the Case of Hydroxypropylcobaloximes: The reaction was carried out for 10 min at 25°C, aerobically or anaerobically after the following treatment. A 100 W tungsten lamp was used as the light source at a distance of 4 cm.

(1) (Dark-Aerobic): A mixture of DBCC, cobaloxime, and apoenzyme was preincubated for 7 min at 25° in the dark prior to the addition of the substrate. The treatment was done in the presence of air.

(2) (Dark-Anaerobic): The same treatment as (1) but under a nitrogen atmosphere.

(3) (Light-Aerobic): A mixture of DBCC, cobaloxime, and apoenzyme was preincubated for 2 min at 25°, followed by irradiation for 5 min at 25°C prior to the addition of the substrate. The treatment was done in the presence of air.

(4) (Light-Anaerobic): The same treatment as (3) but under a nitrogen atmosphere.

In the Case of Substrate Analogs: The reaction was carried out for 2, 3.5, 7.5 (or 8), and 10 min at 25°C in the dark.

Determination of Propionaldehyde: One milliliter of 5 mM 2,4-dinitrophenylhydrazine in methanol containing a small amount of concd. HCl was added to each reaction mixture which was put to stand for 10 min at 37°C. Three milliliters of 80% aqueous pyridine were then added. The amount of hydrazone formed from propionaldehyde was colorimetrically determined at 540 mμ, 20 min or 40 min after the addition of 0.5 m*l* of alcoholic KOH (0.1 g/m*l* of 80% aqueous methanol), with a Hitachi photoelectric spectrophotometer. The absorbance was corrected by using the control solution as a reference, which is the mixture stopped immediately after the addition of apoenzyme solution. Unity of absorbance at 540 mμ, 20 min or 40 min after the addition of KOH corresponds to 0.92 and 1.09 μmol of propionaldehyde in 1 m*l* of the original reaction mixture, respectively.

The velocity was determined with the slope of the straight line passing through the time-conversion plots. The relative activity was determined as follows.

$$\text{Relative activity} = \frac{\text{Amount of aldehyde formed in the presence of additive (inhibitor)}}{\text{Amount of aldehyde formed in the absence of additive (inhibitor)}} \times 100\,(\%).$$

Results and Discussion

Polymeric Cobaloximes, Co(OH)(DH)$_2$(Copoly-AM-VPy). The enzymatic reaction was carried out in the presence of various amounts of polymeric cobaloximes in which

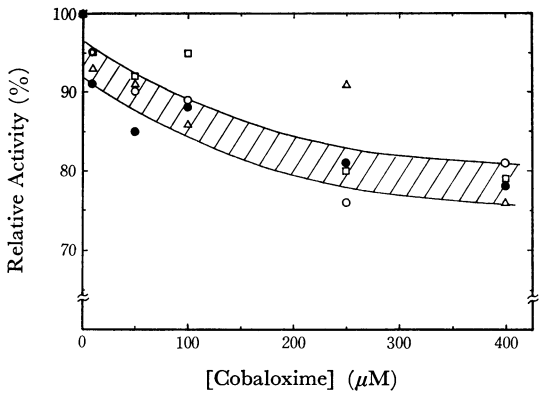

Fig. 1. Relative activity *vs.* concentration of polymeric cobaloxime plots.
The reaction mixture contained 1,2-propanediol 10 μmol, potassium chloride 50 μmol, DBCC 1.5 mμmol, apoenzyme 0.08 unit, potassium phosphate buffer (pH 8.0) 25 μmol, and polymeric cobaloxime (○ HC 025; □ HC 027; ● HC 029; △ HC 030) 0, 10, 50, 100, 250, or 400 mμmol; total volume 1.0 m*l*. The reaction was carried out for 10 min at 37°C in the dark. The average molecular weights of polymeric ligands and AM(acrylamide)/VPy(4-vinylpyridine) ratios are 3000 and 11.9 for HC 025, 2400 and 12.8 for HC 029, 1400 and 13.7 for HC027, 830 and 17.5 for HC030, respectively.

11) N. Yamazaki and Y. Hohokabe, This Bulletin, **44**, 63 (1971).

12) R. A. Baxter and F. S. Spring, *J. Chem. Soc.*, **1945**, 232.

Copoly–AM-VPy[13] coordinates with the Co atom through a pyridine residue and the amide groups are close to the cobaloxime moiety. The results are shown in Fig. 1. Although the relative activities with respect to polymeric cobaloximes decreased with the increase of the amount of cobaloximes, they decreased to *ca.* 80% even at the higher concentration of cobaloximes, and no difference in decrease with respect to the molecular weights of polymers was observed. This suggests that the polymeric cobaloximes employed in this experiment were not sufficiently small to interact with the active site in the enzyme, though their molecular weights are nearly the same as that of coenzyme B_{12} or larger only by a factor less than 2.5. This type of cobaloximes does not seem to interact with the active site in the holoenzyme.

TABLE 1. RELATIVE ACTIVITIES IN THE PRESENCE OF SOME AMIDES.

The reaction mixture contained 1,2-propanediol 10 μmol, potassium chloride 50 μmol, DBCC 1.5 mμmol, apoenzyme 0.10 unit, potassium phosphate buffer (pH 8.0) 25 μmol, and amide 250 mμmol; total volume 1.0 m*l*. The reaction was carried out for 10 min at 37°C in the dark.

Material	Relative activity (%)
None	100
Nicotinamide	90
Acetamide	92
Isobutylamide	93
Benzamide	95
Propionamide	103
Imidazole-4,5-dicarboxyamide[a)]	101

a) nearly insoluble.

Amides. From the viewpoint in the introductory, *i. e.*, some amide groups in cobalamin may take part in the reactions *in vivo*, perhaps as the binding sites to the apoenzyme, the presence of amides is expected to affect the enzymatic reaction. The results of some amides are shown in Table 1. The activity was affected slightly or not at all by the presence of amide. From the results, we see that monoamide is not bound tightly to the site because of weakeness of the hydrogen bonding. The multiple-point attachment of the amide groups in a molecule may favor the single-point attachment by the increase in total entropy and decrease in total energy by each hydrogen bonding. One of the diamide, imidazole-4,5-dicarboxyamide, however, failed to affect the active site, probably because of insolubility. It seems that some amide groups situated at an appropriate distance from each other and the cobaloxime moiety might interact with the active site.

The enzymatic reaction was also carried out in the presence of various monomeric cobaloximes.

Chloro- and Simple Alkyl-cobaloximes. All the chloro- or simple alkyl-cobaloximes were found to exert little effect on the enzymatic reaction: They include $CoCl(DH)_2B$ in which B is water, nicotinamide, pyridine, imidazole, γ-picoline, or *p*-toluidine, $CH_3Co(DH)_2B$ in which B is water, nicotinamide, pyridine, or imidazole, and $RCo(DH)_2B$ in which R is methyl, ethyl, *n*-propyl, or *i*-propyl, and B water or pyridine.

13) Copoly–AM-VPy is a low molecular weight copolymer of acrylamide (AM) and 4-vinylpyridine (VPy).

TABLE 2. RELATIVE ACTIVITIES IN THE PRESENCE OF CYANOCOBALOXIMES.

The reaction mixture contained 1,2-propanediol 10 μmol, potassium chloride 50 μmol, DBCC 1.5 mμmol, cobaloxime 250 mμmol, apoenzyme 0.083 unit (0.69 unit/mg-protein), potassium phosphate buffer (pH 8.0) 25 μmol; total volume 1.0 m*l*. The reaction was carried out for 5, or 10 min at 37°C in the dark.

Material[a)]	Relative activity (%)
None	100
NaCN(250 mμmol)	88
Co (CN)$(DH)_2(H_2O)$	57
Co(CN)$(DH)_2$(py)	90
Co(CN)$(DH)_2$(pico)	98
Co(CN)$(DH)_2$(tolu)	105

a) py: pyridine, pico: γ-picoline, tolu: *p*-toluidine.

Cyanocobaloximes. It is well known that cyanocobalamin is a strong competitive inhibitor in diol dehydratase system and other B_{12}-dependent enzymatic reactions. The effect of cyano cobaloximes was examined in comparison with cyanocobalamin. The results are shown in Table 2. They exerted no inhibition or much less inhibitory effect than cyanocobalamin; only cyanoaquocobaloxime showed a weak inhibition. From an extensive study on cyanoaquocobaloxime, it proved to be non-competitive as shown in Fig. 2. The

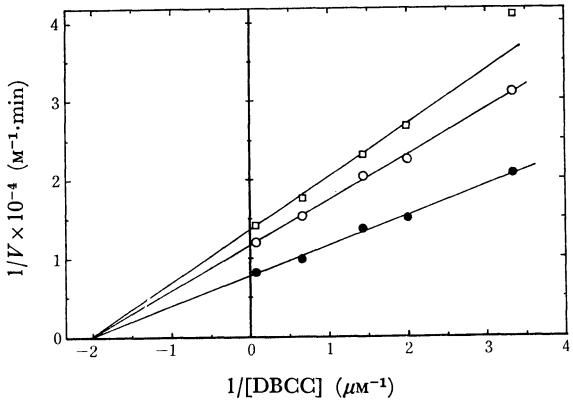

Fig. 2. Non-competitive inhibitory effect of cyanoaquocobaloxime on propanediol dehydratase system.
The reaction conditions are the same as those in Table 2, except that DBCC 0.30, 0.50, 0.70 1.50, or 15.0 mμmol, cyanoaquocobaloxime 0 (●), 200 (○), or 250 (□) mμmol, and apoenzyme 0.14 unit (0.73 unit/mg-protein) were employed.

inhibitor constant K_i was determined to be $3.3\ (\pm 0.1) \times 10^{-4}$M. Since the same amount of sodium cyanide exerted much less inhibition, the weak effect of cyanoaquocobaloxime is not considered to be due to free cyanide ion which might be derived from the cobaloxime. It seems that a slight conformational change of the enzyme protein occurs in the presence of the cobaloxime. A most plausible explanation of the effect is as follows. First, CN linked to cobaloxime interacts with

a given site in the protein, second, another axial ligand of the cobaloxime, *i. e.*, H_2O is displaced by a basic group of the enzyme, resulting in a slight conformational change of the tertiary structure of the enzyme.

Hydroxypropylcobaloximes. The preparation and properties of a series of hydroxyalkylcobaloximes have been reported by Schrauzer and Windgassen.[14] The Co–C bonds are readily cleaved; in mildly acidic media olefins are formed. However, in alkaline solution the products are aldehydes, ketones, or the respective aldols arising from the secondary condensation of the aldehydes. They proposed a mechanism for the action of the corrin coenzyme in diol dehydratase of *Aerobacter aerogenes*, in which they supposed that β-hydroxy-*i*-propyl derivative is the most likely organocobamide as the intermediate in glycol dehydratase system. The corresponding cobaloxime was prepared and found to produce propionaldehyde on decomposition in alkaline medium. Its effect on enzymatic reaction seems to be interesting. β,γ-Dihydroxypropylcobalamin exhibited no coenzyme activity, and exerted an inhibitory effect.[15]

Table 3. Relative activities in the presence of hydroxypropylcobaloximes under various conditions.
The reaction mixture contained 1,2-propanediol 10 μmol, potassium chloride 50 μmol, DBCC 15.0 mμmol, cobaloxime 200 mμmol, apoenzyme 0.068 unit (0.20 unit/mg-protein) for the first series of runs, and 0.086 unit (0.49 unit/mg-protein) for the second, potassium phosphate buffer (pH 8.0) 25 μmol; total volume 1.0 ml. Other conditions are indicated in the experimental section.
The series of runs were carried out twice and the mean values are indicated in the following table.

Material[a]	Dark		Light	
	Aerobic (1) %	Anaerobic (2) %	Aerobic (3) %	Anaerobic (4) %
None	100	133	56	89
$HOCH_2CH(OH)$-$CH_2Co(DH)_2$(py)	100	94	20	87
$HOCH_2CH(CH_3)$-$Co(DH)_2$(py)	98	119	41	101
$CH_3CH(OH)CH_2$-$Co(DH)_2$(py)	99	121	33	136

a) py: pyridine

Three of the possible intermediate models, *i. e.*, β-hydroxy-*i*-opropyl-, β-hydroxy-*n*-propyl-, and β,γ-dihydroxy-*n*-propyl-(pyridine)cobaloxime, were also synthesized and examined in propanediol dehydratase system. As the alkyl moiety of these cobaloximes are very similar to propanediol in structure, some effect could be expected; cobaloxime may block the substrate-binding site, and/or hydroxypropyl radicals arisen from the light irradiation of the cobaloximes may interact with the active coenzyme. As shown in Table 3, however, they also exerted little effect on the reaction.[16] This suggests that the cobaloximes do not contact with the active site of the holoenzyme.

All the cobaloximes described were unable to interact with the active site. Synthesis of 5′-deoxyadenosylcobaloxime failed in isolation by the usual way as carried out in the partial DBCC preparation, presumably because of its alkali and acid lability, as reported by Schrauzer.[17]

Substrate Analogs. Since it is well established that Co(I) species of cobalamin is a powerful nucleophilic agent,[18] the Co(I) nucleophile seems to play an important role in enzymatic conversion of diol to the corresponding aldehyde. Since propanediol and glycol were found not to react with Co(I) species, propanediol is considered to be activated in some form which is able to react with Co(I). Hence, three analogs, *i. e.*, $CH_3CH(OH)CH_2Br$, $CH_3CHBrCH_2OH$, and $HOCH_2CH(OH)CH_2Cl$, which are structurally similar to $CH_3CH(OH)CH_2OH$, are considered to be able to serve as a substrate or an accelerator.

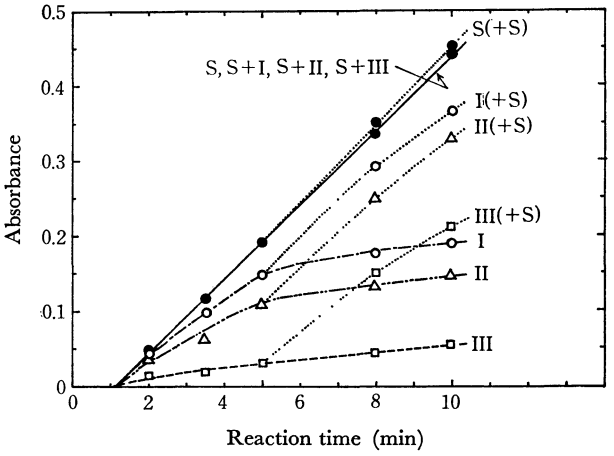

Fig. 3. Effect of substrate anologs on propanediol dehydratase system.
The reaction mixture contained I, II, III, and/or S 10 μmol, potassium chloride 50 μmol, DBCC 15.0 mμmol, apoenzyme 0.10 unit (0.61 unit/mg-protein), potassium phosphate buffer (pH 8.0) 25 μmol; total volume 1.0 or 1.1 ml. The reaction was carried out for 2, 3.5, 5, 8, or 10 min at 25°C in the dark. S (—●—), I (-·-○-·-), II (-···△-···), or III (—□—) represents the reaction in the presence of S, I, II, or III, respectively. S+I (—●—), S+II (—●—), or S+III (—●—) represents the reaction in the co-existence of S and I, S and II, or S and III, respectively. Since the reaction with S+I, S+II, or S+III proceeded in the same way as that with S, they are shown in the same line. S (+S) (···●···), I (+S) (···○···), II (+S) (···△···), or III (+S) (···□···) represents the reaction with the additional 10 μmol 1 of S during the reaction in the presence of S, I, II, or III, respectively. Unity of absorbance corresponds to 1.09 μmol of propionaldehyde. Notations on S, I, II, and III are described in the text.

14) G. N. Schrauzer and R. J. Windgassen, *J. Amer. Chem. Soc.*, **89**, 143 (1967).
15) T. Yamane, S. Shimizu, and S. Fukui, *J. Vitaminol.*, **12**, 10 (1966).
16) The enzymatic reaction was carried out at 25°C, lower than the usual 37°C, in order to minimize the inactivation of holoenzyme by incubation prior to the addition of the substrate. The holoenzyme is known to be inactivated by oxygen in the absence of the substrate.
17) G. N. Schrauzer and J. W. Sibert, *J. Amer. Chem. Soc.*, **92**, 1022 (1970).
18) G. N. Schrauzer and E. Deutsch, *ibid.*, **91**, 3341 (1969).

The following abbreviations will be used for the sake of convenience. S; 1,2-propanediol, I; propylene bromohydrin [a mixture of 1-bromo-2-propanol (79%) and 2-bromo-1-propanol (21%)], II; 2-bromo-1-propanol, III; glycerol α-monochlorohydrin.

When 10 μmol of I, II, or III was added to 10 μmol of S which was considered appropriate to give a maximum velocity V_{max}, the enzymatic reaction proceeded just the same as that in the absence of I, II, or III. I, II, or III did not inhibit the enzymatic reaction. However, they seemed to serve as substrates shown in Fig. 3; even in the absence of S, the velocity of the reaction with I or II was comparable to that with S in the initial stage of the reaction, while that with III was observed to be measurable but much less. I, II, or III is considered to interact with the same site as a normal substrate; the initial velocity of S+I, S+II or S+III would be larger than that of S which gives the V_{max}, if I, II, or III reacted with another site of the enzyme other than the normal substrate-binding site. At low concentration of S not enough to give V_{max}, addition of I or II increased the reaction velocity (Fig. 4), which supports the idea that I and/or II serve as a substrate. However, the active site in the presence of I, II, or III without S was soon inactivated as shown in Fig. 3. When such reactions were carried out anaerobically, the time-course was observed to be the same as that in aerobic conditions. It appears that inactivation is not caused by oxygen, whereas the holoenzyme is known to be inactivated by oxygen in the absence of the substrate.

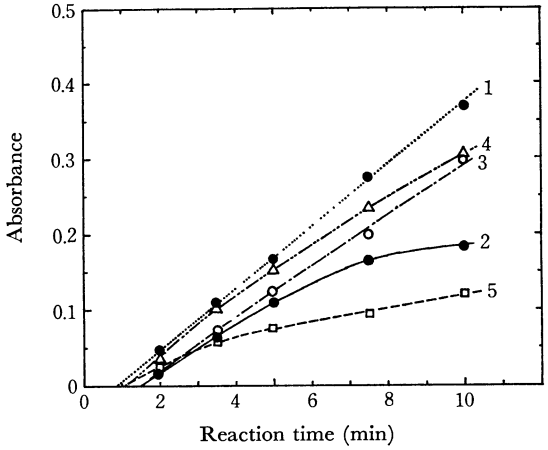

Fig. 4. Effect of substrate analogs on the propanediol dehydratase reaction in the presence of low concentration of the substrate.

The reaction mixture contained S 10 μmol (1; ···●···), S 0.15 μmol (2; —●—), S 0.15 μmol + I 10 μmol (3; -·-○-·-), S 0.15 μmol + II 10 μmol (4; -···△-···-), or S 0.15 μmol+III 10 μmol (5;--□--), potassium chloride 50 μmol, DBCC 15.0 mμmol, apoenzyme 0.080 unit (0.70 unit/mg-protein), potassium phosphate buffer (pH 8.0) 25 μmol; total volume 1.0 ml. The reaction was carried out for 2, 3.5, 5, 7.5, or 10 min 25°C in the dark. Unity of absorbance corresponds to 1.09 μmol of propionaldehyde.

When 10 μmol of S was added during the course of the reaction (5 min after the initiation of the reaction) to the reaction mixture with I, II, or III in the absence of S, the expected inactivation was not observed, but the reaction proceeded with almost the same velocity as that with S which gives V_{max} (Fig. 3). This clearly indicates that the active site is temporarily blocked by some materials, and not completely inactivated in the absence of the substrate. Taking into consideration the fact that the holoenzyme initially gave the product(s) from I, II, or III but was gradually inactivated during the course of the reaction, we could consider that the temporary inactivation was caused by the formation of complex(es) between holoenzyme and the product(s) from I, II, or III. This is plausible, if we assume that the affinity to the holoenzyme decreases in the order S>product(s) from I, II, or III>I, II, or III. In the case of I holoenzyme-I complex is formed in the absence of S, which forms the product. As the amount of the product increases, holoenzyme-product complex is gradually formed, which does not interact with I and gives no more product. When S is added, the holoenzyme-product complex turns into holoenzyme-S complex, again producing the normal product. Since the affinity of S to the holoenzyme is assumed to be greater than that of analogs or product(s), the enzymatic reaction both with S and with I, II, or III proceeds the same as that in the absence of I, II, or III.

The enzymatic reaction is expected to be inhibited by the initial addition of some products estimated to be formed from I, II, or III. When 0.5 μmol of propionaldehyde and/or acetone, which appeared to be enough to block the active site as judged by the amount of the product formed from I, II, or III, were added to the system with I, II, or III, the reaction patterns did not differ from those without the addition of aldehyde and/or acetone. Although the reactions were not inhibited by propionaldehyde or acetone, the hypothesis on blocking the active site by some materials described above might still be reasonable, since other products such as aldols are expected to be produced from I, II, or III. It was recently reported that substrate analogs such as styrene glycol or 1,2-butanediol, protected the holoenzyme against inactivation by oxygen, and by displacement of the analog with the substrate the normal reaction was initiated.[19] Detailed features of the effect of the substrate analogs are not known at present.

We wish to thank Prof. S. Fukui and his coworkers of Kyoto University for kindly supplying the enzyme and giving suggestions.

19) T. Toraya and S. Fukui, *Biochem. Biophys. Res. Commun.*, **36**, 469 (1969).