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STUDY ON THE MECHANISM OF ACTION OF ADENOSYLCOBALAMIN-DEPENDENT GLYCEROL DEHYDRATASE FROM *AEROBACTER AEROGENES*

I. ROLE OF STRUCTURAL COMPONENTS OF ADENOSYLCOBALAMIN IN THE FORMATION OF THE ACTIVE SITE OF GLYCEROL DEHYDRATASE

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Summary

A new method of partial chemical synthesis of adenosylcobalamin (Coα-[α-5,6-dimethylbenzimidazolyl)]-Coβ-adenosylcobamide, AdoCbl) analogs has been developed. A series of derivatives of AdoCbl modified in the nucleoside and nucleotide ligands and corrin macrocycle have been obtained.

The interaction of AdoCl analogs with glycerol dehydratase (EC 4.2.1.30) from *Aerobacter aerogenes* has been investigated. It has been shown that the nucleoside ligand of AdoCbl provides no essential contribution to the binding of apoenzyme but the preservation of the exact structure of the 1-N and 2-C positions of adenine appears essential for the catalysis.

The coordination bond between the Co and nucleotide ligand of AdoCl does not play a decisive role in glycerol dehydratase activity.

To form the active site of the glycerol dehydratase, the nucleotide in the

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Abbreviations: AdoCbl or I, Coα-[α-5,6-dimethylbenzimidazolyl)]-Coβ-adenosylcobamide; II Coα-[α-5,6-dimethylbenzimidazolyl)]-Coβ-(6-exo-*N*-methyl)adenosylcobamide; III, Coα-[α-5,6-dimethylbenzimidazolyl)]-Coβ-(6-exo-*N*-butyl)adenosylcobamide; IV, Coα-[α-5,6-dimethylbenzimidazolyl]-Coβ-(6-exo-*N*-benzoyl)-adenosylcobamide; V, Coα-[α-5,6-dimethylbenzimidazolyl]-Coβ-inosylcobamide; VI, Coα-[α-5,6-dimethylbenzimidazolyl]-Coβ-guanosylcobamide; VII, Coα-[α-5,6-dimethylbenzimidazolyl]-Coβ-(1-*N*-oxide) adenosylcobamide; VIII, Coα-[α-3,5,6-trimethylbenzimidazolyl]-Coβ-adenosylcobamide; IX, Coα[α-3-benzyl-5,6-dimethylbenzimidazolyl]-Coβ-adenosylcobamide; X, Coβ-adenosylcobamide; XI, Coβ-adenosylcobinamide; XII, Coα-(α-5,6-dimethylbenzimidazolyl)-Coβ-adenosyl-a,b,c,d,g-pentaamide cobamic acid; XIII, Coα-(α-5,6-dimethylbenzimidazolyl)-Coβ-adenosyl e-ethoxy-a,b,c,d,g-pentaamide cobamic acid; XIV, Coα-(α-5,6-dimethylbenzimidazolyl)-Coβ-cyanocobamide; XV, Coα-(α-dimethylbenzimidazolyl)-Coβ-cyano ethoxy-a,b,c,d,g-pentaamide cobamic acid; [ ], the corrin macrocycle; Bza, α-5,6-dimethylbenzimidazolylribotide; Pur, purine; Tos, *p*-toluenesulphonyl; DMB, dimethylbenzimidazole.

AdoCbl structure is essential since nucleotide elimination results in a 100-fold increase of K_i for the corresponding analog.

In the binding of AdoCbl with apoenzyme, the main role belongs to the corrin macrocycle, in which the e-propionamide group is significant for binding with apoenzyme, but presumably not essential for catalysis.

Introduction

AdoCbl-dependent enzymes catalyse various conversions of the substrates and play a significant role in the metabolism of microorganisms and animals [1,2]. The chemical mechanism of AdoCbl-dependent reactions is similar, in that they involve transfer of hydrogen between two adjacent carbon atoms of the substrate with simultaneous cleavage of C-O, C-N or C-C bonds. In these reactions, AdoCbl is a direct carrier of hydrogen but acquires this property only as a result of the interaction with the apoenzyme. The hydrogen attachment site is the 5′-CH_2 group of AdoCbl nucleoside ligand [3,4].

A number of fundamental aspects in the mechanism of AdoCbl-dependent enzyme action have not yet been adequately investigated. Thus, practically no attention has been given to the problem of AdoCbl functional groups that are directly involved in the formation of active sites of cobamide enzymes, namely, in the processes of binding with protein, substrates and the final stages of the enzymatic reaction. Studies of the interaction between specifically modified analogs of AdoCbl and apoenzymes might yield relevant information on the subject, but the currently available data are scarce [4—9].

The present study is concerned with AdoCbl-dependent glycerol dehydratase (EC 4.2.1.30) from *Aerobacter aerogenes.* Glycerol dehydratase catalyses the conversion of glycerol, 1,2-propanediol and ethyleneglycol into β-hydroxypropionic, propionic and acetic aldehyde, respectively.

To elucidate the role of the functional groups of nucleoside and nucleotide ligands and the corrin macrocycle of AdoCbl in the formation of the glycerol dehydratase active site, a large number of coenzyme analogs have been synthesized so as to modify the main structural moieties.

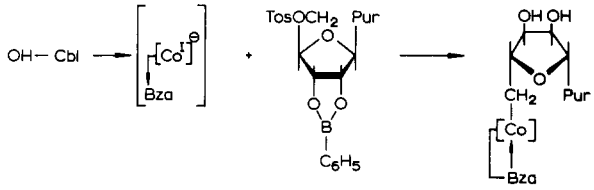
Materials and Methods

Materials. A yeast extract (Merck Darmstadt, G.F.R.), 1,2-propanediol (Gee Lowson Chemical Ltd. U.K.), $NADH_2$ (Boehringer, G.F.R.), crystalline AdoCbl (Reanal, Hungary) and synthesized freeze-dried AdoCbl analogs were employed in these experiments.

Synthesis of AdoCbl analogs. AdoCbl and its analogs (modified on the nucleoside and nucleotide ligands and corrin macrocycle) have been studied. These analogs, except for the coenzyme form of cobinamide analog XI, were synthesized according to a published method [10]. The synthesis comprises several stages which are shown in Scheme I. Reduction with $NaBH_4$ was carried out under strictly anaerobic conditions.

The analogs (II—VII) were obtained by alkylating a nucleophylic Co(I)-cobalamin with corresponding 2′3′-phenylboronic esters of 5′-*O*-tosylnu-

Scheme I



cleoside [11]. The Isobutyl ester of diphenylboronic acid was used instead of phenylboronic acid in the synthesis of 2′,3′-phenylboronic ester of 5′-*O*-tosylinosine and 5′-*O*-tosylguanosine, thereby avoiding the hydrolysis of labile phenylboronates of inosine and guanosine by the water formed during the reaction. The esters, without being isolated from pyridine solutions, were treated with *p*-toluene sulphonylchloride and converted into corresponding tosylates with a good yield [11].

The other tosylates were obtained from 2′,3′-phenylboronic esters of nucleosides by *p*-toluene sulphonylchloride treatment in pyridine [11,12]. Co(I)-cobalamin alkylation with 2′,3′-phenylboronic esters of 5′-*O*-tosylnucleosides was carried out in a hydrogen or argon atmosphere, while the isolation of purine 5′-deoxynucleoside analogs of AdoCbl (II—VII) was effected by phenolic extraction and column chromatography with CM-cellulose in the H^+-form [11].

The absorption spectra of the synthesized purine AdoCbl analogs in the range of 300—600 nm at pH 7.0 resemble the AdoCbl spectrum. With a decrease in pH to 2, the spectra of the analogs, like the AdoCbl spectrum, shifts the long wave maxima from 520—530 nm to 456 nm (Table I). The AdoCbl purine analogs are photosensitive when they are irradiated in solution and undergo conversion into hydroxycobalamin. In the presence of cyanide ions all the 5′-deoxynucleosylcobalamins, as well as AdoCbl, are converted at different rates into dicyanocobalamin [33]. These properties made it possible to confirm the presence the Co-C-bond in the structure of synthesized analogs. In the ultraviolet region the position and configuration of the absorption maxima depend on the structure of the purine nucleus of the nucleoside ligand of the AdoCbl analogs (Table I).

The CD-spectra of the AdoCbl purine analogs were found to be more sensitive to modifications in the AdoCbl molecule than the absorption spectra. Table II shows the main maxima and the intensities of the CD-spectra of the purine 5′-deoxynucleoside analogs of AdoCbl.

Analogs modified on the AdoCbl nucleotide ligand

Analogs VIII, IX and X were synthesized by alkylation of the reduced form of the corresponding factors with 2′,3′-phenylboronic esters of 5′-*O*-tosyladenosine [14]. In turn, factors $B_{12}N_m$ and $B_{12}N_b$ were obtained by methylating dicyanocobalamin with dimethylsulphate and benzylchloride [13]. Factor 1B was synthesized using a new technique, viz., by hydrolysing factor $B_{12}N_m$ in a weak alkaline medium approx. (pH 8.0) [14]. The electrophoretic mobility of this cobamide in different solvent systems is in agreement with its structure and with the data reported for factor Ib previously isolated from

TABLE I
THE PROPERTIES OF AdoCbl AND ITS ANALOGS

Cobalamin	R_F (system B)		Absorption spectra				
			In water		In 0.1 M sodium bicarbonate buffer (pH 10.5)		In 0.1 M HCl
		E	λ_{max},nm	$\epsilon \cdot 10^{-4}$			
1	2	3	4	5	6	7	8
I	1.00	1.00	262	3.58	—	—	264
			289	1.84	—	—	286
			345	1.33	—	—	305
			495	0.67	—	—	383
			525	0.78	—	—	463
II	1.22	1.04	265	3.80	265	3.93	263
			315	1.40	315	1.44	284
			340	1.36	340	1.38	303
			375	1.18	375	1.16	375
			520	0.86	520	0.84	456
III	1.60	0.98	265	4.00	266	3.96	264
			315	1.42	315	1.43	285
			340	1.32	340	1.34	303
			375	1.08	376	1.16	375
			520	0.75	520	0.83	456
IV	0.91	0.66	265	2.32	265	2.36	265
			282	1.95	282	1.99	286
			340	1.35	340	1.37	304
			375	1.05	375	1.05	380
			520	0.85	520	0.87	456
V	0.92	0.58	250	3.10	255	3.20	263
			265	2.80	342	1.32	285
			340	1.28	375	1.16	303
			375	1.08	515	0.82	375
			520	0.84			456
VI	0.91	0.61	253	3.34	265	3.28	263
			278	2.66	315	1.39	285
			315	1.34	340	1.31	303
			340	1.29	375	1.15	380
			375	1.11	520	0.79	456
			524	0.79			
VII	0.98	0.64	266	2.55	263	2.68	264
			290	2.14	280	2.22	286
			288	1.89	288	2.02	303
			345	1.34	340	1.45	375
			378	1.02	375	1.08	456
			520	0.82	520	0.89	
VIII	1.0	1.17	262	4.25	263	—	263
			287	2.40	287	—	287
			303	2.21	303	—	303
			375	0.85	375	—	375
			455	0.92	455	—	455
IX	—	1.0	263	4.04	—	—	—
			288	2.28	—	—	—
			303	0.75	—	—	—
			375	0.82	—	—	—
			455	0.91	—	—	—
X	0.57	0.57	264	7.30	263	—	—
			375	0.83	303	—	—
			460	0.91	460	—	—

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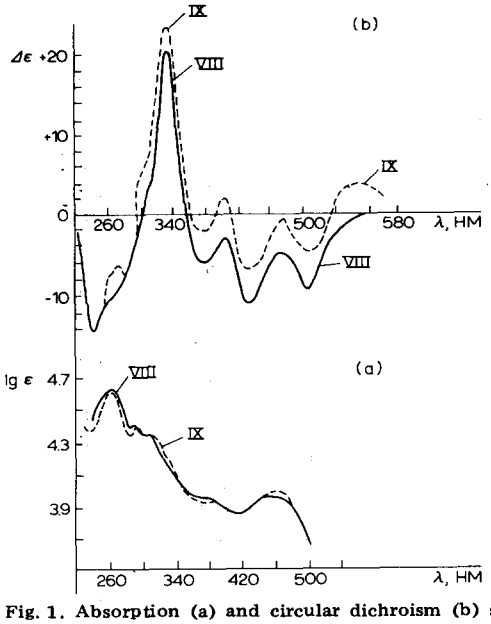
TABLE I (continued)

Cobalamin	R_F (system B)		Absorption spectra				
		E	In water		In 0.1 M sodium bicarbonate buffer (pH 10.5)		In 0.1 M HCl
			$\lambda_{max,nm}$	$\epsilon \cdot 10^{-4}$			
1	2	3	4	5	6	7	8
XI		1.36	261	4.84	259	—	—
			304	2.28	—	—	—
			455	0.93	458	—	—
XII	1.80	1.00	260	3.64	—	—	—
			288	1.89	—	—	—
			372	1.04	—	—	—
			485	0.72	—	—	—
			520	0.80	—	—	—

active sludge [15]. Analog XI was isolated from *Propionibacterium shermanii* cell population grown under anaerobic conditions without addition of 5,6-dimethylbenzymidazole into the fermentation medium, by using Barker's technique [16] modified by Pchelkina et al. [17]. The absence of the coordination bond of the atom Co with 5,6-dimethylbenzimidazole ligand in analogs VIII and IX has been confirmed by the studies of the absorption spectra in the ultraviolet and visible regions neutral pH values (Fig. 1a). Under these condi-



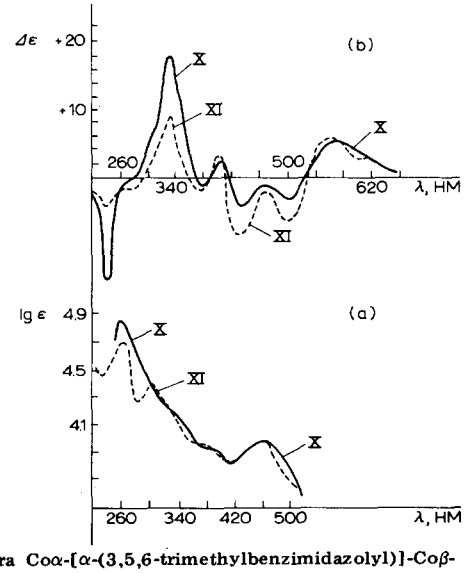


Fig. 1. Absorption (a) and circular dichroism (b) spectra Coα-[α-(3,5,6-trimethylbenzimidazolyl)]-Coβ-adenosylcobamide (VIII), Coα-[α-(3-benzyl-5,6-dimethylbenzimidazolyl)]-Coβ-adenosylcobamide (IX); a, in water; b, in 0.2 M potassium phosphate buffer, pH 8.

Fig. 2. Absorption (a) and circular dichroism (b) spectra. Coβ-adenosylcobamide (X) and adenosylcobinamide (XI); a, in water; b, in 0.2 M potassium phosphate buffer, pH 8.0.

TABLE II

THE CD SPECTRA OF AdoCbl AND ITS PURINE ANALOGS $\lambda_{max,nm}$ ($\Delta\epsilon$ $mol^{-1} \cdot cm^{-1}$)

Cobalamin	λ_1	λ_2	λ_3	λ_4	λ_5	λ_6	λ_7	λ_8	λ_9	λ_{10}
I	550 (—8.3)	482 (+9.9)	426 (—3.7)	385 (+4.6)	360 (—10.0)	332 (+0.8)	320 **	298 (+4.8)	268 (+9.7)	—
II	556 (—8.2)	484 (+10.7)	430 (—2.9)	386 (+6.7)	360 (—9.6)	334 (+2) *	—	300 (+2.9)	273 (6.4)	250 (—2) *
IV	545 (—6.9)	484 (+11.4)	416 (—5.5)	382 (+5.5)	362 (—8.4)	335 (+2.8)	320 (—2) *	397 (+6.1)	276 (+5.5)	254 (—7) *
V	550 (—8.6)	484 (+9.5)	430 (—3.4)	386 (+4.8)	360 (—9.6)	334 (+2) *	—	298 (+7.7)	274 (+6)	258 (—2) *
VI	556 (—7.2)	486 (+9.4)	428 (—2.8)	386 (+3.8)	360 (—9.8)	334 (+1) *	—	298 (+6.6)	275 (+7) *	255 (—7) *
VII	550 (—4.2)	492 (+5.3)	422 (—7.4)	385 (+1.5)	366 ((—3.8)	340 (+2.5)	—	300 (+2.1)	275 (+6) *	250 (—6) *

* $\Delta\epsilon$ was determined with less than 10% relative error.

** The value of dichroic density is comparable with noise level.

tions the spectra of both analogs are identical (λ_{max} = 456 nm) and are similar to the AdoCbl spectrum at pH 2.0, when decoordination of the Co-N_{Bza} bond occurs. The presence of the Co-C-bond in these analogs has been confirmed by studies of their sensitivity to light as well as to CN ions [14].

The absence of the Co-N_{Bza} bond in these analogs can also be confirmed by their CD spectra. The CD curves of analogs VIII and IX (Fig. 1b) show a broad positive maximum at 332 nm with $\Delta\epsilon$ = + 21.4 $M^{-1} \cdot cm^{-1}$ (analog VIII) and +22.7 $M^{-1} \cdot cm^{-1}$ (analog IX) instead of two small positive maxima at 298 and 332 nm with $\Delta\epsilon$ = +4.8 and +0.9 respectively, which are typical of the AdoCbl spectrum.

Similar charges in the positive maximum at 332 nm were observed in the AdoCbl spectra which recorded at pH below 3 [14], as well as at temperatures above 75°C, and appear to be due to the decoordination of the nucleotide ligand. Decoordination of the Co-N_{Bza} bond at elevated temperatures was ascertained earlier, in studies of the AdoCbl absorption spectra from the hypochromic shift of absorption maximum in the visible region [19]. Hogenkamp et al. [20] showed by NMR method that protonation of the 5,6-DMB moiety and its displacement at Co by water at low pH in alkylcobalamins causes a downfield shift of the methine resonances.

The structure of analogs X and XI was shown on the basis of their spectral characteristics in the ultraviolet and visible regions, CD spectra in the range of 250—600 nm, their sensitivity to light, and the CN ion effect, as well as from data their electrophoretic mobility at neutral and acid pH values [14].

In the absorption spectra of both analogs recorded at pH 7.0, the maximum of absorption is observed in the 456 nm range, while there is no absorption maximum at 288 nm associated with the coordinated 5,6-dimethylbenzimidazole in the AdoCbl structure (Fig. 2a).

In the presence of CN ions (pH 10.5), both analogs, like AdoCbl, are converted to their corresponding dicyano derivatives. The comparison of the absorption spectra of dicyano compounds showed that there occurred no significant changes in the structure of the corrin ring of the analogs, as evidenced by the fact that their spectra in the 300—600 nm range were identical to the dicyano-cobalamin spectrum. Likewise, no absorption maximum at 288 nm has been detected in the spectrum of dicyanoderivatives of analogs, this being a further indication of the absence of 5,6-dimethylbenzimidazole in the structure of these derivatives.

The analogs obtained are photolabile and on being irradiated in solution, are converted to their hydroxy derivatives.

There is a broad positive maximum in the range of 332 nm with $\Delta\epsilon$ = +18.0 and $\Delta\epsilon$ = +17.4 $M^{-1} \cdot cm^{-1}$ for analogs X and XI, respectively, in the CD spectra of both analogs (Fig. 2b), as well as in the spectra of analogs VIII and IX. This is further confirmation of the lack of Co bond with the nucleotide ligand.

AdoCbl analogs modified on the e-propionamide group of the corrin ring

Analogs XII and XIII were obtained by alkylating the reduced form of e-monocarboxylic acid of vitamin B_{12} (e-carboxycyanocobalamin, XV) and its ethyl ester, respectively, with the 2′,3′-phenylboronic ester of 5′-*O*-tosyl-

adenosine [21,22]. The e-monocarboxylic acid of vitamin B_{12} was isolated from a mixture of factors accompanying the microbiological synthesis of AdoCbl by *Propionibacterium shermanii* [21], while the ethyl ester of monocarboxylic acid of vitamin B_{12} was obtained by using the mixed anhydrides method, i.e. by treatment of e-carboxycyanocobalamin with the ethyl ester of chloroformic acid in the dimethylformamide and then with ethanol [22]. Diverse physico-chemical properties, viz., electrophoretic mobility at acid pH values, absorption spectra in the ultraviolet and visible regions in neutral and acidic solution CD spectra in the 230—600 nm range (Fig. 3), as well as the constants of the cyanation reaction rates, were found to be practically identical for AdoCbl and its analogs XII and XIII [21,22]. Thus, both analogs subjected to electrophoresis in 1 N CH_3COOH (pH 2.4) acquired a positive charge through adenine and 5,6-dimethylbenzimidazole protonation and moved towards the cathode at the same rate as did AdoCbl. In weak alkaline and neutral buffered systems, analog XII moved towards the anode (ionization of the carboxyl group), while AdoCbl and its analog XIII appeared electroneutral under these conditions. Both analogs are photosensitive and, on being irradiated in solutions, are converted into respective hydroxy derivatives. Like AdoCbl, under the effect of cyanide ions (pH 10.5), analogs XII and XIII formed corresponding dicyano derivatives.

Absorption spectra and circular dichroism of AdoCbl and its analogs

The absorption spectra in the ultraviolet and the visible region were recorded in 1.0-cm quartz cuvettes with a Pye Unicam spectrophotometer SP-800, at room temperature in two solvents, distilled water and 0.1 M HCl.

The CD spectra in 0.2 M potassium-phosphate buffer, pH 8.0, were recorded with the Japanese Jasco ORD/UV/CD-5 spectropolarometer. A 1.0—2 cm light path was employed with analog concentrations of $1 \cdot 10^{-4}$ M in the visible region and $3.3 \cdot 10^{-5}$ M in the ultraviolet range.

Determination of the concentration of AdoCbl and its analogs

The concentrations of AdoCbl and its analogs were determined spectrophotometrically by using the molar extinction coefficient at 522 nm for AdoCbl and its analogs modified on the nucleoside ligand and the corrin macrocycle; in the case of the analogs modified on the nucleotide ligand, a 458 nm wavelength was used.

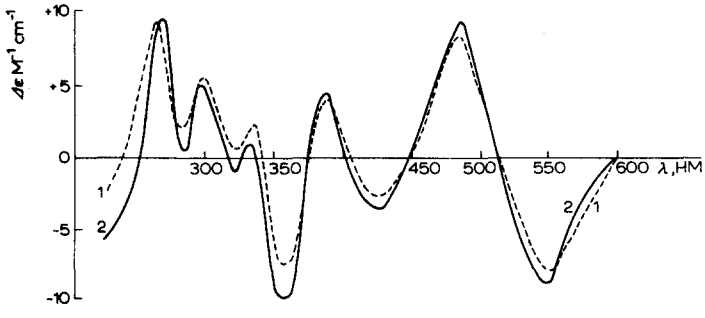


Fig. 3. Circular dichroism spectra of aqueous solutions of analog XII (1) and AdoCbl (2).

To determine the molar extinction coefficient of aqueous solutions of the analogs, they were subjected to photolysis in the presence of 0.1 M KCN, followed by measuring the absorbance of the solutions of the dicyano derivatives at 368 nm. Using a molar extinction coefficient equal to $30.8 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ [24], the molar extinction coefficients at 522 or 458 nm were calculated.

The rate constant of cyanation was determined spectrophotometrically [25].

Preparations of apo-glycerol dehydratase. Partially purified preparations of apo-glycerol dehydratase were isolated from cell extracts of *Aerobacter aerogenes* strain 572 PZH according to the method of Zagalak et al. [26] modified by Prosvetova [27].

Endegenous AdoCbl was removed by adsorption on activated carbon, while the nucleic acids were precipitated by the addition of manganese chloride to the cell-free extract. The main stages of the apoenzyme purification were fractionation by $(NH_4)_2SO_4$, where enzyme activity was concentrated in the fraction sedimenting at 30—50% saturation, and gel filtration on Sephadex G-200.

The obtained preparations of apoenzyme displayed a 25—30 fold degree of purification as compared to the crude cell-free extracts. By special experiments, it was checked that the glycerol dehydratase preparations did not contain enzymes as impurities metabolizing the substrate, product, AdoCbl and NADH [28].

Determination of glycerol dehydratase activity

Glycerol dehydratase activity was determined from the intial rate of glycerol and 1,2-propanediol dehydration into β-hydroxypropionaldehyde and propionaldehyde by using a highly sensitive recording spectrophotometric method [28]. The method is based on the ability of alcohol dehydrogenase to reduce aldehydes formed from glycols by glycerol dehydratase. The rate of the coupled reaction is measured by the decrease in absorbance at 340 nm. The incubation was carried out at 30° C. The reaction mixture contained 120 μmol of potassium phosphate buffer (pH 8.0), 300 μmol of the substrate (glycerol or 1,2-propanediol), 0.6 μmol of $NADH_2$, 80—150 μg of ADG, 0.03—1.0 units of enzyme, 60 nmol of AdoCbl or its analog, in a volume of 3.0 ml. The reaction was initiated by adding 0.1 ml of the coenzyme solution. The reaction mixture absorbance measurements were carried out in a 1.0 cm quartz cuvette on the Pye Unicam SP-800 spectrophotometer with an external recorder SP-22, using 0.1 unit $A/20$ cm scale. The amount of glycerol dehydratase that catalyzes the formation of 1 μmol of propionaldehyde is defined as one unit of glycerol dehydratase activity.

Protein concentration was determined according to Lowry [29].

Results

Role of adenine component of AdoCbl 5'-deoxyadenosyl ligand in the formation of the glycerol dehydratase active site

Since the 5'-deoxyadenosyl ligand of AdoCbl is directly involved in the catalytic reaction, there is particular interest in elucidating the role of specific

atoms and groups of the adenine component at the glycerol dehydratase active site. To do so, the synthesized analogs of AdoCbl were modified in the 1- and 6-positions of adenine, and the analogs that contained inosine or guanosine instead of adenine were synthesized. The influence of each modification on the kinetic parameters of the dehydration reaction of 1,2-propanediol was studied, such as the maximum reaction rate (V) and Michaelis constant (K_m) for the coenzymes and substrates; the nature of inhibition was established for the analogs/inhibitors and the inhibition constant (K_i) was determined.

Examination of the AdoCbl analogs modified in the adenine amino groups demonstrated that the introduction of the electron-donating substitutens, i.e. metnyl or butyl group into the 6-exoposition (Table III, analogs II and III, respectively) does not cause disappearance of the coenzyme properties of these derivatives. The dependence of the initial reaction rates on the concentrations of AdoCbl and analogs II and III obey simple Michaelis kinetics and are linear in reciprocal Lineweaver-Burk and Wolf coordinates [30]. The K_m for AdoCbl is $2.0 \cdot 10^{-8}$ M, while the values are $1.8 \cdot 10^{-7}$—$2.9 \cdot 10^{-7}$ M, for analogs II and III respectively. It was previously shown that the K_m for AdoCbl in the dehydration reaction of 1,2-propanediol is not dependent on pH and temperature within certain limits, while V varies greatly under these conditions. The K_m for AdoCbl in the glycerol dehydratase system is close to the holoenzyme dissociation constant and is characteristic of AdoCbl affinity to the apoenzyme [31].

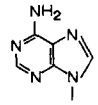
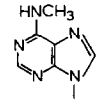
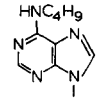
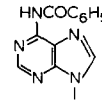
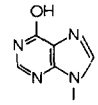
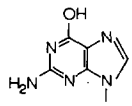
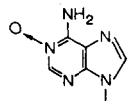
Thus, AdoCbl has an extremely high affinity for the apoenzyme, and the affinity of analogs II and III, though lower by an order of magnitude, nevertheless remains quite high. Both modifications of AdoCbl, however, result in a sharp drop of V, the values for analogs II and III being 12 and 1.5%, respectively, of that for AdoCbl as cofactor (Table III). Coenzyme properties disappear completely upon introduction of a strong electron-accepting benzoyl group in the 6-exo position of adenine, analog IV acquiring in this case the properties of an inhibitor. Fig. 4 shows the reciprocal Lineweaver-Burk coordinates: a plot of initial reaction rates vs. the concentration of AdoCbl in the absence of analog (line 1) and in the presence of three concentrations of analog IV (0.03, 0.01 and 0.3 μM, lines 2, 3 and 4, respectively). In these experiments, AdoCbl and the analog were simultaneously introduced in the reaction mixture. As can be seen from the plot, this analog displays the competitive type of inhibition; K_i was found to be $4.8 \cdot 10^{-8}$ M (Fig. 5). The K_i values were also determined according to Dixon's method.

One of the reasons for the decrease of the V of the enzyme reaction with analogs II and III and for the loss of coenzyme properties of analog IV may be a disturbance in the electron conformation between the structure of the cofactor molecule and that of the corresponding site on the protein molecule. Thus, the methyl and butyl groups, owing to their electron-donating properties, increase the basicity of the adenine-amino group, while the introduction of a strong electron-attracting benzoyl group alters the electron density distribution in the adenine structure.

The results obtained with analog VII, which contains oxygen as a strong electron acceptor in the 1-N position of adenine, showed that such a modification converted AdoCbl into an effective competitive inhibitor with a K_i value

TABLE III

THE EFFECT OF THE PURINE NUCLEUS MODIFICATION IN THE NUCLEOSIDE LIGAND OF AdoCbl ON KINETIC CONSTANTS OF GLYCEROL DEHYDRATASE REACTION AND THE CYANATION RATE CONSTANTS OF FREE COBALAMINES

Structure of the purine nucleus of AdoCbl nucleoside ligand		K_m (M)	$V_{relative}$ (%)	K_i (M)	Cyanation rate constant ($\cdot 10^4 \cdot s^{-1}$)
		2	3	4	5
I		$2.0 \cdot 10^{-8}$	100	—	8.4
II		$1.8 \cdot 10^{-7}$	12.0	—	7.5
III		$2.9 \cdot 10^{-7}$	1.5	—	6.7
IV		—	—	$4.5 \cdot 10^{-8}$	0.44
V		$1.5 \cdot 10^{-7}$	18.0	—	3.9
VI		—	—	$3.3 \cdot 10^{-7}$	2.0
VII		—	—	$3.6 \cdot 10^{-8}$	0.12

very close to the K_m for AdoCbl equal to $3.6 \cdot 10^{-8}$ M. Earlier, Pawelkiewicz et al. showed that the 1-methyladenosine analogue of AdoCbl was inactive in the transformation of glycerol, ethylene glycol and 1,2-propandiol in the corresponding aldehydes [32]. In this case apparently, the effect of substitutions on the adenine ring of either an N-oxide or an N-methyl group at the 1-N-position causes loss of activity through steric hindrance of binding of this portion of the molecule to the enzyme.

To evaluate the role of the AdoCbl adenine aminogroup in the catalytic reaction, the properties of analog V were studied, in which a hydroxyl group is present in place of the 6-NH_2-group. Analog V was found to retain its coenzyme activity. Such a modification of AdoCbl brings about changes in the major kinetic parameters of the reaction, i.e. V diminishes to 18%, while K_m increases by an order of magnitude (Table III). These changes produced an

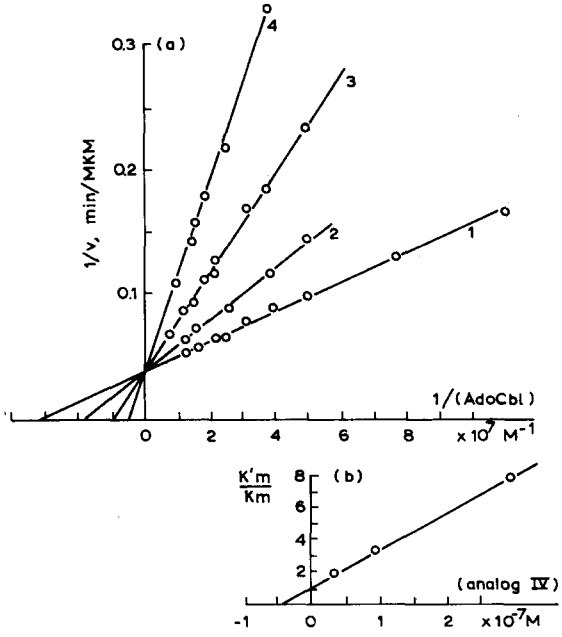


Fig. 4. Initial dehydration rates of 1,2-propanediol vs. AdoCbl concentration without inhibitor (1) and in the presence of 0.03, 0.1 and 0.3 μM of analog IV (2,3 and 4, respectively) in reciprocal coordinates (a). Determination of K_i for analog IV (b). Reaction mixture: apo-glycerol dehydratase, 0.022 units; 1,2-propanediol, $1 \cdot 10^{-2}$ M; ADG, 50 μg, $NADH_2$, $2 \cdot 10^{-4}$ M; other conditions are described in the methods.

effect equivalent to that which results from the introduction of a methyl group instead of a hydrogen in the amino-group.

The introduction of an HN_2-group in the C-2 position of the purine nucleus of analog V (analog VI) results in the elimination of coenzyme properties. Analog VI is a competitive inhibitor with a K_i of $3.3 \cdot 10^{-7}$ M (Table III). Hence, the C-2 position of the AdoCbl purine nucleus is of fundamental importance for the catalysis and appears non-essential for binding with the enzyme.

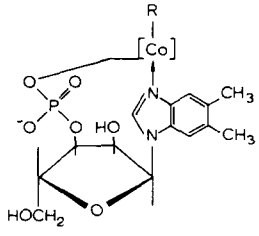
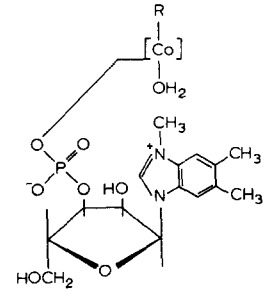
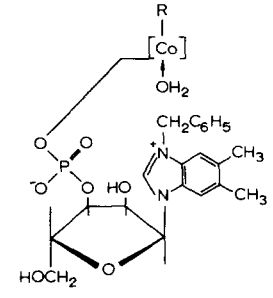
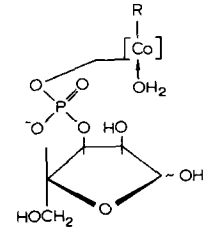
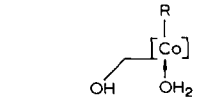
Role of nucleotide AdoCbl ligand in the formation of the glycerol dehydratase active site

To elucidate the role of coordination bonds between the Co atom and the nucleotide ligand of AdoCbl in the enzyme catalytic activity, the coenzyme properties of the two AdoCbl analogs VIII and IX were investigated. The coordination of these analogs with cobalt was due to the introduction of bulky substitutents (methyl or benzoyl groups) into the 3-N position of 5,6-dimethylbenzimidazole.

Kinetic studies devoted to the dehydration reaction of 1,2-propanediol and glycerol with analogs VIII and IX as glycerol dehydratase cofactors showed that both analogs retain coenzyme properties. The V values for analogs VIII and IX were 52 and 48%, respectively, of V found for AdoCbl as cofactor (Table IV). The results obtained suggest that the coordination $Co\text{-}N_{Bza}$ is likely to be of little significance in the binding of these compounds to glycerol

TABLE IV

THE EFFECT OF THE AdoCbl NUCLEOTIDE LIGAND MODIFICATIONS ON THE KINETIC CONSTANTS OF GLYCEROL DEHYDRATASE (R-5′-DEOXYADENOSYL)

Analogs	K_m (M)	$V_{relative}$ (%)	K_i (M)
 I	$2.0 \cdot 10^{-8}$	100	—
 VIII	$4.4 \cdot 10^{-7}$	52	—
 IX	$4.0 \cdot 10^{-7}$	48	—
 X	—	—	$8.0 \cdot 10^{-7}$
 XI	—	—	$1.8 \cdot 10^{-6}$

dehydratase, inasmuch as the absence of this coordination in the AdoCbl analogs studied caused only a two-fold reduction in catalytic activity. A feasible cause for the decreased V value may be an alteration in the geometry of the Co atom of these analogs in relation to the corrin macrocycle, this assumption being confirmed by studies of the physico-chemical properties of the analogs in question [33]. In fact, the K_m values for each of the AdoCbl modifications under saturation conditions are practically identical, viz., $4.4 \cdot 10^{-7}$ M for analog VIII and $4.0 \cdot 10^{-7}$ M for analog IX, these values being 20 times as high as K_m for AdoCbl.

It is assumed that in the process of the interaction of AdoCbl with apoenzyme there occurs a Co-N_{Bza} bond decoordination and 5,6-dimethylbenzimidazole is replaced by a histidine residue or by an other amino acid (probably cystein) of the protein that can coordinate with the Co atom [34, 35]. It may be therefore inferred that the introduction of bulky substitutents into the 3-N position of 5,6-dimethylbenzimidazole in conjuction with the simultaneous positive charge induction, are responsible for the steric and electronic hindraces that prevent these groups from approaching to and thereafter coordinating with the Co atom of AdoCbl. To study the role of 5,6-dimethylbenzimidazole and AdoCbl nucleotide ligands in the process of binding with apoenzyme and in the catalytic activity of holoenzyme, the properties of the coenzyme forms of factor 1b (analog X) and cobinamide (analog XI) were investigated. Analog X does not contain 5,6-dimethylbenzimidazole in its molecule, whereas the entire nucleotide ligand is absent in the structure of analog XI. Both analogs were found to be devoid of any coenzyme activity and to be competitive inhibitors with respect to AdoCbl in the 1,2-propanediol dehydration reaction. Thus, the elimination of 5,6-dimethylbenzimidazole or nucleotide ligand inactivates the coenzyme properties of these analogs. Glycerol dehydratase belongs therefore to the group of cobamide enzymes that require nucleotide ligands in the structure of AdoCbl as a prerequisite for catalytic activity.

Table IV shows the K_i values for analogs X and XI (equal to $8 \cdot 10^{-7}$ M and $1.8 \cdot 10^{-6}$ M, respectively).

As can be seen from the comparison of these constants, the lower ligand plays an essential role in the processes of AdoCbl binding with apoenzyme since its elimination results in a 100-fold increase in the K_i as compared to the K_m for AdoCbl. The K_i value, however, remains $1.8 \cdot 10^{-6}$ M and the affinity of such an analog is accordingly high. These results suggest that the corrin macrocycle is a major factor in AdoCbl binding with glycerol dehydratase. In this connection, it was of interest to assess the role of certain corrin functional groups in the formation of the glycerol dehydratase active site.

Role of e-propionamide group of the AdoCbl corrin ring in the formation of the glycerol dehydratase active site

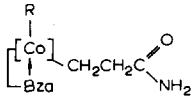
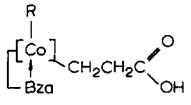
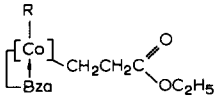
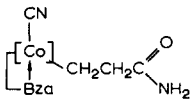
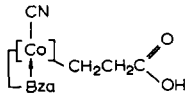
To evaluate the role of the propionamide residue at the e-position of the macrocycle in the processes of binding with apo-glycerol dehydratase and in holoenzyme catalytisis, two AdoCbl analogs were investigated, in which the propionic acid residue (analog XII) or ethyl ether of the propionic acid residue (analog XIII) were substituted for the propionamide group.

In studies of the kinetics of glycerol and 1,2-propanediol dehydration with analogs XII and XIII as glycerol dehydratase cofactors it was found that in both cases, the enzyme retained its catalytic activity; however, each modification brought about a decrease in the *V*.

Thus, the *V* of 1,2-propanediol dehydration was 65% in the case of analog XIII and 12% in the case of analog XII of the *V* value of the same reaction with AdoCbl as cofactor (Table V). It follows from the results obtained that the e-propionamide group of the corrin macrocycle is not essential for the catalytic properties of glycerol dehydratase, since its substitution by the ethyl propionate results in only a 1.5-fold decrease of *V*. However, propionic acid residue substitution for this group decreases the *V* value by almost 90%. Inasmuch as analog XII acquires a negative charge at pH 8.0, due to the dissociation of the carboxyl group, it is reasonable to assume that the latter situation is responsible for the sharp drop in the catalytic activity of the modified holoenzyme in question.

TABLE V

THE EFFECT OF THE E-PROPIONAMIDE GROUP MODIFICATIONS IN AdoCbl CORRIN RING ON THE KINETIC CONSTANTS OF GLYCEROL DEHYDRATASE (R-5'-DEOXYADENOSYL)

AdoCbl analogs	K_m (M)	$V_{relative}$ (%)	K_i (M)
 I	$2.0 \cdot 10^{-8}$	100	—
 XII	$5.0 \cdot 10^{-7}$	12	—
 XIII	$1.8 \cdot 10^{-7}$	65	—
 XIV	—	—	$1.0 \cdot 10^{-8}$
 XV	—	—	$3.5 \cdot 10^{-7}$

In the dehydration reactions of glycerol and 1,2-propanediol, the K_m values were $1.8 \cdot 10^{-7}$ M for analog XIII and $5.0 \cdot 10^{-7}$ M for analog XII (Table V). A modification of the e-propionamide group of the corrin ring diminished the affinity by a factor of 10 and 25 as compared to the affinity of AdoCbl, suggesting that this group plays a significant role in the processes of coenzyme binding with apo-glycerol dehydratase.

This conclusion is substantiated by the correlation of the K_i values of two corrinoids : vitamin B_{12} (analog XIV) and its e-carboxy derivative (analog XV); in the structure of the latter, like in analog XII, the e-propionamide group was substituted by the propionic acid residue. Both corrinoids were found to be competitive inhibitors with respect to AdoCbl. The calculated values of K_i are $1.0 \cdot 10^{-8}$ M for analog XIV and $3.5 \cdot 10^{-7}$ M for analog XV (Table V). In this instance, modifying the e-propionamide group in the structure of the corrin ring of vitamin B_{12} results in a significant decrease (30-fold) of its affinity to the apoenzyme as well.

Role of main structural components of AdoCbl in the formation of substrate binding sites of glycerol dehydratase

To determine the role of certain AdoCbl functional groups in the formation of substrate binding sites of glycerol dehydratase, the influence of a number of coenzyme modifications on the K_m values for 1,2-propanediol and glycerol was investigated.

It could be assumed that the substrate binding site was located close to the binding site of 5′-deoxyadenosyl ligand of AdoCbl since the latter is a direct carrier of substrate hydrogen. It was of interest, therefore, to investigate the influence of adenine modifications on glycerol dehydratase ability to bind the substrate. For that purpose, the K_m values for 1,2-propanediol with AdoCbl and anlogs II and V (modified on the adenine amino group) as glycerol dehydratase cofactors were determined.

The dependence of the initial reaction rate on the substrate concentration determined under the saturation conditions for AdoCbl or one of its analogs is described by the Michaelis-Menten equation. The K_m for 1,2-propanediol in the reaction with AdoCbl as cofactor was equal to $0.8 \cdot 10^{-4}$ M, whereas the K_m in the case of analog II was $1 \cdot 10^{-4}$ M, and in the case of analog V, $1.4 \cdot 10^{-4}$ M. The values obtained differ slightly from one another. Therefore it may be inferred that the AdoCbl adenine amino group is not directly involved in the binding of the substrate and the conformational changes that take place in the holoenzyme formation do not materially affect the substrate binding sites.

In the next series of experiments, determinations of the K_m for the substrate were carried out using analog VIII without the coordination bond Co_{Bza} and analogs XII and XIII modified in the e-propionamide group of AdoCbl as cofactors of glycerol dehydratase. The K_m for glycerol and 1,2-propanediol in the case of analog VIII were $2.2 \cdot 10^{-3}$ and $0.9 \cdot 10^{-4}$ M, respectively, and did not differ from the K_m values for these substrates in the experiments with AdoCbl as cofactor, viz., $1.5 \cdot 10^{-3}$ M and $0.8 \cdot 10^{-4}$ M, respectively. In reactions with analogs XII and XIII, the K_m for 1.2-propanediol was $1.7 \cdot 10^{-4}$ and $0.9 \cdot 10^{-4}$ M, respectively, only slightly different from the K_m value for that

substrate in the case of AdoCbl as cofactor. The K_m for glycerol was the same in reactions with AdoCbl and analog XII ($1.5 \cdot 10^{-3}$ M). Hence, the true and modified holoenzymes have identical affinities for the substrates under examination. The results provide grounds for assuming that conformational changes taking place in the formation of glycerol dehydratase complexes with analogs VIII, XII and XIII do not significantly affect the substrate binding sites of holoenzyme.

Discussion

A method of partial chemical synthesis of AdoCbl analogs using 2′,3′-phenylboronic esters of 5′-*O*-tosylnucleosides as alkylating agents was developed in order to carry out systematic investigations of the influence of AdoCbl modifications on the kinetics of the glycerol dehydratase reaction. It was hoped that the role of the main structural components of AdoCbl in the formation of the holoenzyme active site would be elucidated.

Kinetic studies on the enzymatic reaction with AdoCbl purine analogs showed that the introduction of various substitutes into the 1,2- and 6-positions of the adenine nucleoside ligand slightly alters the affinity of these analogs to the apoenzyme as compared to that of AdoCbl. Thus, the K_m and K_i values remain low enough ($0.4 \cdot 10^{-7}$—$3 \cdot 10^{-7}$ M); the K_m for AdoCbl is $2.0 \cdot 10^{-8}$ M. The substitutents in these positions of the adenine nucleoside ligand do not seem to contribute significantly to the processes of AdoCbl binding with apo-glycerol dehydratase. This conclusion is valid with respect to the nucleoside ligand, since in the case of its replacement by the CN group, the affinity of such corrinoids (analogs XIV and XV) differs little from the affinity of organocobalamins containing nucleoside ligands (AdoCbl and analog XII, respectively).

On the other hand, any modification of AdoCbl adenine brings about either a sharp decrease or a complete loss of the catalytic properties of glycerol dehydratase. Substituting a hydroxyl group for the adenine amino group in analog V does not affect the the coenzyme properties, though the catalytic activity of the enzyme decreases markedly. The adenine amino group appears not to be essential for the catalysis, but its presence in the structure of adenine provides optimal conditions for the formation and decomposition of the holoenzyme-substrate complex. The results obtained with analog V are of interest also because such an analog does not possess coenzyme properties in a number of AdoCbl-dependent reactions [36,37], including glycerol dehydratase (Pawelkiewicz' communication, ref. 32). The discrepancy between our results and those of Pawelkiewicz et al. who failed to detect coenzyme properties of analog V in the glycerol dehydratase system is likely to stem from the fact that they rely on the use of a colorimetric method noted for its low sensitivity for the determination of glycerol dehydratase activity [39]. The results obtained with analogs II and III, which contained the electron-donating methyl or butyl groups in the 6-position of adenine as well as with analog IV containing an electron-attracting benzoyl group, make it possible to assume that a cause for the sharp reduction or complete loss of the glycerol dehydratase catalytic activity could be the difference of electron conformity between the structure

of the modified adenine and the respective site of the protein.

The results warrant the conclusion that the unaltered structure of the adenine nucleoside is a prerequisite for maximal catalytic activity of the enzyme. It is reasonable to assume, on the grounds of the experimentally, demonstrated significance of Co-C bond cleavage in the course of the enzymatic substrate transformation, that the changes of the *V* values occurring when different groups are introduced into the adenine structure are due to their influence on the ability of the Co-C bond to dissociate reversibly. The results of kinetic studies concerned with the cyanide cleavage of AdoCbl and its purine analogs suggest that such an influence is basically feasible [33].

The cyanation reaction provides a convenient model for studies concerning the effect of modifications of trans-axial ligands on the stability of the Co-C bond in free cobalamin [25].

The heterolytic mechanism of Co-C bond cleavage in this reaction appears to be fundamentally distinct from the mechanism of bond cleavage in the course of enzymatic catalysis (homolysis, refs. 40 and 41). However, a comparison of the rate constants for Co-C bond cyanide cleavage in AdoCbl and in its analogs allows the assessment of the relative strength of the Co-C bond in a number of the investigated analogs in the free state. Table III shows the rate constants of Co-C bond cyanide cleavage for different purine analogs of AdoCbl.

Substitution of hypoxanthine (analog V) or guanine (analog VI) for adenine as the deoxynucleoside ligand for AdoCbl was found to bring about a slight increase in the stability of the Co-C bond, as in the case of introducing electron-donating substituents into the adenine amino group (analogs II and III). In contrast, the introduction of strong electron-attracting substituents, in both the 6-exo-N and the 1-N position of adenine, increases the stability of the Co-C bond significantly (analogs IV and VII). A question arises as to how these electron changes inside the adenine nucleus can be transferred to Co-C bonds spatially remote from and unconjugated with the heterocyclic nucleus of the adenine, but no answer has yet been obtained. It may be assumed that mutual electronic interactions of planar corrin and purine rings exist or that the transfer of this effect to the Co-C bond occurs via the oxygen of the ribose ring. That this assumption might be valid is corroborated by some data obtained by the present authors in studying the two AdoCbl analogs containing the alkyl or ether group in place of the ribose in the 5′-deoxyadenosyl ligand, namely [5-(aden-9-yl)pentyl]-cobalamin and β-[(aden-9-yl)methoxyethyl] cobalamin [18]. It appeared that the stability of the Co-C bond of these analogs was different. Thus, the alkyladenyl analog behaves like simple alkyl cobalamins, in which the Co-C bond resists the attack of cyanide in the dark. On the other hand, with the ether analog, the Co-C bond is ruptured by the cyanide ions, though at a lower rate than in the case of AdoCbl [18]. The available data point to an important role played by the oxygen atom in the transfer of substitution effects in the AdoCbl adenine nucleus in the free state. However, in the enzymatic system, Abeles et al. [42] have shown that the oxygen of the ribose ring is not essential in the catalysis, so that the participation of apoenzyme amino acid residues in the transfer of the effects to the Co-C bond cannot be ruled out. The effects of adenine modifications may be

important not only for the facility of homolytic fission but also for the stabilization of the formed fragments (radicals) on the glycerol dehydratase surface, as was shown for ribonucleotide reductase by Hogenkamp et al. [38].

In a series of papers devoted to the physicochemical properties of free cobinamids and the cobalamins, it was suggested that the Co-N_{Bza} bond, in an adequately labile state, might be essential for the orientation of the nucleoside ligand with respect to the corrin ring plane [44].

The nucleotide ligand was shown to play a role which differs for various cobamide enzymes [45—47].

Studies on the kinetics of the glycerol dehydratase reaction with the AdoCbl analogs modified on the nucleotide ligand indicate that the absence of the coordination bond between the Co atom and benzimidazol is not essential for the catalytic activity of glycerol dehydratase. On the other hand, the nucleotide ligand is essential for the formation of the active site of glycerol dehydratase. Moreover, nucleotide elimination results in the complete loss of the coenzyme properties and in a 100-fold increase in the K_i for this analog. Hence, the nucleotide ligand may be regarded one as of the point of AdoCbl binding with apoenzyme. Individual components of the ligand play dissimilar roles in these processes, the main contributor being 5,6-dimethylbenzimidazole, whereas the role of ribose and phosphoric acid residue is less essential.

Analysing the inhibitory properties of cobinamide provided the possibility of establishing that the corrin macrocycle greatly contributes of AdoCbl binding with apo-glycerol dehydratase.

Only a few papers are available concerning the role of functional groups of the corrin macrocyle in the formation of active sites of the cobamide enzyme [7,8,48—51], while for the glycerol dehydratase system there are no published data whatsever.

We have established that the e-propionamide group of the AdoCbl corrin ring is essential for AdoCbl binding with apo-glycerol dehydratase, probably forming the hydrogen bonds with the amino acid residues of protein.

However, this group to hardly contributed to the catalytic activity of the enzyme because its replacement by ethylpropionate caused only a 1.5-fold reduction in the maximum reaction rate.

The findings reported in the present paper indicate that all the groups of AdoCbl studied exert little effect on the formation of substrate-binding sites of the holoenzyme, and provide grounds for assuming that the reduction of V in the case of AdoCbl analogs that retain their coenzyme properties is due to the effect of the modifications on the holoenzyme structure and on the rate of holoenzyme-substrate complex decomposition.

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