Analogs of Deoxyadenosylcobalamin with Alterations in a Side Chain of the Corrin Ring*

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ABSTRACT: An analog of 5′-deoxyadenosylcobalamin having a free carboxyl group on the *e*-propionic acid side chain of the corrin ring has been prepared by mild acid hydrolysis of 5′-deoxyadenosylcobalamin. The analog has been identified with the by-product produced when 5′-deoxy-2′,3′-isopropylideneadenosylcobalamin is cleaved by acid hydrolysis to 5′-deoxyadenosylcobalamin. The structure of the analog was established by degradation studies, by comparison with known cobamides by means of thin-layer chromatography, paper chromatography and electrophoresis, and by amidation of the analog to 5′-deoxyadenosylcobalamin. The following substituted amides of 5′-deoxyadenosylcobalamin were prepared *via* the carboxylic acid analog: the methylamide, ethylamide, anilide, and 2,4-dinitroanilide. These analogs had similar spectra to 5′-deoxyadenosylcobalamin, but differing pK_a values, and whereas I was neither active nor inhibitory in the ribonucleotide reductase system from *Lactobacillus leichmannii*, the other analogs were active to varying degrees.

Hogenkamp and Pailes (1968) showed that their preparations of 5′-deoxyadenosylcobalamin contained another cobamide (I), which was formed during the acid hydrolysis of 5′-deoxy-2′,3′-isopropylideneadenosylcobalamin. This cobamide is inactive in the enzyme systems tested, has a spectrum almost identical with that of 5′-deoxyadenosylcobalamin, but is readily separated from the latter on ion-exchange resins (Hogenkamp and Pailes, 1968). The ease with which certain of the propionamide side chains on the corrin nucleus of cyanocobalamin are hydrolyzed to corresponding propionic acid side chains (Smith, 1965) led to the suggestion (Morley and Blakley, 1967) that compound I may be a derivative of 5′-deoxyadenosylcobalamin containing a propionic acid side chain. It has now been established by various means that I is such a derivative, that is, α-(5,6-dimethylbenzimidazole)-5′-deoxyadenosyl-Co-cobamic acid pentaamide (Figure 1). The best conditions for the preparation of I by acid hydrolysis of 5′-deoxyadenosylcobalamin have been investigated, and the methylamide, ethylamide, anilide, and 2,4-dinitroanilide derivatives of I (Figure 1) have also been prepared and some of their properties investigated.

Experimental Procedure

Materials

Crystalline cyanocobalamin was obtained from Glaxo and 2′,3′-isopropylideneadenosine from Cyclo Chemical Corp., Los Angeles. All reagents to be used under anhydrous conditions were dried over a molecular sieve (type 16A, Union Carbide) for at least 3 days before use. The Dische diphenylamine reagent was prepared and used according to Blakley (1966). Reduced lipoic acid was prepared from lipoic acid (Sigma, St. Louis) according to the method of Gunsalas and Razzell (1957). 5′-Deoxyadenosylcobalamin was prepared by the method of Morley and Blakley (1967) and purified by gel filtration on G-15 Sephadex. Thioredoxin and thioredoxin reductase were prepared from *Escherichia coli* by the method of Laurent *et al.* (1964) and Moore *et al.* (1964). Ribonucleoside triphosphate reductase was prepared from *Lactobacillus leichmannii* as previously described (Vitols *et al.*, 1967). The enzyme had a specific activity of 125 μmoles of ATP[1] reduced/mg of protein per hr at 37° under the standard conditions previously used (Ghambeer and Blakley, 1966).

Methods

Chromatography of Cobamides. Thin-layer chromatography was carried out on Eastman Kodak precoated silica gel sheets K301R using solvent system 1, *sec*-butyl alcohol–water–ammonia solution (25%) (100:36:14, v/v), or solvent system 2, water-saturated *sec*-butyl alcohol. Paper chromatography was performed by the ascending technique on Whatman No. 1 paper with solvent system 3, *n*-butyl alcohol–acetic acid–water (5:2:3, v/v), or solvent system 4, 1 M sodium acetate–0.2 M boric acid–95% ethanol (1:1:2). The separation of adenosine-5′-aldehyde from other nucleosides and adenine was best achieved after con-

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[1] Abbreviations used: ATP, adenosine triphosphate; GTP, guanosine triphosphate; NADPH, reduced nicotinamide–adenine dinucleotide phosphate.

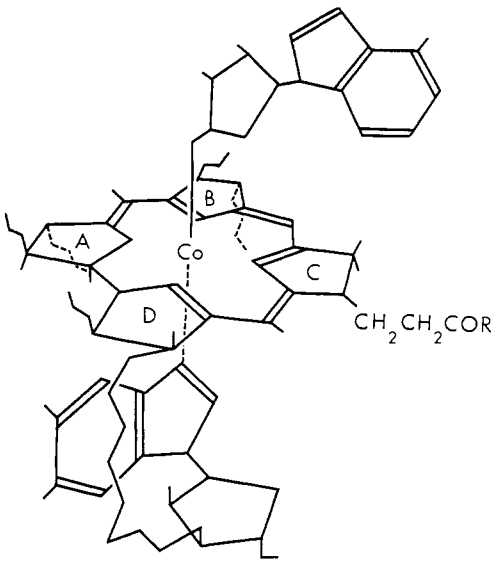

FIGURE 1: Cobamide structures. 5′-Deoxyadenosylcobalamin, R = NH_2; analog I, R = OH; methylamide, R = NHMe; ethylamide, R = NHEt; anilide, R = NHC_6H_5; and 2,4-dinitroanilide, R = $NHC_6H_3(NO_2)_2$.

version of the aldehyde to bisulfite addition compound using the following method. The paper was sprayed with 0.05 M sodium bisulfite and dried at 60° for 1 hr before sample application. The chromatogram was then developed with solvent system 3.

Spectra of Cobamides. Visible and ultraviolet absorption were determined on a Cary 15 spectrophotometer. The extinction coefficients of the cobamide analogs were calculated from absorbance with the aid of concentration determinations performed by the method of Barker *et al.* (1960). In the latter method the cobamide is converted to dicyanocobalamin by treatment with 0.1 M KCN at room temperature for 1 hr. It is assumed that the molar extinction coefficients at 367 mμ for the analogs of dicyanocobalamin are identical with that of dicyanocobalamin itself, that is 30.4×10^3.

Purification of I. Compound I was isolated from acid hydrolysates of 5′-deoxyadenosylcobalamin by the method of Hogenkamp and Pailes (1968) on Dowex 50W-X2 (200–400 mesh) (30 × 2.5 cm column for 50 μmoles of material). The solution of I from the Dowex 50W column was extracted with phenol, adjusted to pH 10 with 2 N NaOH, and adsorbed on Dowex 1-X2 (Cl^- form, 200–400 mesh, 30 × 2.5 cm column). The column was washed thoroughly with water to remove traces of 5′-deoxyadenosylcobalamin and then with 0.03 M ammonium acetate (pH 6) to remove traces of the monocarboxylic acid of hydroxocobalamin, which is also formed during the hydrolysis. I was then eluted with 0.1 M ammonium acetate (pH 6), desalted by extraction into phenol, and crystallized from aqueous acetone.

Synthesis of Cobamides from I. The analog I (1.6 μmoles) was treated with anhydrous dimethylformamide, triethylamine, and ethyl chloroformate as described by Armitage *et al.* (1953), excess of the appropriate dry amidation reagent was then added slowly with agitation at −5°, and the solution was left at −5° for 10 min and then for a further specified period of time at room temperature before proceeding with the appropriate extraction procedure.

Purification of Cobamides Synthesized from I. The solution of the extracted products from the amidation reactions (see sections on individual analogs) was adjusted to pH 10 with 2 N NaOH and applied to a Dowex 1-X2 column (Cl^- form, 200–400 mesh, 11 × 1 cm for 3 μmoles of material). The required product was eluted from the column with water whereas unchanged I and other anionic impurities were retained. The aqueous solution of the cobamide was acidified to pH 3 with 2 N HCl and adsorbed on a Dowex 50W-X2 column (200–400 mesh, 8 × 0.5 cm for 3 μmoles of material, at pH 3). The column was washed thoroughly with water and the analog was eluted from the column with 0.1 N sodium acetate (pH 6.5), hydroxocobalamin being retained on the resin (Hogenkamp and Pailes, 1968). The cobamide solution was desalted by extraction with phenol and crystallized by addition of acetone to the concentrated aqueous solution (Barker *et al.*, 1960). Unchanged I could be recovered from the Dowex column by elution with 0.1 M ammonium acetate (pH 6.0).

Determination of pK_a Values for the Benzimidazole Base of Cobamides. The pK_a values determined are a measure of the dissociation constant of the chromophoric corrin ring system and represent the dissociation of the bond between cobalt and a nitrogen of the dimethylbenzimidazole base. The method employed is that of Ladd *et al.* (1961), in which the pK_a is determined from a plot of absorbance at 520 or 460 mμ against pH.

Determination of the Effect of Cobamides on Enzymic Ribonucleotide Reduction. Initial tests of the effect of the analogs on the activity of the enzyme from *L. leichmannii* were performed by use of the colorimetric determination of ribonucleotide reductase activity (Blakley, 1966). The reaction mixture was as described elsewhere (Blakley, 1966), except that it contained, instead of 5′-deoxyadenosylcobalamin, the cobamide to be tested at a final concentration of 8 μM. The ability of the cobamide to replace deoxyadenosylcobalamin as cofactor in the enzymic reduction was determined by comparing the initial velocity of ATP reduction in the presence of the cobamide with the velocity in the presence of 5′-deoxyadenosylcobalamin. A more detailed analysis was carried out by using the procedure of Vitols and Blakley (1965) with slight modification. The complete reaction system contained, in a total volume of 0.5 ml, 0.1 M potassium phosphate buffer, pH 7.3, 4 mM EDTA, 0.2 mM NADPH, 7.2 μM thioredoxin, 10 μg of thioredoxin reductase, 3 mM GTP, 16 μg of ribonucleotide reductase, and the cobamide at concentrations from 0.11 to 2.3 μM. The reference cuvette contained the complete system except for the cobamide, and the enzymic reduction was followed spectrophotometrically by observing the oxidation of NADPH at 340 mμ with a Cary 14 spectrophotometer. K_m and V_{max} values were obtained from two initial

velocity determinations at each of five cobamide concentrations by use of a FORTRAN IV computer program to fit a standard hyperbola (Cleland, 1963).

Incorporation of Tritium from Water into Cobamides by the Reductase System. The procedure used followed that of Hogenkamp *et al.* (1968) in which the cobamide, after incubation with the appropriate mixture containing tritiated water, was isolated on a Dowex 50-X2 column (Hogenkamp and Pailes, 1967) and counted.

The incubation mixture contained 1 mg of enzyme, 2 mM dGTP, 30 mM reduced lipoic acid, 0.2 M sodium dimethylglutarate (pH 7.3), approximately 1 μCi of tritium oxide, and 0.05 mM cobamide in 0.5 ml. After incubation at 37° for 15 min, the mixture was cooled in ice and adjusted rapidly to pH 3 with 1 N HCl, and the precipitate was removed by centrifugation. The cobamide analog was adsorbed from the supernatant solution on a Dowex 50-X2 (pH 3) column (2 × 0.4 cm) and the column was washed with 10 ml of water. The cobamide was then eluted with 0.1 N NH_4OH, the eluate was evaporated to dryness, and the residue was taken up in 0.5 ml of water. The solution was allowed to stand for 2 hr to permit exchange of hydrogen in the cobamide with the solvent before reevaporation. This procedure was repeated four times. The cobamide was finally dissolved in a known volume of water for measurement of the radioactivity and the concentration estimated from the absorbance of the solution at 525 mμ. The purity of the isolated cobamide was verified by thin-layer chromatography (Table IV).

TABLE I: Electrophoretic Mobilities[a] of I, Degradation Products of I, and Related Compounds.

Compound	Migration Distance (cm) pH 3.2[b]	pH 6.5[b]	pH 10.3[b]
Cyanocobalamin	+1.10	+0.75	+1.25
Hydroxocobalamin	+3.75	+3.00	+1.10
5′-Deoxyadenosylcobalamin	+2.25	+0.25	+0.06
I	+2.10	−1.90	−0.60
Photolyzed 5′-deoxyadenosylcobalamin	+3.70	+2.90	+1.00
Photolyzed I	+2.25	−1.00	+0.15
Cyanide-treated 5′-deoxyadenosylcobalamin	+1.10	+0.75	+1.50
Cyanide-treated I	+0.20	−1.45	−0.80
II[c]	+0.15	−1.45	−0.90
III[c]	+0.10	−3.5	−2.25

[a] Whatman No. 3MM paper. Negative signs indicative movement to anode. [b] Conditions at pH 3.2: 0.1 M NaH_2PO_4 buffer, 4.4 V/cm for 7 hr. Conditions at pH 6.5: 0.1 M sodium acetate buffer, 3.4 V/cm for 7 hr. Conditions at pH 10.3: 0.1 M sodium pyrophosphate buffer, 2.4 V/cm for 9 hr. [c] Compounds II and III are the derivatives of cyanocobalamin in which one and two of the propionamide side chains have been hydrolyzed, respectively.

Results

Synthesis of I. The analog I was originally obtained as a by-product in the synthesis of 5′-deoxyadenosylcobalamin by acid hydrolysis of 5′-deoxy-2′,3′-isopropylideneadenosylcobalamin (Hogenkamp and Pailes, 1968; Morley and Blakley, 1967). To ascertain the optimum conditions for the formation of I by acid hydrolysis of 5′-deoxyadenosylcobalamin, hydrolytic studies were carried out. It was found that the best conditions for maximal formation of I were either 0.5 N HCl at 37° for 3 hr or 0.1 N HCl at room temperature for 2 days. Acids of normality greater than 1 N yielded chiefly hydroxocobalamin, even at room temperature, and glacial acetic acid had no effect on 5′-deoxyadenosylcobalamin, at room temperature, for periods up to 1 week. Dowex-50 (H^+ form) has been used by Bernhauer *et al.* (1966), with some success, in preparing the monocarboxylic acid derivative of cyanocobalamin in good yield, but when used with 5′-deoxyadenosylcobalamin at various temperatures for varying periods of time gave lower yields of I than with HCl under similar conditions. Experiments on the formation of I by acid hydrolysis of 5′-deoxy-2′,3′-isopropylideneadenosylcobalamin showed less formation of I than from 5′-deoxyadenosylcobalamin under identical conditions. In all experiments where significant quantities of I were formed it was observed that the yield of I increased to a maximum and thereafter decreased, whereas the formation of hydroxocobalamin (monocarboxylic acid) continued until all the deoxyadenosylcobalamin had disappeared (Figure 2).

Routine Synthesis of I. 5′-Deoxyadenosylcobalamin (32.80 μmoles) was treated with 0.5 N HCl (250 ml) and incubated at 37° for 3 hr. The solution was cooled in ice and adjusted to pH 3 with NaOH, and I was obtained from the hydrolysate as described in the Methods section. The average yield of crystalline I was 7.9 μmoles (24% theoretical). Unchanged 5′-deoxyadenosylcobalamin was also collected and could be used for further preparation of I, after desalting the solution by the phenol extraction procedure.

Proof of Structure. Electrophoresis of I, at three pH values, gave the results shown in Table I. At pH 6.5 and 10.3, I, in contrast to 5′-deoxyadenosylcobalamin, behaves as an anion and at these pH values shows behavior like the analog (II) of cyanocobalamin in which one propionamide residue has been hydrolyzed to a propionic acid side chain. At pH 3.2, I is more basic than II but slightly less basic than 5′-deoxyadenosylcobalamin. These data suggest that I is a product of 5′-deoxyadenosylcobalamin in which one of the propionamide side chains has been hydrolyzed to a propionic acid residue, and such an identification is supported by the similar behavior of I and II in thin-layer chromatography on precoated silica gel sheets (Table II).

Evidence that the structural difference between I

TABLE II: Comparison of Cobamides by Thin-Layer Chromatography.[a]

Compound	R_F Values			
	Solvent System 1[a]		Solvent System 2[a]	
	Red Spots	Ultraviolet-Absorbing Spots	Red Spots	Ultraviolet-Absorbing Spots
Cyanocobalamin	0.30		0.17	
Hydroxocobalamin	0.05		0.03	
5′-Deoxyadenosylcobalamin	0.22		0.13	
I	0.11		0.05	
Photolyzed 5′-deoxyadenosylcobalamin	0.05	0.58, 0.70	0.02	0.46, 0.66
Photolyzed I	0.04	0.57, 0.69	0.02	0.46, 0.64
Cyanide-treated 5′-deoxyadenosylcobalamin	0.28	0.70	0.15	0.68
Cyanide-treated I	0.15	0.70	0.09	0.65
II	0.12		0.07	
III	0.07		0.03	

[a] See Methods section.

and 5′-deoxyadenosylcobalamin is confined to the corrin nucleus was obtained by investigation of the products obtained by treatment of I with cyanide and by photolysis of I. In photolysis experiments, samples of I and 5′-deoxyadenosylcobalamin (3 μmoles) were irradiated in dilute solution with light from a 100-W lamp at a distance of 6 cm for 3 hr at 0°. The solutions were concentrated and examined by electrophoresis and chromatography. Cyanolysis was carried out on approximately 3-μmole samples of each compound in dilute solution, by addition of excess solid potassium cyanide. After 1 hr in the dark the purple solutions were adjusted to pH 5 with 0.1 N HCl and evaporated to dryness under reduced pressure at 25°. The solid residue was extracted with methanol and the compounds present, after concentration of the extracts under reduced pressure, were examined by electrophoresis and chromatography.

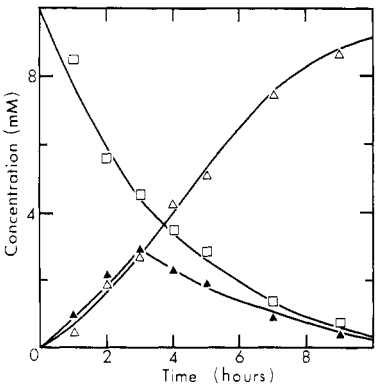

FIGURE 2: Hydrolysis of 5′-deoxyadenosylcobalamin with 0.5 N HCl at 38°. (△) Hydroxocobalamin, (▲) analog I, and (□) 5′-deoxyadenosylcobalamin. All results are means of duplicate experiments. Millimolar concentrations of compounds were obtained by separation of hydrolytic products on Dowex resins as described in Methods section, and measurement of absorption of solutions at 525 mμ.

Cyanolysis of I gave one colorless product which absorbed ultraviolet light. This compound was shown by thin-layer and paper chromatography (Tables II and III) to be identical with that formed from 5′-deoxyadenosylcobalamin under similar conditions and was identified as adenine, in agreement with the report of Johnson and Shaw (1962). Photolysis of I yielded two major colorless products which absorbed ultraviolet light. These compounds were shown by thin-

TABLE III: Comparison by Paper Chromatography of Purine Products Obtained by Cyanolysis and by Photolysis of I and of 5′-Deoxyadenosylcobalamin.

Compound	R_F Values	
	Solvent System 3[a,b]	Solvent System 4[b]
Adenine	0.60	
Adenosine-5′-aldehyde	0.16	
5′, 8-Cyclic adenosine	0.52	0.40
Photolyzed 5′-deoxyadenosylcobalamin	0.15, 0.52, trace 0.59	
Photolyzed I	0.16, 0.53, trace 0.58	
Adenosine	0.54	0.52
Photolyzed and reduced I	0.52–0.53, trace 0.59	0.41, 0.52, trace 0.60
Cyanide-treated 5′-deoxyadenosylcobalamin	0.60	
Cyanide-treated I	0.60	

[a] Paper pretreated with bisulfite as described in Methods section. [b] Details described in Methods section.

TABLE IV: Thin-Layer Chromatography of Cobamide Analogs.[a]

Compound	R_F Values (Solvent System I)[a]	
	Authentic	Synthetic
5′-Deoxyadenosylcobalamin	0.22	0.21[c]
Methylamide of 5′-deoxyadenosylcobalamin		0.23[c]
Ethylamide of 5′-deoxyadenosylcobalamin		0.28[c]
Anilide of 5′-deoxyadenosylcobalamin	0.31[d]	0.31[c]
2,4-Dinitroanilide of 5′-deoxyadenosylcobalamin		0.35[c]
Cyanocobalamin	0.30	0.30[b]
Methylamide of cyanocobalamin	0.35[d]	0.36[b]
Ethylamide of cyanocobalamin	0.42[d]	0.40[b]
Anilide of cyanocobalamin	0.58[d]	0.58[b]

[a] Details of the procedure are given in the Methods section. [b] Obtained by cyanolysis of synthetic analogs of 5′-deoxyadenosylcobalamin. [c] Synthesized from I. [d] Samples supplied by Dr. E. Lester Smith.

layer and paper chromatography (Tables II and III) to be identical with the products formed, under similar conditions, from 5′-deoxyadenosylcobalamin, and identified as adenosine-5′-aldehyde (Hogenkamp *et al.*, 1962) and 5′,8-cyclic adenosine (Hogenkamp, 1963). These facts support the contention that I and 5′-deoxyadenosylcobalamin do not differ in their nucleoside moieties. To confirm this view, the mixture of photolytic products from I was reduced with sodium borohydride (Hogenkamp *et al.*, 1962) and the resulting solution then desalted on Dowex 50 (H^+) and examined by paper chromatography in two solvent systems (Table III). As expected, the compound behaving like adenosine-5′-aldehyde on chromatography of the original mixture of photolytic products was converted to a substance that chromatographed with authentic adenosine after reduction.

Cyanolysis of I gave a cobamide which migrated with II during electrophoresis at each of three pH values (Table I) and on thin-layer chromatography (Table II). Photolysis of I yielded a red product, not identical with hydroxocobalamin (the corrinoid photolysis product of 5′-deoxyadenosylcobalamin), but migrating as an anion at pH 6.5 and as a cation, although slower than hydroxocobalamin, at pH 3.2 (Table I). This suggests the presence in I of a carboxylic acid moiety with a pK_a of about 3.2.

The above evidence suggests that analog I is α-(5,6-dimethylbenzimidazole)-5′-deoxyadenosyl-Co-cobamic acid *abcdg*-pentaamide (see Discussion). As additional proof of structure, 5′-deoxyadenosylcobalamin was synthesized from I as follows. Analog I

TABLE V: Molar Extinction Coefficients of Cobamides at the Absorbance Maxima.

Compound	Absorbance Maxima (mμ)												
	pH 6							pH 1					
	262	266	290	340	375	520	525	262	264	285	303	380	460
5′-Deoxyadenosylcobalamin[a]	34.6		18.4	11.2	9.0		7.6	43.5		25.5	21.4		8.64
5′-Deoxyadenosylcobalamin[b]	34.7		18.1	12.3	10.9		8.0						
Analogs of 5′-deoxyadenosylcobalamin													
I	35.0		18.4	11.1	9.8		7.6	40.6		24.0	22.8	7.8	9.0
Methylamide		46.4	23.0	14.1	9.0	7.3			54.0	25.8	22.5		8.5
Ethylamide	46.8		22.7	15.9	11.5		9.5		52.5	29.2	25.8		10.7
Anilide	43.6		24.0	14.0	10.8	8.4		48.1		29.2	25.2		9.2
Anilide[c]	46.0		23.0	13.6	10.6	7.8		49.5		24.7	21.4		9.1
2,4-Dinitroanilide		49.5	23.3	15.7	11.5		8.4	53.4		30.3	23.6		8.4

[a] Synthetic. [b] Values from Barker *et al.* (1960). [c] Values determined from a sample supplied by Dr. E. Lester Smith.

TABLE VI: pK_a Values for the Benzimidazole Base of Cobamides.[a]

Compound	pK_a Value	Ref Value
5′-Deoxyadenosylcobalamin (synthesized from I)	3.50	3.50[b]
I	3.31	
Methylamide of 5′-deoxyadenosylcobalamin	2.98	
Ethylamide of 5′-deoxyadenosylcobalamin	2.78	
Anilide of 5′-deoxyadenosylcobalamin	3.53	3.57[c]
2,4-Dinitroanilide of 5′-deoxyadenosylcobalamin	3.49	

[a] For procedure, see Methods section. [b] Ladd *et al.* (1961). [c] Determined from a sample supplied by Dr. E. Lester Smith.

TABLE VII: Values of Apparent K_m and Apparent V_{max} Obtained for Ribonucleoside Triphosphate Reductase with Various Cobamides.[a]

Compound	App K_m ($\times 10^7$ M)	App V_{max}
5′-Deoxyadenosylcobalamin	4.71 ± 0.51	0.073 ± 0.003
I	Inactive	
Methylamide of 5′-deoxyadenosylcobalamin	6.12 ± 0.92	0.060 ± 0.003
Ethylamide of 5′-deoxyadenosylcobalamin	22.80 ± 1.04	0.049 ± 0.001
Anilide of 5′-deoxyadenosylcobalamin	16.80 ± 3.70	0.032 ± 0.005
Anilide of 5′-deoxyadenosylcobalamin[b]	17.10 ± 2.78	0.028 ± 0.001
2,4-Dinitroanilide of 5′-deoxyadenosylcobalamin	12.48 ± 1.64	0.008 ± 0.001

[a] For procedure, see Methods section. [b] Values determined from a sample supplied by Dr. E. Lester Smith.

(1.6 μmoles) was treated with anhydrous dimethylformamide, triethylamine, and ethyl chloroformate as described in the Methods section. Following treatment with ethyl chloroformate at −5°, an excess of dry liquid ammonia was added slowly with shaking at −5° and the solution was left at this temperature for 10 min. The solution was then brought to room temperature and stood for 1 hr, followed by 10 min at 50°. The solution was treated with water (2 ml), the aqueous solution was washed with ether (2 × 5 ml), and a sample was electrophoresed. The major reaction product migrated as a cation at pH 6.5. The bulk of the solution was purified as described in the Methods section. The yield of crystalline 5′-deoxyadenosylcobalamin was 1.05 μmoles (66% theoretical). The chromatographic properties (Table IV), spectral properties (Table V), pK_a values (Table VI), and coenzymic properties of the product were shown to be the same as those of authentic 5′-deoxyadenosylcobalamin.

Formation of a Tetraamide Product. On electrophoresis of the hydrolytic products from 5′-deoxy-2′,3′-isopropylideneadenosylcobalamin at certain pH values, a small amount (approximately 10%) of a third cobamide was detectable as a contaminant of I. This cobamide was tentatively identified as the analog of 5′-deoxyadenosylcobalamin in which two propionamide groups have been hydrolyzed. The amount of this third hydrolysis product varies according to the conditions of hydrolysis.

Properties of I. The chromatographic properties of I are shown in Tables III and IV. Its spectrum is similar to that of 5′-deoxyadenosylcobalamin (Table V); its pK_a value is somewhat lower than that of 5′-deoxyadenosylcobalamin (Table VI). In the ribonucleotide reductase system, I was shown to be inactive and to cause no inhibition at concentrations up to 200 μM in the presence of 4 μM 5′-deoxyadenosylcobalamin.

Synthesis of the Methylamide of 5′-Deoxyadenosylcobalamin. A solution of methylamine in anhydrous dimethylformamide was prepared by gently heating methylamine hydrochloride (2 g) with solid KOH (0.5 g) and passing the methylamine gas evolved over CaO and into dimethylformamide (4 ml). The solution was allowed to stand over a molecular sieve for 12 hr at room temperature before use.

A solution of I (3 μmoles), in anhydrous dimethylformamide, and triethylamine was treated with ethyl chloroformate as described in the Methods section, and the dimethylformamide solution of methylamine (4 ml) was then added slowly, with shaking, and the mixture was left at −5° for 5 min, at room temperature for 1 hr, and at 50° for 30 min. The resulting solution was treated with 10 ml of water, extracted with water-saturated phenol, and purified as described in the Methods section. The average yield of crystalline methylamide was 1.60 μmoles (53.3% theoretical).

Synthesis of the Ethylamide of 5′-Deoxyadenosylcobalamin. A solution of I (3 μmoles), in anhydrous dimethyl formamide, and triethylamine was treated with ethyl chloroformate as described in the Methods section, followed by excess anhydrous ethylamine at −5°, and the resulting solution was allowed to stand at −5° for 10 min, followed by 12 hr at room temperature; the solution was then extracted as above and purified as described in the Methods section. A preparation was also carried out under similar conditions using a dimethylformamide solution of ethylamine (prepared as for the solution of methylamine above). The average

yields of crystalline ethylamide, by the two methods were 1.6 (55% theoretical) and 1.2 μmoles (40%), respectively.

Synthesis of the Anilide of 5′-Deoxyadenosylcobalamin. A solution of I (3 μmoles) in anhydrous dimethylformamide and triethylamine was treated with ethyl chloroformate as described in the Methods section, followed by anhydrous aniline (3 ml, freshly distilled and dried over a molecular sieve) at −5°, and the mixture was left at room temperature for at least 24 hr. The solution was then diluted with ether (5 ml) and shaken repeatedly with small samples of water in order to extract red compounds into the water layer. The aqueous layers were washed with ether and purified as before on Dowex resins. The average yield of crystalline anilide was 1.2 μmoles (40% theoretical).

Synthesis of the 2,4-Dinitroanilide of 5′-Deoxyadenosylcobalamin. A solution of I (3 μmoles), in anhydrous dimethylformamide, and triethylamine was treated with ethyl chloroformate as described in the Methods section, followed by an anhydrous solution of 2,4-dinitroaniline (2 g) in dimethylformamide (4 ml) at −5°, and the mixture was then left at room temperature for 3 days. The solution was diluted with water (50 ml) and the deposit of excess 2,4-dinitroaniline was removed by centrifugation. The remaining solution was extracted with phenol and purified on Dowex 50 resins as described above. The average yield of crystalline 2,4-dinitroanilide was 1.0 μmole (33% theoretical).

Properties of the Cobamide Analogs. The purity of the analogs was demonstrated by thin-layer chromatography (Table IV) and electrophoresis at three pH values, when only one spot was detectable for each analog. Cyanolysis of the 5′-deoxyadenosylcobalamin (synthesized from I) and the amide analogs gave the expected corrin products (Table IV). The amide analogs had spectra similar to that of 5′-deoxyadenosylcobalamin, the chief differences being in the extinction coefficients at various absorbance maxima which were somewhat greater than those of 5′-deoxyadenosylcobalamin at the same maxima, especially for the highest absorbance maximum at 258–265 mμ (Table V). The pK_a value for the dimethylbenzimidazole base of the cobamides (Table VI) is unexpectedly low in the case of the methylamide and ethylamide analogs. Experiments on the analogs showed the ease of hydrolysis to I by 2 N HCl was in the order 2,4-dinitroanilide = anilide > ethylamine > methylamide. Complete hydrolysis to I and hydroxocobalaminmonocarboxylic acid required about 12 hr at room temperature for the 2,4-dinitroanilide and anilide, approximately 24 hr for the ethylamide, and 72–80 hr for the methylamide.

Studies carried out to determine the coenzymic properties of the cobamide analogs in the ribonucleotide reductase system gave the results shown in terms of apparent K_m and apparent V_{max} values in Table VII. It is of interest to compare the values for the analogs with those for the probable physiological coenzyme for the reductase, 5′-deoxyadenosylcobalamin. Surprisingly, the anilide and 2,4-dinitroanilide are active despite the presence of the additional buky aromatic group.

It has been demonstrated (Hogenkamp *et al.*, 1967) with 5′-deoxyadenosylcobalamin that tritium from water can be incorporated in the 5′-methylene group of the cobamide by the reductase in the presence of dihydrolipoate and a nucleotide. In order to determine whether such incorporation into the anilide also occurs, experiments similar to those performed by Hogenkamp *et al.* (1967) were carried out. Previous results (Hogenkamp *et al.*, 1967) had indicated that the maximum incorporation of tritium from water into 5′-deoxyadenosylcobalamin is 1.4 atoms of tritium/molecule of cobamide. The results of tritium incorporation shown in Table VIII indicate that in this experiment incorporation reached 60% of the equilibrium value. Nevertheless these results give an approximate measure of the inital velocity of tritium incorporation into the cobamides. It may be seen that the rate of incorporation into the anilide analog is similar to that into 5′-deoxyadenosylcobalamin despite the fact that the apparent V_{max} for the reaction in the presence of the anilide is one-half that in the presence of 5′-deoxyadenosylcobalamin.

Discussion

The positive identification of I as a pentaamide derivative of 5′-deoxyadenosylcobalamin leaves unresolved the uncertainty as to which of the propionamide side chains on the corrin ring has been hydrolyzed. In the light of recent work on the monocarboxylic acid derivative of cyanocobalamin by Bernhauer *et al.* (1966), Nockholds *et al.* (1967), and Moore *et al.* (1967), it is very probable that, as in the case of cyanocobalamin, it is the propionamide group on C-13 of the corrin nucleus (*e* side chain on ring C) of 5′-deoxyadenosylcobalamin which is readily hydrolyzed. Some support for this hypothesis comes from the fact that the cobalamin obtained from the cyanolysis of I behaves in an identical fashion, on thin-layer chromatography, with the monocarboxylic acid derivative of cyanocobalamin (Table II). It is therefore very likely that I is α-(5,6-dimethylbenzimidazole)-5′-deoxyadenosyl-Co-cobamic acid *abcdg*-pentaamide.

Although a small quantity of α-(5,6-dimethylbenzimidazole)-5′-deoxyadenosyl-Co-cobamic acid tetraamide is formed under certain conditions, no other polybasic acids have been detected, and it seems unlikely that these could be formed from 5′-deoxyadenosylcobalamin since the more drastic conditions necessary for their formation would readily remove the deoxyadenosyl moiety of the parent compounds (5′-deoxyadenosylcobalamin).

The method for the synthesis of the amide analogs of 5′-deoxyadenosylcobalamin described in this paper is limited in its applications by the moderate yields obtainable. Although the amidation method employed gave almost 100% yields when tested with monocarboxylic acid of cyanocobalamin (Armitage *et al.*, 1953), it was impossible to obtain yields better than 70% from analog I, and they were often lower. The reasons for the low yields are uncertain since few side reactions occur and much unchanged I may be recovered. However, the direct amidation of I is a somewhat more convenient method of obtaining the amide analogs of 5′-deoxy-

TABLE VIII: Comparison of Tritium Exchange from Water into 5′-Deoxyadenosylcobalamin with Exchange into the Anilide Analog.[a]

Cobamide Added	Tritium Incorpd in Cobamide (cpm/ μmole)[b]	Atoms of Tritium/ Molecule of Cobamide[b]
Boiled enzyme with 5′-deoxyadenosylcobalamin	270	0.03
5′-Deoxyadenosylcobalamin	5300	0.81
Anilide analog	5050	0.78

[a] For procedure, see Methods section. [b] All results are means of four experiments.

adenosylcobalamin than the previous route to these analogs *via* the amidation of cyanocobalamin carboxylic acid derivatives and their subsequent conversion to the 5′-deoxyadenosylcobalamin derivative.

The lower pK_a values of the ethylamide and methylamide analogs (Table VI) are unexpected. They cannot be due to inductive effects attributable to the additional methyl or ethyl groups, since these effects would have to operate through too many carbon atoms to affect the cobalt–nitrogen bond. In any case, an additional methyl or ethyl group should increase the pK_a since the cobalt–nitrogen bond would be weakened by the additional electrons donated to the cobalt atom *via* the inductive effects.

Although the apparent K_m values in Table VII cannot be related directly to the affinity of the cobamides for the enzymes in this complex reaction for which the enzymic mechanism is not known, some conclusions about the binding of these analogs to the enzyme are possible. It appears that I does not bind to the enzyme since it is neither active nor inhibitory at concentrations up to 200 μM, and this failure to bind is presumably due to the negatively charged carboxylate ion on the side chain attached to C-13 of the corrin nucleus. It is possible to suggest an explanation of the fact that the anilide and 2,4-dinitroanilide are active, despite the additional relatively bulky aromatic group, by assuming that attachment to the enzyme is strengthened through formation of a charge-transfer complex between the anilide moiety and an aromatic amino acid residue on the enzyme. However, in the case of 2,4-dinitroanilide there was no observable change in spectrum of this cobamide in the presence of substrate quantities of enzyme, so that this hypothesis is improbable.

The lower apparent V_{max} for the reaction obtained in the presence of the anilide and 2,4-dinitroanilide analogs indicates a decreased rate for the rate-limiting step of the reductase reaction, or rather for the step that is rate limiting in the reaction involving these analogs. In order to test whether this step is closely related to the incorporation of tritium from tritiated water into the anilide analog, the rate of such incorporation was compared with incorporation into 5′-deoxyadenosylcobalamin (Table VIII). Since the results suggest that the rate of tritium incorporation into the anilide analog is similar to that of incorporation into 5′-deoxyadenosylcobalamin under identical conditions, it seems unlikely that the rate-limiting step of the reductase reaction is closely linked with the hydrogen-transfer step *via* the coenzyme. This is in agreement with previous suggestions regarding the rate-limiting step of the ribonucleotide reductase reaction in *L. leichmannii* (Hogenkamp *et al.*, 1967).

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References

Armitage, J. B., Cannon, J. R., Johnson, A. W., Parker, L. F. J., Smith, E. L., Stafford, W. H., and Todd, A. R. (1953), *J. Chem. Soc.*, 3849.

Barker, H. A., Smyth, R. D., Weissbach, H., Toohey, J. I., Ladd, J. N., and Volcani, B. E. (1960), *J. Biol. Chem. 235*, 480.

Bernhauer, K., Wagner, F., Beisbarth, H., Rietz, P., and Vogelmann, H. (1966), *Biochem. Z. 344*, 289.

Blakley, R. L. (1966), *J Biol. Chem. 241*, 176.

Cleland, W. W. (1963), *Nature 198*, 463.

Ghambeer, R. K., and Blakley, R. L. (1966), *J. Biol. Chem. 241*, 4710.

Gunsalas, I., and Razzell, W. (1957), *Methods Enzymol. 3*, 941.

Hogenkamp, H. P. C. (1963), *J. Biol. Chem. 238*, 477.

Hogenkamp, H. P. C., Ghambeer, R. K., Vitols, E., Brownson, C., and Blakley, R. L. (1967), *J. Biol. Chem.* in press.

Hogenkamp, H. P. C., Ladd, J. N., and Barker, H. A. (1962), *J. Biol. Chem. 237*, 1950.

Hogenkamp, H. P. C., and Pailes, W. H. (1968), *Biochem. Prepn.* (in press).

Johnson, A. W., and Shaw, N. (1962), *J. Chem. Soc.*, 4068.

Ladd, J. N., Hogenkamp, H. P. C., and Barker, H. A. (1961), *J. Biol. Chem. 236*, 2114.

Laurent, T. C., Moore, E. C., and Reichard, P. (1964), *J. Biol. Chem. 239*, 3436.

Moore, E. C., Reichard, P., and Thelander, L. (1964), *J. Biol. Chem. 239*, 3445.

Moore, F. M., Willis, B. T. M., and Hodgkin, D. C. (1967), *Nature 214*, 130.

Morley, C. G. D., and Blakley, R. L. (1967), *Biochemistry 6*, 88.

Nockholds, C. K., Waters, T. N. M., Ramaseshan, S., Waters, J. M., and Hodgkin, D. C. (1967), *Nature 214*, 129.

Smith, E. L. (1965), Vitamin B_{12}, London, Methuen, p 38.

Vitols, E., Brownson, C., Gardiner, W., and Blakley, R. L. (1967), *J. Biol. Chem. 242*, 3035.

The Reaction of Trypsin with Bromoacetone*

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ABSTRACT: The reaction of trypsin and certain trypsin derivatives with bromoacetone has been investigated. The resultant inactivation of the enzyme, measured by the rate of hydrolysis of ester substrates or by reaction with [^{32}P]diisopropylphosphofluoridate ([^{32}P]DFP), correlated with loss of one histidine residue. Histidine also reacted in diisopropylphosphoryl- (DIP) trypsin but not in L-1-chloro-2-tosylamido-7-amino-2-heptanone (TLCK) trypsin. Experiments were also carried out using [1,3-^{14}C]bromoacetone. A chromatographic procedure, based on the high affinity of trypsin for soybean trypsin inhibitor, was employed to separate active from inactive trypsin in the reaction mixture. The inactivated trypsin contained 1.06 moles of [^{14}C]-acetone more than did the active material and showed a loss of 0.8 residue of histidine when compared with the internal control. Thus inactivation resulted from modification of a single residue of histidine. The radioactive peptides present in the inactive but not in the active bromoactone-treated trypsin contained 1.1 moles of ^{14}C and included histidine-46, while the peptide including histidine-27 contained a negligible quantity of ^{14}C. Bromoacetone was also found to react almost exclusively with histidine-46 in DIP-trypsin. Thus of the three histidine residues present in trypsin, only histidine-46 reacted to a significant extent with bromoacetone. The rate of reaction of histidine-46 in trypsin was comparable to that of the model compound α-*N*-benzoyl-L-histidine methyl ester. Furthermore, the introduction of an acetonyl group into histidine-46 abolished the unusual reactivity of serine-183, thus providing chemical evidence in support of the hypothesis that interaction between these residues is essential for the catalytic function of trypsin.

The involvement of histidine in the catalytic function of trypsin has long been inferred fron the pH dependence of the hydrolysis of specific substrates (Gutfreund, 1955) and from the reaction of the enzyme with *p*-nitrophenyl acetate (Dixon and Neurath, 1957). More recently, Shaw *et al.* (1965) have demonstrated that trypsin will react stoichiometrically with the substrate analog TLCK[1] to form an inactive derivative in which one histidine residue has been alkylated.

Trypsin contains three histidine residues. Two of these, in positions 29 and 46, are brought into close proximity in the linear sequence (Walsh *et al.*, 1964) by virtue of a disulfide bridge which cuts off a segment of the peptide chain containing 15 amino acid residues. A similar "histidine loop" occurs in the amino acid sequence of chymotrypsin (Walsh and Neurath, 1964; Hartley, 1964) and other serine proteases (Hartley *et al.*, 1965) with the two histidine residues located in the same relative position. On the basis of these homologies, it has been suggested that the two histidine residues might have been conserved during evolution in order to fulfill a functional role in enzyme catalysis (Walsh *et al.*, 1964). Bender and Kézdy (1964) have shown that the involvement of two histidine residues might be mechanistically favorable. No functional role has yet been proposed for the third histidine residue in trypsin which does not occur in the homologous enzymes chymotrypsin A or B (Desnuelle and Rovery, 1961).

The single histidine residue that becomes alkylated upon reaction with TLCK is histidine-46 (Shaw and Springhorn, 1967). This residue corresponds to histidine-57 in chymotrypsin, which likewise is singularly alkylated by reaction with the analogous chloromethyl ketone TPCK (Ong *et al.*, 1965). This situation is qualitatively different from ribonuclease where, by use of alkylating agents such as bromoacetate and iodoacetate, either one of two histidine residues of the active site (19 and 112) can become alkylated (Barnard and Stein, 1959; Crestfield *et al.*, 1963). Ribonuclease may represent a special case, however, since the histidine residues of the active center react considerably more rapidly

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[1] Abbreviations used: DIP, diisopropylphosphoryl; DPC, diphenylcarbamyl; TLCK, L-1-chloro-2-tosylamido-7-amino-2-heptanone; BAEE, *N*-α-benzoyl-L-arginine ethyl ester; STI, soybean trypsin inhibitor; PTI, pancreatic trypsin inhibitor; BA-trypsin, bromoacetone-treated trypsin.