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Adenosylcobalamin Analogues as Inhibitors of Ribonucleotide Reductase and Vitamin B_{12} Transport

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SUMMARY

JACOBSEN, D. W., DIGIROLAMO, P. M. & HUENNEKENS, F. M. (1975) Adenosylcobalamin analogues as inhibitors of ribonucleotide reductase and vitamin B_{12} transport. *Mol. Pharmacol.*, 11, 174-184.

Nucleoside analogues of 5'-deoxy-5'-adenosylcobalamin have been synthesized by reduction of cyanocobalamin with zinc in ammonium chloride, followed by reaction with the 5'-chloro derivatives of inosine, thioinosine (6-mercaptopurine riboside), adenine arabinoside, cytosine arabinoside, 2-fluoroadenosine, homoadenosine, 1,N^6-ethenoadenosine, tubercidin, and formycin. These analogues, several alkylcobalamins (CH_3-, CCl_2F-, $CClF_2$, aminoethyl-, and carboxypentyl-), and adenosyl- and methylcobinamide were tested as inhibitors of the adenosylcobalamin-dependent ribonucleotide reductase (EC 1.17.4.2) from *Lactobacillus leichmannii*. The fluoroadenosine derivative had full coenzymatic activity ($K_m = 6.7 \times 10^{-7}$ M), but all the other cobalamin analogues were inhibitory; most effective were the formycin ($K_i = 1.0 \times 10^{-6}$ M) and adenine arabinoside ($K_i = 3.0 \times 10^{-6}$ M) analogues. The cobinamides were very weak inhibitors. When tested as inhibitors of the transcobalamin II-mediated transport of vitamin B_{12} into L1210 cells, all the cobalamins were effective competitors (50% inhibition at analogue to vitamin B_{12} ratios of less than 10). The cobinamides, however, were poor inhibitors of vitamin B_{12} transport.

INTRODUCTION

The symptoms of vitamin B_{12} deficiency in man (anemia, neurological disorders, and suppression of growth) indicate that the vitamin is involved in essential metabolic reactions (2, 3), some of which may still be undiscovered. Analogues of the vitamin, and particularly of its coenzyme forms, may be useful in delineating these processes and in examining the mechanism of known corrinoid-dependent enzyme systems.

In the present investigation several new analogues of the coenzyme, 5'-deoxy-5'-adenosylcobalamin, have been synthesized, and these, along with some previ-

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ously available cobalamin and cobinamide analogues, have been examined as inhibitors of two representative systems: (a) Ado-Cbl[3]-dependent ribonucleotide reductase from *Lactobacillus leichmannii* and (b) transcobalamin II-mediated transport of vitamin B_{12} into L1210 cells. The results obtained provide some insight into the structural requirements of the enzymatic and transport systems and establish guidelines for the synthesis of additional analogues.

MATERIALS AND METHODS

Materials

The following were obtained from commercial sources: adenosine, inosine, and cyanocobalamin (Sigma); [^{57}Co]cyanocobalamin (250 Ci/mmole) (Amersham/Searle); CH_3Br, CCl_3F (Freon 11), and CCl_2F_2 (Freon 12) (Matheson); 2-chloroethylamine hydrochloride, hexamethylphosphoramide, and 6-bromohexanoic acid (Aldrich); chloroacetaldehyde (K & K Laboratories); phosphocellulose (Cellex-P) and polyacrylamide beads (P-2; 50–200 mesh) (Bio-Rad); Soluene 100 (Packard Laboratories); sterile human serum (Miles); RPMI 1640 culture medium lacking vitamin B_{12} but containing 10% fetal calf serum, penicillin, and streptomycin (Associated Biomedic Systems); *Lactobacillus leichmannii*, ATCC 7830 (American Type Culture Collection). The following compounds were gifts from Dr. Florence White, National Cancer Institute, National Institutes of Health: cytosine arabinoside, adenine arabinoside, formycin, thioinosine (6-mercaptopurine riboside), 2-fluoroadenosine, tubercidin, and homoadenosine.

Aquacobalamin was prepared from cyanocobalamin by the procedure of Hogenkamp and Rush (4). Aquacyanocobinamide (Factor B) was synthesized by the procedure of Friedrich and Bernhauer (5). The method of Barrio *et al.* (6) was used for the preparation of ϵ-adenosine.

5'-Deoxy-5'-adenosylcobalamin. Step A: 5'-Chloro-5'-deoxyadenosine was prepared by the following procedure, based upon the method of Kikugawa and Ichino (7). Adenosine (7.74 g, 29.0 mmoles) was stirred with 77.5 ml of hexamethylphosphoramide containing 12.4 ml of $SOCl_2$ for 15 hr at room temperature. After dilution to 800 ml with water, 160 ml (packed volume) of Dowex 50 (200–400 mesh, H^+ form) were added and the mixture was stirred for 30 min. The resin was collected by filtration on Whatman No. 1 paper, washed with 3 liters of water, and extracted three times with 400 ml of warm (45°) 1 N NH_4OH. The combined extracts were concentrated by evaporation under vacuum, and the product crystallized from water at 5°. Yield, 6.60 g (80%). On thin-layer chromatography in solvent D (composition given below) the product exhibited an R_f of 0.66 (quenching).

$C_{10}H_{12}N_5O_2Cl \cdot 0.7H_2O$

Calculated: C 40.3, H 4.50, N 23.5, Cl 12.0
Found: C 39.8, H 4.87, N 23.8, Cl 12.3

Step B: Cyanocobalamin (2.00 g, 1.40 mmoles) and 100 g of NH_4Cl were dissolved in 1 liter of water, and the solution was purged vigorously with argon for 20 min to remove dissolved oxygen. With rapid stirring and continued argon purging, 50 g of powdered zinc were added. After the solution had been stirred under anaerobic conditions for about 30 min, the cyanocobalamin was completely reduced to cob(I)alamin, as judged by the black color. In a dark room equipped with a red light, 1.00 g (3.5

[3] The abbreviations used are: Ado-Cbl, 5'-deoxy-5'-adenosylcobalamin; CN-Cbl, cyanocobalamin (vitamin B_{12}); CCl_2F-Cbl, dichlorofluoromethylcobalamin; $CClF_2$-Cbl, chlorodifluoromethylcobalamin; carboxypentyl-Cbl, 5-carboxypentylcobalamin; aminoethyl-Cbl, 2-aminoethylcobalamin; HomoAdo-Cbl, 5',6'-dideoxy(9-β-D-*ribo*-hexofuranosyl)-6'-adenylcobalamin, TC-II, transcobalamin II; Ino-Cbl, 5'-deoxy-5'-inosylcobalamin; AraA-Cbl, 5'-deoxy(9-β-D-arabinofuranosyl)-5'-adenylcobalamin; AraC-Cbl, 5'-deoxy(1-β-D-arabinofuranosyl)-5'-cytosylcobalamin; Sno-Cbl, 5'-deoxy-5'-thioinosylcobalamin; ϵ-Ado-Cbl, 5'-deoxy-5'-(1,N^6-etheno)adenosylcobalamin; F-Ado-Cbl, 5'-deoxy-5'-(2-fluoro)adenosylcobalamin; tubercidinyl-Cbl, 5'-deoxy-5'-tubercidinylcobalamin; formycinyl-Cbl, 5'-deoxy-5'-formycinylcobalamin; Me-Cbl, methylcobalamin; Adocobinamide, 5'-deoxy-5'-adenosylcobinamide; Mecobinamide, methylcobinamide.

mmoles) of 5'-chloro-5'-deoxyadenosine dissolved in 100 ml of deoxygenated 1 N NH_4OH was added (with vigorous stirring and argon purging) over a 3-min period to the cob(I)alamin solution. The disappearance of cob(I)alamin, or the appearance of Ado-Cbl, was monitored by changes in the absorbance spectrum of the solution. After the reaction had been completed (approximately 40 min), the mixture was filtered under vacuum through Whatman No. 1 paper. The filtrate was desalted by extraction with phenol–chloroform as described by Johnson *et al.* (8). Ado-Cbl was back-extracted from the organic phase with water, concentrated to dryness under vacuum, and dissolved in 15 ml of 0.01 M acetic acid. This solution was passed through a 4 × 40 cm column of phosphocellulose which had been equilibrated with 0.01 M acetic acid. The column was washed with 1.0 liter of 0.01 M acetic acid to remove minor impurities, including cyanocobalamin. Ado-Cbl was eluted from the column with 0.025 M sodium acetate, pH 6.0. Fractions containing Ado-Cbl were pooled and concentrated under vacuum to approximately 25 ml. Final purification and desalting was accomplished by passage through a 4 × 95 cm column of P-2 polyacrylamide, using water as the eluent. Aquacobalamin, a minor contaminant, was retained on the column. The column could be regenerated by washing with 0.01 M acetic acid, followed by water. Ado-Cbl was crystallized from aqueous acetone at 0°. Yield, 1.98 g of hydrated crystals (H_2O content approximately 10.2%) = 80%. On thin-layer chromatography the product had the following $R_{CN\text{-}Cbl}$ values: in solvent A (see below), 0.65; in solvent B, 0.63; in solvent C, 0.70 (quenching). λ_{max} (0.1 N HCl) = 265 nm (ϵ_{mM} 40.4), 284 nm (ϵ_{mM} 22.6), 304 nm (ϵ_{mM} 21.6), 380 nm (ϵ_{mM} 8.0), 458 nm (ϵ_{mM} 9.2). λ_{max} (0.1 M Na_2HPO_4, pH 7.0) = 261 nm (ϵ_{mM} 33.5), 317 nm (ϵ_{mM} 12.3), 337 nm (ϵ_{mM} 12.2), 376 nm (ϵ_{mM} 10.4), 523 nm (ϵ_{mM} 8.0).

$C_{72}H_{100}N_{18}O_{17}PCo \cdot 10H_2O$

Calculated: C 49.10, H 6.87, N 14.32
Found: C 49.07, H 6.92, N 14.22

Nucleoside analogues of 5'-deoxy-5'-adenosylcobalamin. The procedure given above for adenosine was used to prepare the 5'-chloro (or, in the case of homoadenosine, the 6'-chloro) derivatives of the following nucleosides: adenine arabinoside, cytosine arabinoside, inosine, thioinosine (6-mercaptopurine riboside), 2-fluoroadenosine, homoadenosine, tubercidin, and formycin. Products were characterized by elemental analysis, thin-layer chromatography and absorbance spectra. Because of its base lability, ϵ-Ado-Cbl was prepared by a slight modification of the above procedure. In step A, Dowex 50 treatment was omitted and 5-chloro-5'-deoxy-ϵ-adenosine was crystallized directly from a concentrated aqueous solution. In step B, 5'-chloro-5'-deoxy-ϵ-adenosine was added to the cob(I)alamin preparation in aqueous solution rather than in 1 N NH_4OH.

Alkylcobalamins. Methylcobalamin was synthesized by the following modification of the procedure of Wood *et al.* (9). Cyanocobalamin (0.5 g, 0.35 mmole) in 500 ml of 1.87 M NH_4Cl was reduced to cob(I)alamin by the addition of powdered zinc (25 g). Methyl bromide was bubbled through the reaction mixture for 15 min; after removal of the zinc by filtration, methylcobalamin was purified by phenol extraction and successive chromatography on phosphocellulose and polyacrylamide, as described above for Ado-Cbl. Yield, 76%.

CCl_2F-Cbl, $CClF_2$-Cbl, aminoethyl-Cbl, and carboxypentyl-Cbl were prepared in a similar manner, except for replacement of methyl bromide by CCl_3F, CCl_2F_2, 2-chloroethylamine, and 6-bromohexanoic acid, respectively.

Cobinamides. 5'-Deoxy-5'-adenosylcobinamide and methylcobinamide were synthesized from aquacyanocobinamide and 5'-chloroadenosine or methyl bromide according to the procedure given above for Ado-Cbl. Purification was achieved by chromatography on phosphocellulose. Yields, 53% and 77%, respectively.

Methods

Elemental analyses were performed by Spang Laboratories, Ann Arbor, Mich., and PCR, Inc., Gainesville, Fla. Absorbance spectra were obtained with a Cary recording spectrophotometer, model 14.

Extinction coefficients for Ado-Cbl, HomoAdo-Cbl, the alkylcobalamins, and the cobinamides were determined by quantitative conversion of these compounds to the corresponding dicyano corrinoids [ϵ_{mM} = 30.8 at 368 nm (10)]. By this procedure ϵ_{mM} for Ado-Cbl was found to be 8.0 at 523 nm. Extinction coefficients for the other nucleoside analogues of Ado-Cbl were calculated on the assumption that ϵ_{mM} = 8.0 for their longest wavelength absorbance band.

Thin-layer chromatography of corrinoids was performed on silica gel plates (Eastman No. 6061), using solvent systems similar to those reported by Firth *et al.* (11): A, *sec*-butyl alcohol–water (95:40); B, *sec*-butyl alcohol–glacial acetic acid–water (95:1:40); and C, *sec*-butyl alcohol–concentrated NH_4OH–water (95:6.6:40). Mobilities are reported as $R_{CN\text{-}Cbl}$ values, i.e., migration relative to that of cyanocobalamin (11). Thin-layer chromatography of nucleosides was performed on cellulose plates (Eastman No. 6064) using solvent system D, 1-butanol–water (84:16).

L. leichmannii was maintained by monthly transfers on litmus milk. The medium used for large-scale cultures was that of Blakley (12), except that thymidine was decreased to 2 mg/liter. Cells were grown to the late logarithmic phase at 35° in 150-liter cultures after inoculation with two 500-ml cultures grown on the same medium (with thymidine at 5 mg/liter). Cells were harvested by centrifugation, and the cell paste (approximately 525 g/150-liter fermentation) was stored frozen at −80°. A cell-free extract of this paste contained 0.08 unit (1 unit = 1 μmole of GTP reduced per minute) of ribonucleotide reductase activity per milligram of protein.

Ribonucleotide reductase was purified to homogeneity from *L. leichmannii*, using the procedure of Vitols *et al.* (13), followed by two cycles of chromatography on DEAE-cellulose; in the latter steps elution was accomplished with a linear gradient (0.05 → 0.3 M) of Trizma chloride buffer, pH 7.0, that also contained 5 mM mercaptoethanol and 1 mM EDTA. Specific activity of the enzyme, assayed by the procedure described below, was 1.2–1.4 units/mg of protein.

The assay mixture for ribonucleotide reductase contained 1.0 mM GTP, 20.0 mM sodium dihydrolipoate, 2.5 μM Ado-Cbl, vitamin B_{12} analogue (where indicated), 50 mM potassium phosphate (pH 7.5), 0.15 M KF, 1.0 mM EDTA, and 5 μg of ribonucleotide reductase in 0.25 ml. After incubation at 37° for 30 min, the reaction was terminated by rapid cooling to 0°. Excess dihydrolipoate (which interferes with color development) was removed by heating for 20 min at 100° in the presence of 0.20 ml of 0.5 M chloroacetamide containing 0.25 M potassium phosphate, pH 7.5, and 0.1 mM aquacobalamin. The solution was cooled to 4°, and 1.0 ml of diphenylamine reagent (14) was added prior to incubation at 37° for 60 min. The colored adduct formed between deoxyribose and diphenylamine was determined spectrophotometrically at 580 nm against a blank that had been kept at 0° during the enzyme assay.

Transport of vitamin B_{12} into L1210 cells was measured by the following method. Midlogarithmic phase L1210 cells, harvested by centrifugation after 48 hr of growth in the modified RPMI 1640 medium (see *Materials*), were suspended in phosphate-buffered NaCl (15) containing 0.1% glucose, at a density of 1×10^7 cells/ml. To 1.5 × 10 cm reaction tubes the following were added in order: 0.1 ml of 0.4nM [^{57}Co]cyanocobalamin, 0.1–0.4 ml of nucleoside or alkyl analogue (where indicated), and an aliquot of human serum whose vitamin B_{12}-binding capacity (see below) equaled the amount of labeled cyanocobalamin. The volume was brought to 1.0 ml with phosphate-buffered NaCl, and the mixture was incubated at room temperature for 20 min to allow for complete binding of the corrinoids to the serum TC-II. To start the reaction, 1 ml of cell suspension was added to the vitamin B_{12}–serum mixture and, after a 20-min incubation at room temperature, 8 ml of ice-cold 0.9% NaCl were added to stop uptake. Cell pellets, recovered by centrifugation, were washed with 5 ml of 0.9% NaCl, digested overnight at 37° with 0.6 ml of Soluene 100, and then added to 10 ml of scintillation fluid (5 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis[2-(4-methyl-

5-phenyloxazolyl)]benzene per liter of toluene). After addition of 0.15 ml of glacial acetic acid to each sample, radioactivity was determined in a Beckman liquid scintillation counter, model LS 233. Uptake was expressed as picomoles of labeled CN-Cbl per 10^9 cells.

The amount of serum to be used in the above procedure was determined by measuring vitamin B_{12}-binding capacity according to the method of Gottlieb *et al.* (16), except that 0.1 M potassium phosphate buffer (pH 7.5) containing 0.1% bovine serum albumin was used instead of 0.9% NaCl, and incubation was carried out for 20 min at room temperature. The vitamin B_{12}-binding capacity of various samples of human serum averaged about 1.0 ng/ml.

RESULTS AND DISCUSSION

Synthesis and Properties of Adenosylcobalamin

The coenzyme, 5'-deoxy-5'-adenosylcobalamin, was originally synthesized by the reaction of 2',3'-*O*-isopropylidene-5'-tosyladenosine with cob(I)alamin followed by removal of the protective isopropylidene group (8, 17). The latter process, however, produced some cleavage of the carbon-cobalt bond and hydrolysis of the *e*-amide of the corrin ring. Better yields of the coenzyme were obtained subsequently by using *p*-dimethylaminobenzaldehyde (18) or *p*-anisaldehyde (19) as the blocking groups; these groups could be removed after tosylation, thereby allowing 5'-tosyladenosine to react directly with cob(I)alamin. Another direct synthesis of deoxyadenosylcobalamin was reported by Murakami *et al.* (20), who treated cob(I)alamin with 5'-iodoadenosine. These procedures have also been used to prepare various nucleoside analogues of 5'-deoxy-5'-adenosylcobalamin (21-23).

In the present investigation a modification of the procedure of Murakami *et al.* (20) was used to prepare a series of nucleoside analogues of adenosylcobalamin.[4] Treatment of the nucleosides [inosine, thioinosine (6-mercaptopurine riboside), adenine arabinoside, cytosine arabinoside, 2-fluoradenosine, homoadenosine, ϵ-adenosine, tubercidin, and formycin] with $SOCl_2$ in hexamethylphosphoramide (7) gave the 5'-chloro derivatives in good yield (approximately 80%). The latter were then allowed to react with cob(I)alamin that had been prepared by reduction of cyanocobalamin or aquacobalamin with zinc and NH_4Cl (25). The products were purified by phenol extraction, chromatography on phosphocellulose, or gel filtration on polyacrylamide P-2, and in some instances were crystallized from acetone-water. For comparative purposes, several alkyl analogues of adenosylcobalamin and two cobinamides were also synthesized. Alkylcobalamins were prepared by the general method of Wood *et al.* (9), in which an excess of the alkyl halide is allowed to react with cob(I)alamin. For the synthesis of adenosyl- and methylcobinamide, aquacyanocobinamide (factor B) was reduced with zinc and NH_4Cl to the +1 oxidation state and then treated with 5'-chloroadenosine or methyl iodide. Yields of products ranged between 60% and 80%. Purity was verified by elemental analysis (for crystalline products), thin-layer chromatography, and absorbance spectra.

Elemental analyses for the analogues were in good agreement with the empirical formulae, provided that allowances were made for hydration. In the case of Ado-Cbl the amount of hydration was 10%. Chromatographic analysis utilized three solvent systems similar to those described previously by Firth *et al.* (11), *viz*, *sec*-butyl alcohol containing glacial acetic acid, water, and concentrated ammonium hydroxide, respectively. Each analogue gave a single, well-defined spot in all three systems; $R_{CN\text{-}Cbl}$ values are given in Table 1.

Absorbance spectra of the analogues were determined at pH 1 and pH 7. Extinc-

[4] While this manuscript was in preparation, a similar procedure, involving reaction of the 5'-chloronucleosides [prepared by the method of Kikugawa and Ichino (7)] with Cob(I)alamin (prepared by reduction of aquacobalamin with borohydride), was reported by Hogenkamp (24).

tion coefficients were determined by conversion to the dicyanocorrinoid or were calculated on the assumption that ϵ_{mM} for the α-band was the same as that for Ado-Cbl, i.e., 8.0. The spectra of Ado-Cbl (21), AraA-Cbl (21), Ino-Cbl (21), Me-Cbl (26), CCl_2F-Cbl (27), $CClF_2$-Cbl (9), aminoethyl-Cbl (28), Ado-cobinamide (29), and Me-cobinamide (30) were in good agreement with previously reported values. Spectral characteristics of the new analogues (AraC-Cbl, Sno-Cbl, F-Ado-Cbl, HomoAdo-Cbl, ϵ-Ado-Cbl, tubercidinyl-Cbl, and carboxypentyl-Cbl) at pH 1 and 7 are summarized in Tables 2 and 3, respectively. At the acidic pH the visible absorbance bands of the alkyl- and nucleoside cobalamins were shifted to lower wavelengths, owing to protonation and uncoordination of the lower axial ligand. All the analogues showed the customary photolability of alkylated corrinoids (31); aquacobalamin and diaquacobinamide were demonstrated by thin-layer chromatography to be the products of aerobic photolysis of the cobalamin and cobinamide analogues, respectively.

TABLE 1

Thin-layer chromatography of corrinoid compounds

Chromatography in solvent systems A, B, and C was performed as described under *Methods*.

Compound	$R_{CN\text{-}Cbl}$ in system		
	A	B	C
Ado-Cbl	0.65	0.63	0.70
AraA-Cbl	0.88	0.83	0.69
AraC-Cbl	0.70	0.49	0.46
Ino-Cbl	0.72	0.46	0.45
Sno-Cbl	1.04	0.71	0.55
F-Ado-Cbl	1.00	0.99	0.96
HomoAdo-Cbl	0.71	0.70	0.47
ϵ-Ado-Cbl	0.86	0.61	0.57
Formycinyl-Cbl	0.87	0.62	0.39
Tubercidinyl-Cbl	1.06	0.78	0.84
Me-Cbl	1.76	1.10	1.12
CCl_2F-Cbl	1.76	1.15	1.13
$CClF_2$-Cbl	1.84	1.29	1.19
Aminoethyl-Cbl	0.0	0.24	0.16
Carboxypentyl-Cbl	0.95	1.29	0.74
Ado-cobinamide	0.83	0.90	0.81
Me-cobinamide	1.00	1.29	1.07

Adenosylcobalamin Analogues as Inhibitors of Ribonucleotide Reductase

The *L. leichmannii* ribonucleotide reductase, which catalyzes the reduction of GTP (and other nucleoside triphosphates)

TABLE 2

Absorbance spectra of adenosylcobalamin analogues at pH 1.0

Analogue	Main absorption bands (millimolar extinction coefficients)							
	nm	*nm*	*nm*	*nm*	*nm*	*nm*	*nm*	*nm*
AraC-Cbl	265	273	285		315sh[a]		379	457
	(35.0)	(32.8)	(32.1)		(19.3)		(7.7)	(8.7)
Sno-Cbl	265	278	287	305	316		379	456
	(32.4)	(28.4)	(30.0)	(28.1)	(27.1)		(8.3)	(8.9)
F-Ado-Cbl	262		284	303	315sh	329sh	380	457
	(41.3)		(21.4)	(22.0)	(19.9)	(16.5)	(8.3)	(9.1)
HomoAdo-Cbl	264		285	303			380	458
	(40.8)		(23.3)	(24.8)			(9.0)	(9.2)
ϵ-Ado-Cbl	265	276sh	285	303	317sh		380	458
	(36.7)	(32.3)	(28.1)	(22.5)	(19.9)		(8.0)	(9.1)
Formycinyl-Cbl	265	278	287	302			380	458
	(30.5)	(25.8)	(28.2)	(28.6)			(7.9)	(8.4)
Tubercidinyl-Cbl	265	274	284	301			374	456
	(38.5)	(31.0)	(28.2)	(22.2)			(8.2)	(9.0)
Carboxypentyl-Cbl	264	275sh	286	302			380	450
	(28.3)	(20.3)	(21.8)	(28.9)			(9.6)	(8.6)

[a] Shoulder.

TABLE 3

Absorbance spectra of adenosylcobalamin analogues at pH 7.0

Analogue	Main absorption bands (millimolar extinction coefficients)								
	nm	*nm*	*nm*	*nm*	*nm*	*nm*	*nm*	*nm*	*nm*
AraC-Cbl		266	278sh[a]		315	335	375	431	523
		(27.7)	(24.9)		(12.0)	(12.0)	(10.0)	(4.0)	(8.0)
Sno-Cbl		267	282	289	316		376	433	523
		(26.7)	(28.6)	(28.5)	(20.0)		(10.8)	(4.2)	(8.0)
F-Ado-Cbl		262	280sh	289sh	315	339	376	431	523
		(36.0)	(19.1)	(17.3)	(12.1)	(12.5)	(10.1)	(4.2)	(8.0)
HomoAdo-Cbl		262		289sh	317	342	376	432	520
		(32.3)		(17.7)	(13.9)	(12.6)	(10.4)	(4.0)	(8.6)
ϵ-Ado-Cbl	257sh	264	274	288sh	315sh	340sh	375	431sh	523
	(24.7)	(26.1)	(23.4)	(20.1)	(14.0)	(12.4)	(10.2)	(4.1)	(8.0)
Formycinyl-Cbl		266	284	289	305sh	341	375	433	523
		(24.8)	(27.2)	(27.0)	(19.1)	(12.7)	(10.0)	(4.12)	(8.0)
Tubercidinyl-Cbl		264	277		315	838	374	429	520
		(26.7)	(24.4)		(12.2)	(12.3)	(10.1)	(4.3)	(8.0)
Carboxypentyl-Cbl		265	279	289	312	343	380sh	442	509
		(21.3)	(18.9)	(18.2)	(15.7)	(13.1)	(9.1)	(4.4)	(9.5)

[a] Shoulder.

by a dithiol (Eq. 1), was selected as a representative Ado-Cbl-dependent

$$GTP + R\begin{matrix}SH\\SH\end{matrix} \xrightarrow{\text{Ado-Cbl}} dGTP + R\begin{matrix}S\\S\end{matrix} + H_2O \quad (1)$$

system in which the analogues could be tested for inhibitory activity. In contrast to most vitamin B_{12}-dependent systems, which are purified as holoenzymes, the *L. leichmannii* ribonucleotide reductase is isolated as the apoenzyme. Results of inhibition assays performed with Ado-Cbl present at a concentration just below saturation (2.5 μM) and with nucleoside analogues of Ado-Cbl present at equivalent and higher concentrations are shown in Fig. 1.

The most potent inhibitor was formycinyl-Cbl, which caused a 50% decrease in activity at a concentration of 2.5 μM (equimolar with the concentration of Ado-Cbl). AraA-Cbl produced 50% inhibition at a concentration of 19 μM (approximately 8 times the concentration of Ado-Cbl). Tubercidinyl-Cbl, HomoAdo-Cbl, Adocobinamide, ϵ-Ado-Cbl, Ino-Cbl, Sno-Cbl, and AraC-Cbl caused 50% inhibition at concentrations of 56, 78, 119, 120, 168, 170, and 255 μM, respectively.

A detailed study was made of the inhibitory action of AraA-Cbl and formycinyl-Cbl (Fig. 2). Both analogues exhibited

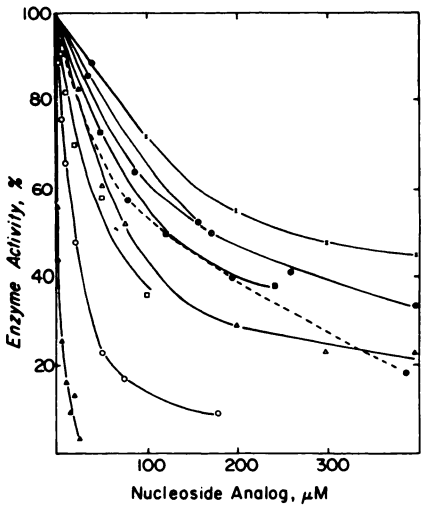

FIG. 1. *Inhibition of ribonucleotide reductase by nucleoside and analogues of adenosylcobalamin*

The assay system is described under *Methods*. Ado-Cbl was present at 2.5 μM and the concentration of nucleoside analogues was varied as indicated. Results are expressed as a percentage of control (i.e., activity in the absence of any analogue). ▲——▲, formycinyl-Cbl; O——O, AraA-Cbl; □——□, tubercidinyl-Cbl; Δ——Δ, HomoAdo-Cbl; ●- - -●, Adocobinamide; ■——■, ϵ-Ado-Cbl; ●——●, Ino-Cbl; ◐——◐, Sno-Cbl; ×——×, AraC-Cbl.

competitive inhibition, with K_i values of 3.0×10^{-6} M and 1.0×10^{-6} M, respectively. These values are close to the K_m (0.8×10^{-6} M) for the coenzyme, 5′-deoxy-5′-

adenosylcobalamin (13). Vitols *et al.* (13) and Tamao and Blakley (32) previously reported K_i values for inhibition of this enzyme by other nucleoside analogues of Ado-Cbl: 4-(adenin-9-yl)butylcobalamin (7×10^{-6} M); 2',5'-dideoxyadenosylcobalamin (2×10^{-5} M), and 2',3'-isopropylidene adenosylcobalamin (3×10^{-5} M). Deoxycytidylcobalamin was stated to be a poor inhibitor (13).

In contrast to the nucleoside analogues of Ado-Cbl, none of the alkyl analogues were very good inhibitors of Ado-Cbl-dependent ribonucleotide reductase (Fig. 3). Thus CCl_2F-Cbl, $CClF_2$-Cbl, Me-Cbl, and aminoethyl-Cbl produced 50% inhibition at concentrations of 72, 95, 98, and 195 μM, respectively. These may be compared with the inhibition caused by aquacobalamin and cyanocobalamin (50% at 78 and 190 μM, respectively). With Me-cobinamide and carboxypentyl-Cbl it was difficult or impossible to achieve 50% inhibition.

The present results demonstrate that there is a high degree of specificity at the coenzyme site of ribonucleotide reductase. The best inhibitor of the nucleoside analogues, formycinyl-Cbl, contains a modified purine ring in which C-8 and N-9 are exchanged; however, replacement of nitrogen by carbon at N-7 of the purine ring, as found in tubercidin and hence in tubercidinyl-Cbl, decreases the inhibitory activity considerably (Fig. 1). The 6-amino group of adenosine also has an important function in the Ado-Cbl-dependent ribonucleotide reductase, since the 6-hydroxyl (Ino-Cbl) and the 6-sulfhydryl (Sno-Cbl) analogues are relatively weak inhibitors and are totally inactive as coenzymes (see below). It is perhaps not surprising that only very weak inhibition is seen with AraC-Cbl, which contains both a pyrimidine base and an arabino sugar. However, two other analogues, modified only in their sugar components, were fairly potent inhibitors: AraA-Cbl, with an inverted hydroxyl at C-2, and HomoAdo-Cbl, with an additional methyl-

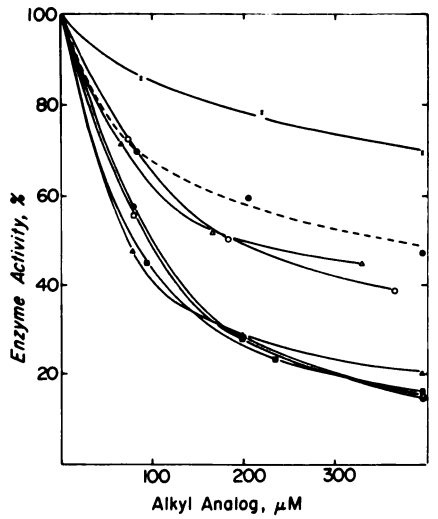

FIG. 3. *Inhibition of ribonucleotide reductase by alkyl analogues*

The assay system is described under *Methods*. Ado-Cbl was present at 2.5 μM, and the concentration of the alkyl analogues was varied as indicated. Results are expressed as a percentage of control (i.e., activity in the absence of any analogue). □——□, $CClF_2$-Cbl; ●——●, Me-Cbl; ■——■, aquacobalamin; ▲——▲, CCl_2F-Cbl; ○——○, CN-Cbl; △——△, aminoethyl-Cbl; ●- - -●, Me-cobinamide; ×——×, carboxypentyl-Cbl.

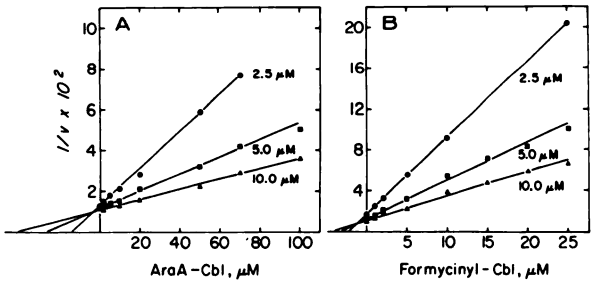

FIG. 2. *Kinetic analysis of inhibition of ribonucleotide reductase by AraA-Cbl and formycinyl-Cbl*

The assay system is described under *Methods*. Ado-Cbl was present at 2.5, 5.0, and 10.0 μM, and the concentration of analogues was varied as indicated. Velocity, v, is expressed as nanomoles of dGTP formed in 30 min.

ene group between the nucleoside and the cobalt. Ado-cobinamide, an analogue which lacks the lower nucleotide, was a poor inhibitor; this suggests that the lower axial ligand may play an important role in binding of the coenzyme or its analogues to the active site.

The coenzyme analogues containing small alkyl substituents (-CCl_2F, -$CClF_2$, -CH_3, or H_2O as in aquacobalamin) were better inhibitors of ribonucleotide reductase than analogues containing larger alkyl substituents [-$CH_2CH_2NH_3$ and -$(CH_2)_5COOH$]. With the latter compounds, steric hindrance could be responsible for the lowered inhibition. Electronic contributions to the cobalt may also be a factor in this process, since CN-Cbl is a poorer inhibitor than would be anticipated by comparison with Me-Cbl and aquacobalamin. Again, the alkylcobinamide analogue Me-cobinamide was a much poorer inhibitor than its cobalamin counterpart, Me-Cbl.

Analogues were also tested for coenzymatic activity (i.e., as a substitute for Ado-Cbl) in the ribonucleotide reductase system. As shown in Fig. 4, 2-fluoroadenosyl-Cbl was fully active as a coenzyme (K_m = 6.7 × 10^{-7} M). However, except for tubercidinyl-Cbl and formycinyl-Cbl, which had about 10% and 6% of the activity of Ado-Cbl (Fig. 4), none of the other analogues showed any activity. The low activity might have been due to traces of Ado-Cbl, although the starting materials in the synthetic procedure, tubercidin and formycin, were free from adenosine as determined by thin-layer chromatography. It was somewhat surprising to find so little coenzyme activity (even at high concentrations) with analogues such as tubercidinyl-Cbl, formycinyl-Cbl, and AraA-Cbl, which, because of their inhibitory properties, must fit reasonably well into the coenzyme site. Since coenzymatic function in the ribonucleotide reductase-catalyzed reaction is accompanied by an exchange of hydrogen at C-5′ (33), and possibly by rupture of the carbon-cobalt bond (32), there would appear to be no reason a priori why these nucleoside analogues, capable of binding strongly to the enzyme, are unable to fulfill these requirements.

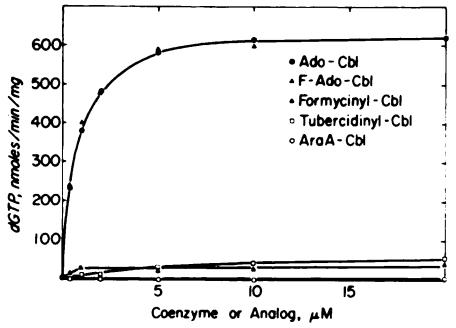

FIG. 4. *Coenzymatic activity of Ado-Cbl and its analogues for ribonucleotide reductase*

The assay system is described under *Methods*. The concentration of Ado-Cbl or analogue was varied as indicated.

Adenosylcobalamin Analogues as Inhibitors of Vitamin B_{12} Transport

In contrast to bacterial cells, which require only the carrier systems in their membranes for the uptake of vitamin B_{12} (34, 35), mammalian cells appear to be incapable of transporting CN-Cbl unless a serum protein, transcobalamin II, is also present. Despite its extremely low concentration of TC-II (approximately 0.1 nM), unfractionated human serum can be used to facilitate the uptake of ^{57}Co-labeled CN-Cbl into mammalian cells *in vitro* (36). Total uptake is very small (about 1 pmole/10^9 cells) but readily measured with labeled vitamin B_{12}.

The adenosylcobalamin analogues were tested as inhibitors of the TC-II-mediated uptake of vitamin B_{12} by L1210 cells (Table 4). All the nucleoside and alkyl analogues appear to have approximately equal inhibitory ability, but the two cobinamides are ineffective. These results are consistent with the previous work of Perlman and Giuffre (37), who found that aquacobalamin, Ado-Cbl, and Me-Cbl (but not cyanocobinamide) inhibited the ascitic fluid-mediated uptake of labeled vitamin B_{12} by Ehrlich ascites cells. Although transport of vitamin B_{12} into mammalian cells appears to involve a complex, multi-step sequence of events, the details of which are still not well understood, it is likely that the analogues inhibit this process by competing with CN-Cbl for the

TABLE 4

Inhibition of CN-Cbl transport into L1210 Cells by corrinoid compounds

Transport was measured as described under *Methods*. Results are expressed relative to a control containing only CN-Cbl (no analogue added).

Compound	Inhibition at molar ratios relative to [^{57}Co]CN-Cbl of		
	1	10	100
	%	%	%
Ado-Cbl	39	89	98
AraA-Cbl	35	87	97
AraC-Cbl	16	83	97
Ino-Cbl	19	87	97
Sno-Cbl	45	82	96
F-Ado-Cbl	35	86	99
HomoAdo-Cbl	35	87	99
ϵ-Ado-Cbl	28	85	97
Formycinyl-Cbl	30	86	97
Me-Cbl	64	90	98
CCl_2F-Cbl	58	93	97
$CClF_2$-Cbl	26	87	94
Aminoethyl-Cbl	30	71	94
Carboxypentyl-Cbl	50	87	99
Ado-cobinamide	0	7	32
Me-cobinamide	4	12	24

binding site on TC-II. This hypothesis is supported by an earlier observation (38) that cobalamins were more effective than cobinamides in blocking the binding of labeled CN-Cbl to human serum. Although it is likely that TC-II-bound analogues would be transported subsequently into the cells, verification of this point must await the synthesis of appropriately labeled analogues. Transport of the analogues would be a necessary prerequisite for inhibition of intracellular corrinoid-dependent enzyme systems.

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