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The Chemical Synthesis and Nuclear Magnetic Resonance Spectroscopy of Adenosylcobalamin Selectively Enriched with Carbon-13†

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ABSTRACT: Adenosylcobalamin selectively enriched with 90% carbon-13 in the 5′-methylene carbon attached to cobalt has been synthesized from 90% enriched potassium [^{13}C]cyanide. The reaction sequence involves the condensation of labeled cyanide with L-erythrose to yield a mixture of L-[1-^{13}C]ribonic acid and L-[1-^{13}C]arabonic acid. L-[1-^{13}C]Ribonic acid is then inverted to D-[5-^{13}C]ribose by a series of reactions involving the oxidation of the 5-hydroxymethyl group to an aldehyde to become C-1 of D-ribose and reduction of C-1 of the acid to a hydroxymethyl group to become C-5 of D-ribose. The conversion of D-[5-^{13}C]ribose to [5′-^{13}C]adenosylcobalamin is then accomplished by published procedures. The proton and carbon-13 nuclear magnetic resonance spectra of [5′-^{13}C]adenosylcobalamin and its labeled precursors are also presented.

Adenosylcobalamin functions as a coenzyme in enzymatic reactions involving the transfer of hydrogen. For instance, in the dioldehydrase reaction a hydrogen from C-1 of propanediol is transferred *via* the 5′-methylene carbon of adenosylcobalamin to C-2 of propionaldehyde (Abeles, 1971). Similar hydrogen transfer has been shown with glyceroldehydrase, ethanolamine ammonia-lyase, and the adenosylcobalamin-dependent mutases (Barker, 1972; Stadtman, 1972). Ribonucleotide reductase also catalyzes such a transfer but in this reaction the hydrogen donor and acceptor are different compounds (Blakley, 1966).

Electron spin resonance (esr) data have indicated that during catalysis the carbon–cobalt bond of adenosylcobalamin is alternately opened and closed by a homolytic mechanism. Several authors have suggested that the 5′-deoxyadenosyl radical, formed in the initial homolytic fission, abstracts a hydrogen from the substrate to form 5′-deoxyadenosine and a substrate radical. The latter then combines with cob(II)alamin to form a new carbon–cobalt bond. Rearrangement of this substrate–cobalamin complex and cleavage of the carbon–cobalt bond yields cob(II)alamin and a product radical (Babior *et al.*, 1972; Cockle *et al.*, 1972; Finlay *et al.*, 1973). However, Blakley and coworkers (Tamao and Blakley, 1973; Orme-Johnson *et al.*, 1974) have pointed out that these paramagnetic species, also observed in the ribonucleotide reductase system, do not have the kinetic behavior expected of an intermediate in the reaction. Indeed, using stop-flow techniques with ribonucleotide reductase they were able to demonstrate the reversible formation of intermediates in the first 100 msec. The uv–visible spectrum of these rapidly formed species resembles that of cob(II)alamin, while the esr spectrum is quite distinct from that formed more slowly. The spin concentration is consistent with the production of two radicals, presumably a cob(II)alamin type paramagnetic species and a 5′-deoxyadenosyl radical.

In order to provide another technique for the study of the mechanism of the reaction, and more specifically to provide a probe for the adenosyl moiety, we have synthesized adenosylcobalamin enriched with ^{13}C in the 5′-methylene carbon. Recently carbon-13 enriched cobalamins and cobinamides have been prepared and analyzed by ^{13}C nuclear magnetic resonance (cmr) spectroscopy. These results have shown that cmr is a very sensitive technique for the study of the corrinoids (Needham *et al.*, 1973; Walker *et al.*, 1974).

This paper describes the chemical synthesis and the physical and chemical properties of [5′-^{13}C]adenosylcobalamin.

Experimental Section

Materials. Brucine, cyanocobalamin, and L-rhamnose were purchased from Sigma Chemical Co. Carbon-13 potassium cyanide, 90% enriched, was a gift from Dr. M. Goldblatt of LASL. Ribonucleotide reductase from *Lactobacillus leichmannii* was kindly supplied by Dr. R. L. Blakley. Aquocobalamin, 2,3-*O*-isopropylidene-L-rhamnose, L-erythrose, D-riburonic acid, and chloromercuri-*N*-benzoyladenine were

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prepared by published procedures (Hogenkamp and Rush, 1968; Baker and Hewson, 1957; Perlin, 1962; Heyns and Lenz, 1961; Davoll and Lowy, 1951).

Methods. Melting points were measured on a hot stage equipped with a microscope and are not corrected. Ultraviolet spectra were recorded on a Cary Model 15 spectrophotometer. Optical rotation measurements were made in 1-dm tubes with a Zeiss–Winkel polarimeter. Gas–liquid chromatography of the trimethylsilyl derivatives was performed on a column (5 ft × 0.125 in.) of poly(ethylene glycol)-sebacate on Chromosorb Q maintained at 150 or 180° with helium at 25 ml min^{-1} as the carrier gas. Descending chromatography on Whatman No. 1 paper was conducted with the following solvent systems: solvent I, ethyl acetate–acetic acid–water (18:4:4); II, ethyl acetate–*n*-propyl alcohol–water (5:3:1); III, *n*-butyl alcohol–acetic acid–water (7:0.7:2.3). Nucleosides and cobalamins on chromatograms were located by their absorption of ultraviolet or visible light, respectively. Sugars were located by spraying with a 4% solution of *p*-anisidine hydrochloride in *n*-butyl alcohol, or with 0.5% sodium periodate and 0.5% benzidine solutions. Carbohydrates in solution were measured by the phenol–sulfuric acid reaction (Dubois *et al.*, 1956) or by the ferricyanide reduction method (Park and Johnson, 1949). Aldonic acids in 0.5 M acetic acid were first neutralized, treated with sodium periodate, and assayed for formaldehyde using the procedure of Frisell and Mackenzie (1958). Pulse ^{13}C (25.2 MHz) nuclear magnetic resonance spectra were obtained at 32° using a Varian XL-100-15 spectrometer locked to the resonance (15.4 MHz) of internal D_2O and interfaced to a Supernova computer. The transients resulting from the application of 45-μsec pulses in a spectral width of 5000 Hz were accumulated as 4096 points in the time domain and transformed into a 2048 point real spectrum (frequency domain). The data acquisition time was 410 msec and most of the spectra were obtained under conditions of simultaneous broad band (3000 Hz) proton noise decoupling. Peak positions were determined by computer examination of the final Fourier transformed spectrum. Chemical shifts were measured with respect to a tetramethylsilane external standard. Proton magnetic resonance spectra at 220 MHz were determined using a Varian HR-220 spectrometer, and tetramethylsilane or sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$ as reference standards. Spectra at 100 MHz were determined using a Varian HA-100 spectrometer; tetramethylsilane was used as reference standard and lock signal. Proton chemical shifts are reported on the δ scale.

L-[*1*-^{13}*C*]*Ribonic Acid* (**2**) *and* L-[*1*-^{13}*C*]*Arabonic Acid* (**3**). L-Erythrose (54 mmol) was treated with 2.64 g (40 mmol) of potassium [^{13}C]cyanide, 40 mmol of sodium hydroxide, and 2.54 g (24 mmol) of sodium carbonate in a total volume of 120 ml of water as described by Frush and Isbell (1953). The potassium aldonate solution was then treated with charcoal, concentrated to 15 ml, saturated with methanol and seeded with potassium L-arabonate. The crystals were collected and recrystallized from aqueous methanol to give 3.8 g (18.5 mmol) of potassium L-[1-^{13}C]arabonate. The combined mother liquors were evaporated to dryness. The residue was dissolved in water and applied to a (2.5 × 50 cm) column of Dowex 1-X8 (acetate) (200–400 mesh). The column was washed with 200 ml of water and the aldonic acids were eluted with 0.5 M acetic acid. L-[1-^{13}C]Ribonic acid (1.78 g, 10.7 mmol) was eluted in fractions (15 ml) 111–145 while L-[1-^{13}C]arabonic acid (1.39 g, 8.3 mmol) was eluted in fractions 165–240. The total yield based on 40 mmol of potassium [^{13}C]cyanide was 94% (L-ribonic acid 27% and L-arabonic acid 67%). An analytical sample of cadmium L-[1-^{13}C]ribonate was obtained by adding solid cadmium hydroxide to an aqueous solution of L-[1-^{13}C]ribonic acid, which had been previously dried over potassium hydroxide and phosphorus pentoxide.

Epimerization of L-*Arabonic Acid.* To a solution of 4.60 g (27.5 mmol) of L-[1-^{13}C]arabonic acid in 40 ml of water was added 4 ml of pyridine. The mixture was sealed in a stainless steel pressure cell and heated in an oven at 126° for 5 hr. The resulting dark brown solution was evaporated to 10 ml, treated with 5 ml of 6 N sodium hydroxide, and evaporated again to remove the pyridine. Water (50 ml) was then added and the solution was treated with charcoal and filtered through Celite. The resulting yellow solution was then applied to a Dowex 1-X8 (acetate) column and eluted with 0.5 M acetic acid as described above, yield, 1.05 g (6.3 mmol, 23%) of L-[1-^{13}C]ribonic acid and 2.19 g (13.1 mmol, 48%) of L-[1-^{13}C]-arabonic acid.

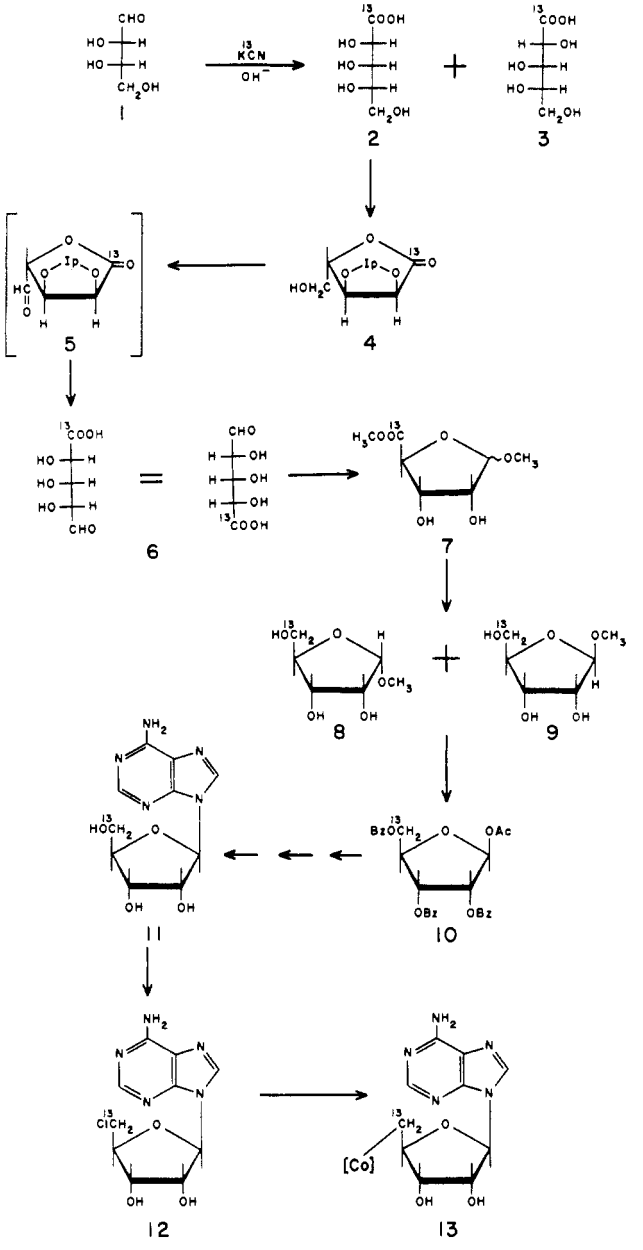
*2,3-O-Isopropylidene-*L-[*1*-^{13}*C*]*ribonolactone* (**4**). A solution containing 3.39 g of L-[1-^{13}C]ribonic acid in 40 ml of 0.05 N hydrochloric acid was evaporated to dryness. The residue was evacuated on a vacuum pump for 15 min at room temperature and for 10 min at 100°. The resulting syrup was then stored for 5 hr in a vacuum desiccator over phosphorus pentoxide and potassium hydroxide. The residue (3.25 g, 21.8 mmol) was dissolved in 80 ml of dry acetone and stirred vigorously with 0.34 ml of sulfuric acid and 4 g of anhydrous copper sulfate. After 16 hr the reaction mixture was neutralized with 0.84 ml of ammonium hydroxide. The inorganic salts were removed by filtration and the filtrate was evaporated to dryness. The residue was dissolved in 200 ml of ethyl acetate and the solution was extracted twice with 20 ml of water. The organic phase was dried over anhydrous sodium sulfate, decolorized with charcoal, and evaporated to dryness. Crystallization of the residue from chloroform–hexane yielded 2.70 g (14.3 mmol, 66%) of **4**. The yield could be raised to 76% by repeating the synthesis using the original water extract. Compound **4**: mp 136–139°, mixture melting point with the D isomer 100–135°; retention time 12 min by glc at 180°; $[\alpha]_D^{27}$ +61.7° (*c* 2.4, water) D isomer, mp 138–139°, $[\alpha]_D^{25}$ −57.5°, initial value (Hough *et al.*, 1958). The proton nmr spectrum of **4** was identical to that of the unlabeled D isomer (Abraham *et al.*, 1963).

D-[*5*-^{13}*C*]*Riburonic Acid* (**6**). 2,3-*O*-Isopropylidene-L-[1-^{13}C]ribonolactone (**4**) (3.74 g, 19.8 mmol) was oxidized with the dimethyl sulfoxide–dicyclohexylcarbodiimide reagent as described before (Walker and Hogenkamp, 1974). Removal of the isopropylidene group with 0.1 N hydrochloric acid and chromatography on Dowex 1-X8 (acetate) gave 2.33 g (14.1 mmol, 71%) of **6** as a colorless viscous syrup. An analytical sample of the brucine salt gave mp 195–197°; the melting point was undepressed in a mixture with the authentic brucine salt. R_F, solvent I 0.18; solvent III 0.21.

Methyl α-D-[*5*-^{13}*C*]*Ribofuranoside* (**8**) *and Methyl* β-D-[*5*-^{13}*C*]*Ribofuranoside* (**9**). A solution of 2.33 g (14.1 mmol) of **6** in 0.5% methanolic hydrogen chloride was boiled under reflux for 10 min. The cooled solution was neutralized with silver carbonate and filtered and the filtrate evaporated to a colorless syrup (**7**) yield 2.39 g (12.4 mmol, 88%). Glc of the trimethylsilyl derivatives showed both anomers of **7**; retention times 8.8 min (β anomer) and 11.2 min (α anomer) at 150°, R_F solvent I 0.75.

The colorless syrup (**7**) (2.39 g, 12.4 mmol) was reduced with sodium bis(2-methoxyethoxy)aluminum hydride as described before (Walker and Hogenkamp, 1974). Chroma-

SCHEME I



tography on Dowex 2-X8 (OH^-) gave two well-resolved compounds which were identified as the α and β anomers of methyl D-ribofuranoside: α anomer (**8**), 350 mg (2.12 mmol, 17%), R_F solvent II 0.45; retention time 5.2 min by glc at 150°; $[\alpha]_D^{27}$ +99° (*c* 1.80, water) (reported R_F solvent II 0.55; $[\alpha]_D$ +146° (Barker and Fletcher, 1961; Bishop and Cooper, 1963)); β anomer (**9**) 1.01 g (6.12 mmol, 49%) R_F solvent II 0.53; retention time 5.2 min by glc at 150°, $[\alpha]_D^{27}$ −54° (*c* 2.03, water) (reported R_F solvent II 0.61; $[\alpha]_D$ −50° (Baker and Fletcher, 1961)). A crystalline sample of the β anomer had mp 77–79°; a mixture melting point with an authentic sample was undepressed. The pmr spectra for **8** and **9** were in agreement with those published for the unlabeled L isomers (Walker and Hogenkamp, 1974). The ^{13}C–H coupling constant was 143 Hz for both anomers.

*1-O-Acetyl 2,3,5-Tri-O-benzoyl-β-*D*-[5-^{13}C]ribofuranoside* (**10**). Compound **10** was prepared from 1.11 g (6.7 mmol) of a mixture of **8** and **9** according to the method of Recondo and Rinderknecht (1959). Crystallization from 2-propanol gave 1.60 g (3.15 mmol, 47%); mp 127–130°, $[\alpha]_D^{27}$ +39.5° (*c* 1.5, chloroform) (reported mp 131–132°, $[\alpha]_D^{22}$ +45.1° (Recondo and Rinderknecht, 1959)). A mixture melting point with an authentic sample was undepressed.

*9-β-*D*-[5′-^{13}C]Ribofuranosyladenine* (**11**). Compound **10** (1.10 g, 2.18 mmol) was converted to 2,3,5-tri-*O*-benzoyl-D-[5-^{13}C]ribosyl chloride which in turn was condensed with chloromercuri-6-benzamidopurine (1.78 g, 3.81 mmol) according to the method of Ryan and Acton (1968). After deblocking with sodium methoxide in methanol, **11** was chromatographed on a (2 × 50 cm) column of Dowex 2-X8 (OH^-) (200–400 mesh) (Dekker, 1965). The nucleoside was crystallized from water; yield 190 mg (709 μmol, 33%), mp 229–231°; a mixture melting point with an authentic sample was undepressed. By lyophilizing the mother liquor another 90 mg of [5′-^{13}C]adenosine could be obtained increasing the overall yield to 45%.[1]

5′-Deoxy-5′-chloro-[5′-^{13}C]adenosine (**12**). To a solution of 0.15 ml of thionyl chloride in 10 ml of hexamethylphosphoramide was added 95 mg (350 μmol) of **11** and the reaction mixture was stirred at room temperature for 12 hr. The desired product (**12**) was then isolated as a chromatographically homogeneous preparation (335 μmol, 96%) as described by Kikugawa and Ichino (1971).

[5′-^{13}C]Adenosylcobalamin (**13**). Aquocobalamin (800 mg, 529 μmol) in 20 ml of water was reduced with sodium borohydride (200 mg) to cob(I)alamin by the method of Dolphin (1971), a deaerated solution of **12** (335 μmol) in 10 ml of methanol was then added, and the reaction mixture was stirred at room temperature for 2 hr. The cobalamin was purified as described before (Hogenkamp and Pailes, 1967) and crystallized from aqueous acetone, yield 434 mg (284 μmol, 85%).

Results and Discussion

Synthesis of [5′-^{13}C]Adenosylcobalamin. The synthesis of the desired cobalamin requires the synthesis of D-[5-^{13}C]-ribose. Although carbon-1 labeled sugars are readily prepared from labeled cyanide by the cyanohydrin method, the lower reactivity of primary hydroxyls precludes the simple introduction of the label at carbon-5. The reaction sequence shown in Scheme I involves the introduction of the label as cyanide in carbon-1, followed by an inversion of the entire molecule to produce the carbon-5 labeled sugar. Because the ribo configuration is symmetrical, inversion of the L isomer yields the desired D-ribo configuration. Thus the reaction of L-erythrose with potassium [^{13}C]cyanide gave L-[1-^{13}C]ribonic acid, which could be converted to D-[5-^{13}C]ribose by the methods developed in this laboratory for the synthesis of L-ribofuranose from D-ribono-1,4-lactone (Walker and Hogenkamp, 1974).

Since the cyanohydrin reaction yields both L-ribonic acid and L-arabonic acid (the latter being the predominant product) methods had to be found for the separation of the aldonic acids as well as for the epimerization of L-arabonic acid. Although pure potassium L-arabonate could be obtained by crystallization, the mother liquors always contained approximately equal amounts of L-arabonate and L-ribonate. Crystallization of L-ribonate as the cadmium salt from this mixture was not feasible. However, the aldonic acids were readily separated by chromatography on Dowex 1-X8, described as an analytical procedure by Samuelson and Thede (1967).

[1] The yield of labeled adenosine could be further increased by reacting the crude mother liquors of 10 with chloromercuri-6-benzamidopurine.

TABLE I: Carbon-13 Chemical Shifts in [5′-^{13}C]Adenosylcobalamin and ^{13}C-Labeled Intermediates.[a]

Compound	Carbon Positions[b]					
	OCH_3	C-1	C-2	C-3	C-4	C-5 (5′)
Potassium L-[1-^{13}C]arabonate (**3**)		180.1[c]	72.5	73.1	72.1	64.0
Cadmium L-[1-^{13}C]ribonate (**2**)		179.0[c]	74.0	74.1	72.2	63.8
2,3-*O*-Isopropylidene-L-[1-^{13}C]ribonolactone (**4**)[d]		178.1[c]	76.1	85.0	79.2	61.4
Methyl α-D-[5-^{13}C]ribofuranoside (**8**)	56.6	104.3	72.2	70.8	87.3	62.7[c]
Methyl β-D-[5-^{13}C]ribofuranoside (**9**)	55.9	108.6	75.0	71.5	83.6	63.5[c]
[5′-^{13}C]Adenosine (**11**)		88.3	74.7	71.4	85.3	62.3,[c] $J_{^{13}C-H}$ = 144
[5′-^{13}C]Adenosylcobalamin (**13**)						25.3,[c] $J_{^{13}C-H}$ = 138
[5′-^{13}C]Adenosylcobalamin (**13**)[e] pH 0.82						19.8[c]

[a] Spectra were obtained in D_2O solution. [b] Chemical shifts are measured downfield from Me_3Si = 0. [c] Indicates enriched carbon-13. [d] Isopropylidene carbons at 114.5, 26.7, and 25.4 ppm. [e] Natural abundance spectra were not obtained.

The epimerization of L-arabonate was accomplished in aqueous pyridine at high temperature as described by Fischer and Piloty (1891). Although this reaction yields another equilibrium mixture, with L-arabonate as the predominant epimer, the unreacted L-arabonate can be recovered in approximately 50% yield by column chromatography on Dowex 1-X8 and reepimerized. Using this procedure 25 mmol of L-[1-^{13}C]ribonic acid were obtained from 69 mmol of [1-^{13}C]-arabonic acid by eight repeated epimerizations. The conversion of L-[1-^{13}C]ribonic acid to D-[5-^{13}C]ribose, its conversion to [5′-^{13}C]adenosine and [5′-^{13}C]adenosylcobalamin, was accomplished by published procedures. The overall yield of [5′-^{13}C]adenosine from 7.9 g (120 mmol) of potassium [^{13}C]cyanide was 2.8% (3.35 mmol).

Characterization of the ^{13}C-Enriched Compounds. As expected, the physical properties such as melting points, chromatographic behavior and uv–visible spectra of the carbon-13 enriched compounds do not differ from those of the unlabeled compounds. The uv spectra of [5′-^{13}C]adenosine and the uv–visible spectra of [5′-^{13}C]adenosylcobalamin were identical with those of the authentic compounds over a wide range of pH values. The labeled cobalamin was active as a coenzyme with ribonucleotide reductase from *Lactobacillus leichmannii*. Under identical assay conditions the labeled and unlabeled coenzyme have virtually identical kinetic constants (K_m = 4.9 ± 0.3 × 10^{-6} M; V_{max} = 2.78 ± 0.07 μmol of dATP mg of protein^{-1} hr^{-1} and K_m = 4.7 ± 0.3 × 10^{-6} M; V_{max} = 2.80 ± 0.06 μmol of dATP mg of protein^{-1} hr^{-1} respectively).

The approximate ^{13}C enrichment (90 ± 10%) of the 5′-carbon atom of adenosylcobalamin was determined from the proton-coupled cmr spectrum by comparing the intensities of the 5′-carbon C–H multiplets with those of other single atom resonances present at natural abundance (1.1%) in the coenzyme. The enrichment of the precursors (**2**–**4**, **8**, **9**, and **11** in Scheme I) was found to be 90 ± 3% by analysis of the ^{13}C–^{13}C spin spin coupling patterns in the proton noise decoupled spectra of these simpler molecules (Matwiyoff and Burnham, 1973).

Nuclear Magnetic Resonance Spectroscopy. The cmr spectral data for the carbon-13 enriched compounds are summarized in Table I, while the proton noise decoupled cmr spectrum of one of the intermediates methyl β-D-[5-^{13}C]ribofuranoside is reproduced in Figure 1. It is clear from this spectrum and also from other spectra not shown that only one carbon in each compound is labeled. The spectra of the two [1-^{13}C]-aldonates and of 2,3-*O*-isopropylidene-L-[1-^{13}C]ribonolactone exhibit one prominent peak in the carbonyl region of the spectrum (approximately 180 ppm) showing that the label is indeed at carbon-1. Carbon-2 can be readily assigned to the doublet caused by the ^{13}C–^{13}C coupling to carbon-1, while carbon-5 shows the high field shift common to the hydroxymethyl functions.

The aldonates are diastereomeric at C-2 and exhibit large chemical shift differences only for carbon atoms 1, 2, and 3. Carbon-13 chemical shift effects induced by molecular asymmetry have been noted for other carbon atoms which are not part of cyclic systems, *e.g.*, asymmetric alcohols (Kroschwitz *et al.*, 1969) and diastereomeric ascorbic acid derivatives (Matwiyoff and Tolbert, 1974). These shifts can result from steric interactions (steric compression) between carbon atoms constrained to gauche orientations (Grant and Cheney, 1967; Reich *et al.*, 1969; Stothers, 1972). In the case of the aldonates, one must consider also intramolecular hydrogen bonding effects. For instance in arabonic acid (**3**) the disposition of the C-2 and C-3 hydroxyl groups allows intramolecular hydrogen bonding to the carboxyl group. In ribonic acid (**2**), however, a strong hydrogen bond can occur only between the C-2 hydroxyl and the carboxylic acid groups. Although we are not certain of the origin of the chemical shift differences between **2** and **3**, we note that the conformation of **3** that yields optimum hydrogen bonding results in slightly eclipsed conformations at C-2 and C-3. These eclipsed conformations could induce, by steric compression, the upfield shifts observed for C-2 and C-3 in arabonic acid relative to ribonic acid.

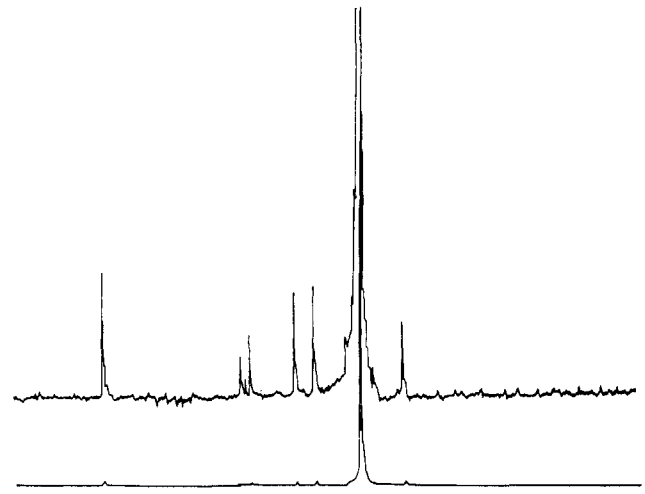


FIGURE 1: Cmr spectrum of methyl β-D-[5-^{13}C]ribofuranoside (**8**).

The cmr spectrum of methyl β-D-[5-^{13}C]ribofuranoside (Figure 1) is virtually identical with that reported by Breitmaier *et al.* (1972) and Mantsch and Smith (1972), who were able to make all the assignments. The intense resonance at 62.7 ppm and the doublet at 87.3 ppm confirm the assignments of carbons 5 and 4, respectively. The cmr spectrum of methyl α-D-[5-^{13}C]ribofuranoside is similar to that of the β anomer and only simple comparison of the two spectra is needed to make the proper assignments. As expected on the basis of steric effects in cyclic systems (Grant and Cheney, 1967; Reich *et al.*, 1969) the resonance of carbon-1 is shifted 4.3 ppm upfield for the α anomer, analogous to the 4.7 ppm upfield shift for the D-ribofuranoses (Breitmaier *et al.*, 1972), and the two reasonances at 72.2 and 70.8 ppm can be assigned to C-2 and C-3 of the α anomer, respectively. The cmr spectrum of [5′-^{13}C]adenosine shows one prominent resonance at 62.3 ppm. The assignment of the resonance to C-5′ is in agreement with the assignments made from the natural abundance cmr spectra by Breitmaier *et al.* (1972), 61.3 ppm, and Jones *et al.* (1970), 62.7 ppm. The pmr spectrum of [5′-^{13}C]adenosine is identical with that of unlabeled adenosine, except for the extra resonances due to the coupling of the 5′-protons with carbon-13. These ^{13}C-satellite peaks were partially obscured by the solvent peak and by the C-2′ and C-3′ resonances such that the ^{13}C–H coupling constant could not be accurately determined. The coupling constant determined from the proton coupled cmr spectrum ($J_{^{13}C-H}$ = 144 Hz) is in good agreement with the values obtained for a hydroxymethyl carbon (Stothers, 1972).

The proton noise decoupled cmr spectrum of [5′-^{13}C]-adenosylcobalamin shows a single, intense resonance at 25.3 ppm for the 5′-methylene carbon. The peak width at half-maximum intensity is 12.4 Hz, similar to that found for [^{13}C]-methylcobalamin. In contrast, Doddrell and Allerhand (1971) were unable to detect the 5′-methylene carbon in the natural abundance cmr spectrum of adenosylcobalamin and suggested that this was due to line broadening by the ^{59}Co nucleus. At pH 0.82 the resonance of the 5′-methylene carbon shifts 5.5 ppm upfield to 19.8 ppm; this shift reflects the displacement of the 5,6-dimethylbenzimidazole moiety by water at low pH (Ladd *et al.*, 1961). A similar trans effect has been noted for [^{13}C]methylcobalamin where the methyl resonance shifted 7.7 ppm upfield upon acidification (Needham *et al.*, 1973). The proton coupled cmr spectrum of [5′-^{13}C]adenosyl-cobalamin shows a triplet due to the splitting by the two 5′-methylene protons ($J_{^{13}C-H}$ = 138 Hz). The coupling constant for the "base off" form in acid could not be determined because the cobalamin would decompose during the time required to obtain a coupled spectrum. An identical coupling constant was found for [^{13}C]methylcobalamin suggesting that the electronegativity of the cobalt in both cobalamins is very similar ($E_{Co} \approx 2.23$).

In the 220-MHz pmr spectrum of [5′-^{13}C]adenosylcobal-amin in D_2O or in acid the 5′-methylene protons could not be detected. Brodie and Poe (1972) have assigned these two protons to broad resonances at approximately 1.1 and 0.57 ppm in dimethyl-d_6 sulfoxide while Cockle *et al.* (1970) show them at 1.0 and 0.63 ppm in D_2O. If these assignments are correct, the pmr spectrum of the ^{13}C-labeled cobalamin should have shown the ^{13}C-satellite peaks. One of these resonances, corresponding to half a proton, should have appeared 69 Hz upfield from the parent peak at approximately 0.3 ppm, a region containing no other resonances. However, our pmr spectra of [5′-^{13}C]adenosylcobalamin are identical with those published for the unlabeled cobalamin and no extra satellite peak could be detected. These observations suggest that the assignments made by Brodie and Poe (1972) and Cockle *et al.* (1970) for the 5′-methylene hydrogens are incorrect and that these resonances appear further downfield such that the ^{13}C-satellite peaks are obscured by other resonances. Work is in progress to resolve this question with double resonance (indor) (Baker, 1962; Philipsborn, 1971) techniques. Preliminary results obtained with these techniques also suggest that the high field proton resonances are *not* coupled to the 5′-^{13}C atom.

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