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Chemical and Biological Studies with Fluoroalkylcobalamins*

M. W. Penley, Dennis G. Brown,† and J. M. Wood‡

ABSTRACT: A new homologous series of fluoromethylcobalamins has been synthesized by reacting a variety of Freons with Cob(I)alamin (B_{12-s}). A comparative study of the physical and chemical properties of the nephelauxetic series $CF_3 > CF_2Cl > CF_2H > CH_3$ shows that there is a linear relationship between the electronegativity of each ligand and the stability of the Co–C σ bond to light. Also, a linear relationship exists between the stability of the 5,6-dimethylbenzimidazole (Bz)–Co complex and the electronegativity of each ligand. However, ligands which contain more than one chlorine atom (*e.g.*, $CFCl_2$) are more photolabile than any of the above derivatives, and evidence is available that these *gem*-dihalides generate carbenes upon photolysis. The authenticity of these fluoromethylcobalamins was rigorously confirmed by fluorine nuclear magnetic resonance and 220-MHz proton nuclear magnetic resonance. Cobalamin analogs containing $CFCl_2$, CF_2Cl, and CF_3 in place of CH_3 were shown to be competitive inhibitors for methylcobalamin in enzymatic methane formation by cell extracts of the methanogenic bacterium (MOH). Ligands containing more than one chlorine atom were shown to be more potent inhibitors than those which contained one or zero chlorine atom. Difluoromethylcobalamin replaces methylcobalamin as a substrate in the methane system; in the presence of ATP and hydrogen as a source of electrons, this analog yields methane. The physical and chemical properties of these fluoromethylcobalamins allow us to predict why the difluoromethyl analog is a substrate for the methane enzyme when all the other fluorine-containing cobalamins are inhibitors. The significance of the lack of specificity of the methane enzyme system for methylcobinamide, methyl factor III, methylcobalamin, and the abiogenic methylcobaloximes is discussed.

Bauchop (1967) made the initial observation that Antifoam A causes inhibition of methane formation in the rumen of sheep. Inhibition of methanogenesis was shown to be accompanied by immediate concomitant evolution of hydrogen. Methane formation in the rumen may be considered by the following general reaction

$$CO_2 + 4H_2 \rightleftharpoons CH_4 + 2H_2O$$

Clearly this equilibrium in the rumen is affected by Antifoam A. It was discovered subsequently that Freon 12 (CF_2Cl_2), which is present as a propellant in Antifoam A, was responsible for the inhibition of methane formation in the rumen ecosystem. The involvement of vitamin B_{12} in methane formation prompted us to examine whether strongly nucleophilic Cob(I)alamin could be the site for this inhibition (Wood and Wolfe, 1966; Wood *et al.*, 1968).

Freons are not generally regarded as alkylating agents, but Cob(I)alamin is such a strong nucleophile that a homologous series of fluoromethylcobalamins can be synthesized in good yield by standard procedures used for the synthesis of alkylcobalamins (Pailes and Hogenkamp, 1968; Buckman *et al.*, 1969). Details of the synthesis of this homologous series are outlined in Scheme I.

This homologous series of compounds has been used to examine some of the parameters which determine the stability of the Co–C σ bond, and how these parameters relate to our interpretation of both the ultraviolet–visible spectra and the nuclear magnetic resonance spectra of alkylcobalamins in general.

We have already reported the details of the synthesis of a series of halomethylcobalamins which contain chlorine, bromine, and iodine (Wood *et al.*, 1968). One interesting feature of the properties of these halomethylcobalamins is that the *gem*-dihalides photolyze to give carbenes, whereas ligands containing only one halogen atom (excluding fluorine) give radicals by homolytic cleavage (Scheme II) (Hogenkamp, 1966; Schrauzer *et al.*, 1968; Kennedy *et al.*, 1969).

A study of the first-order photolysis rates of these fluoromethyl derivatives provides an ideal model system for studying the effect of electron withdrawal from cobalt on the stability of the Co–C σ bond. A comparison of the ligands CF_3, CF_2H, and CH_3 is of particular interest since fluorine has a similar Van der Waals radius to hydrogen and strain introduced by bulky electron-withdrawing substituents need not be considered (Hogenkamp *et al.*, 1965). The present study helps to confirm the different photolysis mechanisms which are proposed for mono- and dichloromethylcobalamins (Scheme II).

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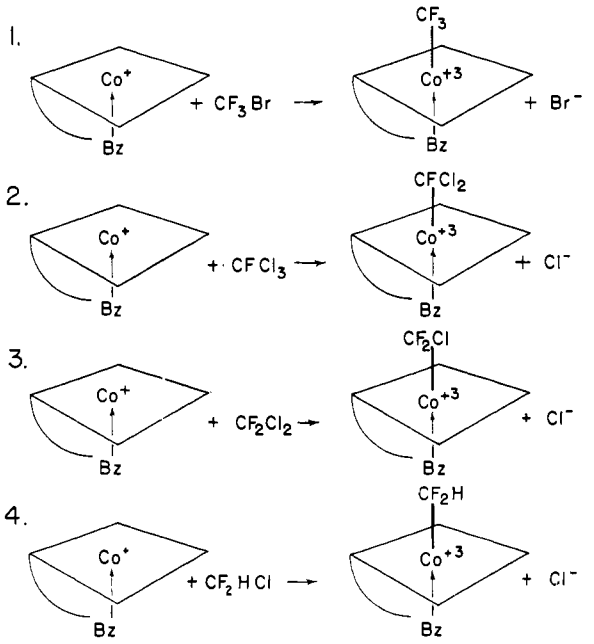
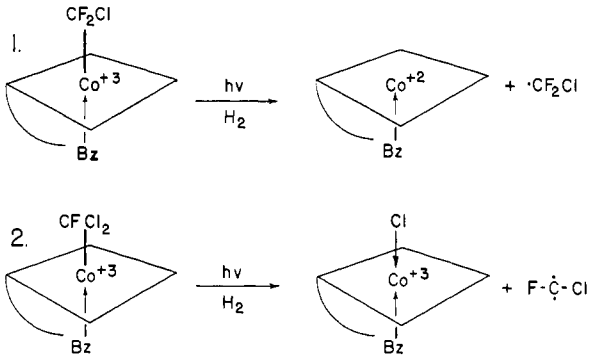
SCHEME I



SCHEME II



We have used fluorine nuclear magnetic resonance to characterize this series of fluoromethylcobalamins. Enormous chemical shifts (upfield) are observed for these fluoromethylcobalamins relative to those observed for a B_{12} model compound trifluoromethyl-Co-(pyridine)bis(dimethylglyoxime) in which pyridine is the axial base instead of Bz[1] (Schrauzer, 1968). A 220-MHz nuclear magnetic resonance study of CF_3-CF_2H-, and CH_3-cobalamin has helped us to confirm the authenticity of these fluoroalkylcobalamins.

Our discovery that difluoromethylcobalamin is a substrate in the methane system, but that trifluoromethylcobalamin is an inhibitor, suggests that the pK_A for the displacement of Bz may be important in methane formation from cobalamins. Cobalamins which have been found to be active in the methane system have pK_A values for the displacement of Bz between 2.5 and 2.7, whereas trifluoromethylcobalamin has a pK_A value of 2.1, and both ethyl- and propylcobalamin, with pK_A values of 3.87 and 3.84, respectively (Hogenkamp *et al.*, 1965), are not substrates in this enzyme system (Wood *et al.*, 1966; Wolfe *et al.*, 1966). It must be emphasized that although abiogenic methylcobaloximes function as methyl donors in the methane system, activity for these compounds can only be demonstrated when reactions are performed in the presence of catalytic amounts of Cob(II)alamin ($B_{12\text{-}r}$) (McBride *et al.*, 1968; Sibert and Schrauzer, 1970). Thus, these compounds do not apparently function as substrates in this enzymatic system. Possible mechanisms for the transfer of methyl and difluoromethyl substituents in the methane system are discussed, and the significance of methyl transfer from methylcobaloximes reappraised.

[1] Abbreviations used are: Bz, 5,6-dimethylbenzimidazole; Cob(I)alamin ($B_{12\text{-}s}$), Co^+-5,6-dimethylbenzimidazolylcobamide; Cob(II)alamin ($B_{12\text{-}r}$), Co^{2+}-5,6-dimethylbenzimidazolylcobamide; methyl factor III, CH_3-Co^{3+}-5-hydroxybenzimidazolylcobamide.

Materials and Methods

Culture Methods and Preparation of Extracts. The methanogenic organism (*Methanobacterium* strain MOH) was cultured and harvested as described by Bryant *et al.* (1968). Cell-free extracts were prepared by exposing 1 g of cells (wet wt)/ml of 0.5 M potassium phosphate buffer (pH 7.0) to the maximum frequency output of a Branson sonic probe for 2-min intervals to a total of 10 min at 0°. Cell debris was removed by centrifugation at 23,000*g* for 20 min at 0°. The extracts were stored under an H_2 atmosphere at −20° prior to use. Protein was determined by the method of Lowry *et al.* (1951).

Chemical and Analytical Methods. Cyanocobalamin was purchased from Sigma Chemical Co. Methylcobalamin was prepared by the method of Müller and Müller (1962). Aquocobalamin was prepared from methylcobalamin through aerobic photolysis. The Freons were purified through distillation. Methyliodide (Aldrich) was purified by shaking with dilute aqueous $Na_2S_2O_3$ as described by Perrin *et al.* (1966). Methane was assayed by gas chromatography on a silica gel column by the method of Wolin *et al.* (1963).

The fluoromethylcobalamins were obtained by a modification of the procedure of Wood *et al.* (1968). The appropriate Freon (Matheson) was used to saturate an aqueous solution of aquocobalamin (1.0 g) previously reduced to Co(I) with zinc dust (10 g) and ammonium chloride (100 ml of 10% w/v solution).

After filtration to remove the excess zinc, the reaction mixtures were phenol extracted to remove inorganic salts (Johnson *et al.*, 1963). The remaining aqueous solution of alkylcobalamin was lyophilized to dryness. The lyophilized powder was dissolved in a minimal volume of 0.05 N HOAc and applied to a phosphocellulose column (cellulose phosphate cation exchanger, Sigma). This resin was generated in the phosphate form by the method of Lezius and Barker (1965). The column (40 × 2.5 cm) was eluted with 0.015 N NaOAc buffer (pH 4.1). The eluent containing the alkyl derivative was extracted with phenol and lyophilized. Aquocobalamin was removed on the phosphocellulose column and a DEAE-cellulose column was used to remove a trace contamination of cyanocobalamin. The product was dissolved in 0.005 M Tris-HCl buffer (pH 8.9) and applied to a DEAE-cellulose column (DEAE-cellulose anion exchanger, Sigma). This resin (0.94 mequiv/g) was previously passed through acid and alkali cycles, washed with water, and equilibrated with 0.005 M Tris-HCl buffer. The

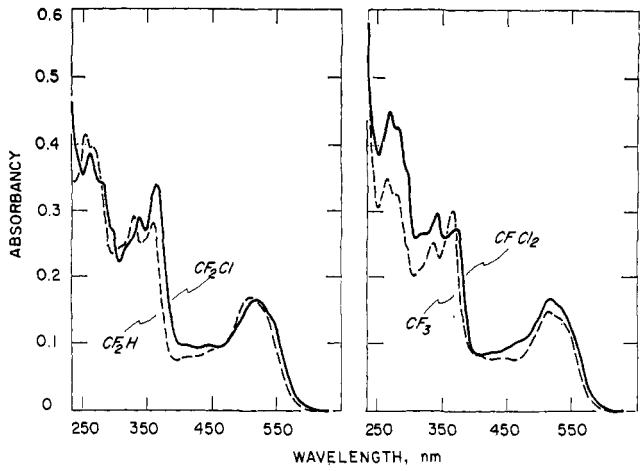


FIGURE 1: Ultraviolet–visible spectra of the fluoromethylcobalamins recorded in 0.05 M phosphate buffer (pH 7.0). Each cuvet contained approximately a 2×10^{-5} M solution of the respective derivative.

column (35 × 1 cm) was eluted with 0.05 M Tris-HCl (pH 9.0). The first band eluted was extracted with phenol and lyophilized to dryness. The lyophilized powder was dissolved in a minimal amount of water and crystallized from acetone–water. The crystals were isolated, washed with acetone, and allowed to air-dry. Then the crystals were dried over P_2O_5 at 57° and 1.5 mm for 2.5 hr. The final crystalline product was stored over activated silica gel desiccant in a freezer at −15°. Yields of 20% are routine.

The purity of each derivative was established by thin-layer chromatography in four solvent systems. Thin-layer cellulose chromatography was carried out on Eastman Chromagram sheets (6065 Cellulose) by the ascending technique with the following solvent systems: solvent I, *sec*-butyl alcohol–water (9.5:4, v/v); solvent II, *sec*-butyl alcohol–acetic acid–water (100:1:50, v/v); solvent III, *sec*-butyl alcohol–0.88 ammonia–water (9.5:.68:4, v/v); solvent IV, *n*-butyl alcohol–acetic acid–water (4:1:5, v/v) (upper layer). The results are expressed (Table I) in terms of $R_{B_{12}}$ as defined by Firth *et al.* (1968). The R_F value of cyanocobalamin is given in each case.

Aerobic photolysis of the fluoromethylcobalamins was carried out on unbuffered aqueous solutions (approximately 4.0×10^{-5} M) in 1.5-ml quartz cuvets by irradiation from a 200-W tungsten lamp at a distance of 45 cm. A stream of cold air was used to prevent heating of the solutions during the photolysis process. The rate of aquocobalamin formation was measured by the increase in absorbance at 350 nm.

TABLE I

Solvent:	I	II	III	IV
R_F of B_{12}:	0.420	0.458	0.390	0.586
		$R_{B_{12}}$ of Derivative		
CN	1.00	1.00	1.00	1.00
$CFCl_2$	1.40	1.35	1.31	1.06
CF_2H	1.02	1.02	0.80	1.04
CF_2Cl	1.31	1.25	1.37	1.12
CF_3	1.28	1.23	1.37	1.09

TABLE II

Compound	Chemical Shift (ppm)[a]
Trifluoromethylcobaloxime[b]	30.6
Trifluoromethylcobalamin[c]	94.2
Difluorochloromethylcobalamin[c]	94.2
Difluoromethylcobalamin[d]	212.0
Fluorodichloromethylcobalamin[d]	213.5

[a] Referenced to an internal $CFCl_3$ standard. [b] Solvent: dimethyl sulfoxide. [c] Solvent: water. [d] Solvent: phenol–water.

The pK_A for the displacement and protonation of benzimidazole from each fluoromethyl derivative was determined by the method of Hogenkamp *et al.* (1965).

Absorption spectra were recorded with a Beckman Model DB-G spectrophotometer using matched 1-cm quartz cells. Other spectral measurements were made with a Zeiss PMQII spectrophotometer.

^{19}F nuclear magnetic resonance spectra were run on a Varian HA-100 spectrometer operating at 94.1 MHz. For most samples it was necessary to use a computer of average transients in order to improve the signal to noise ratio. All fluorine spectra were referenced to a $CFCl_3$ standard.

^1H nuclear magnetic resonance spectra were run on a Varian HR-220 operating at 220 MHz. Deuterium oxide was employed as the solvent in all cases. All spectra were recorded using a single scan.

Results

Ultraviolet–Visible Spectra. This homologous series of fluoromethylcobalamins exhibits very similar spectra both in the visible and ultraviolet regions (Figure 1). However, the position of the γ band reflects the extent to which electrons are withdrawn by the fluorine-containing ligand from the cobalt atom. As would be predicted, the position of the γ band falls between that observed for methylcobalamin (340 nm) and that for cyanocobalamin (368 nm). Although slight changes for the extinction coefficients of these fluoromethylcobalamins for the γ band occur, the extinction at 520 nm remains constant at 7.6×10^{-3} M^{-1} cm^{-1} (Firth *et al.*, 1967).

Nuclear Magnetic Resonance Spectra. Figure 2 shows ^{19}F nuclear magnetic resonance spectra for trifluoromethylcobaloxime and fluorodichloromethylcobalamin. As can be seen, very good signal to noise ratios can be achieved for fluoromethylcobalamins using time averaging. The spectra of all the cobalamins were obtained using the CAT while the spectrum of the cobaloxime was obtained with a single scan. Table II gives the chemical shifts for the fluorine-containing derivatives under consideration. The cobalamin spectra were obtained in either water or in water–phenol mixtures. The line widths of the compounds run in phenol–water were larger than those run in water, possibly because of the high viscosity of these solutions.

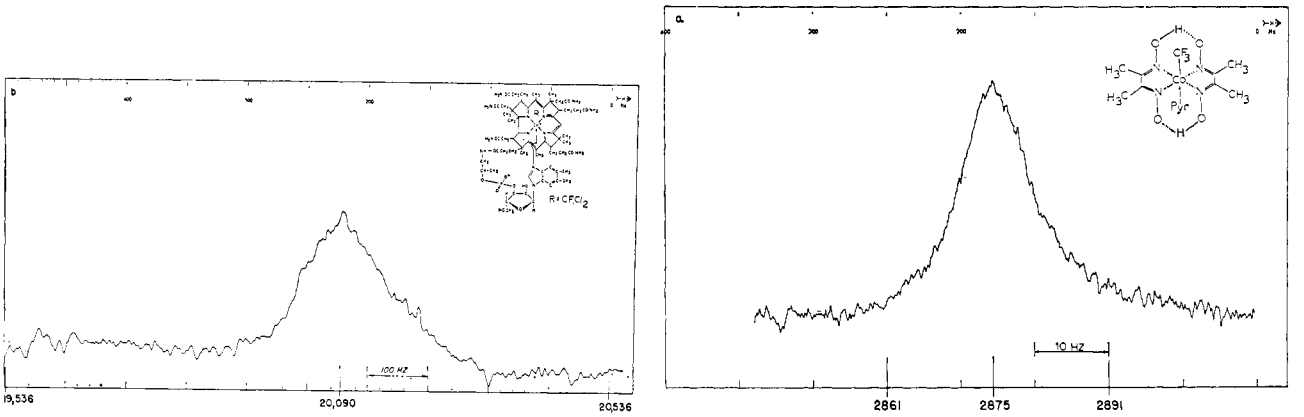


FIGURE 2: ^{19}F nuclear magnetic resonance spectra of trifluoromethylcobaloxime and dichlorofluoromethylcobalamin. The spectra were run on a Varian HA-100 spectrometer operating at 94.1 Hz and are referenced to a $CFCl_3$ standard. Solvents are listed in Table II.

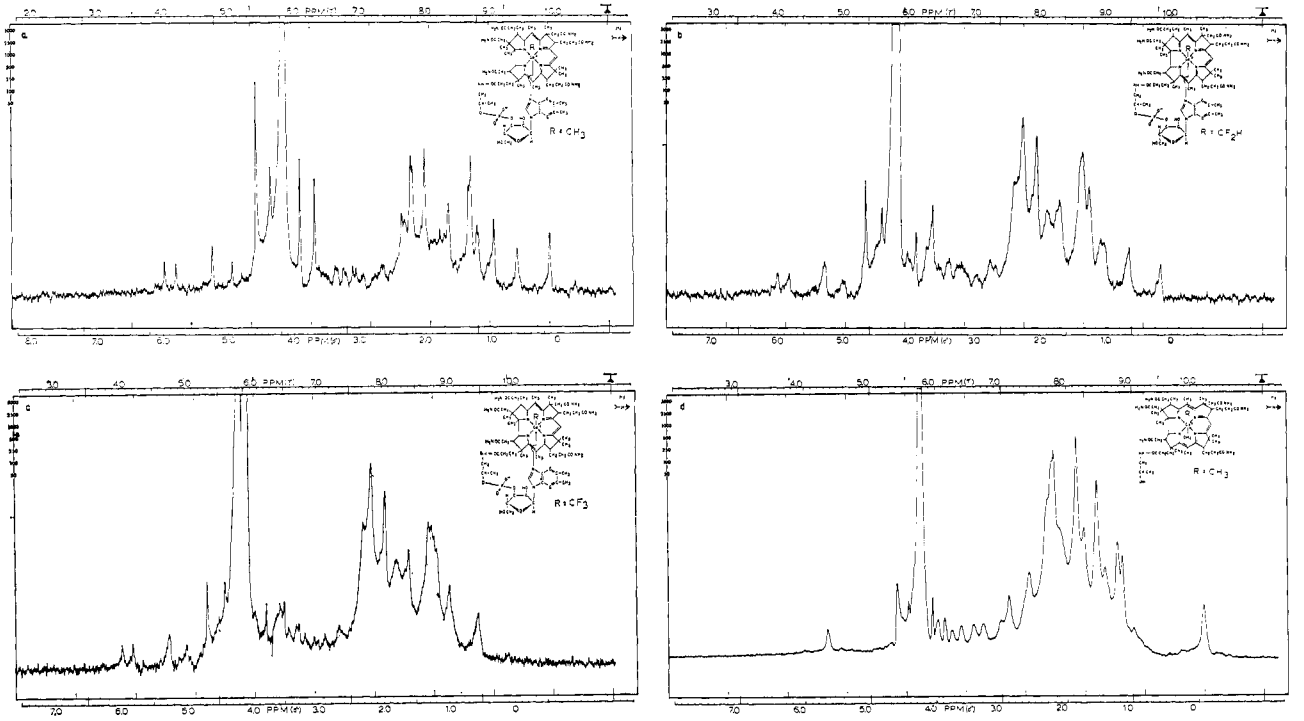


FIGURE 3: ^1H nuclear magnetic resonance spectra of methylcobalamin, difluoromethylcobalamin, trifluoromethylcobalamin, and methylcobinamide run on a Varian HR-220 operating at 220 Hz.

Figure 3 shows the ^1H nuclear magnetic resonance spectra for methylcobalamin, difluoromethylcobalamin, trifluorocobalamin, and methylcobinamide. The peaks at highest field for methyl- and difluoromethylcobalamin correspond to the CH_3 and CF_2H proton resonances, respectively. As would be expected, this peak is absent in the spectrum of trifluoromethylcobalamin. The resonance occurring at about τ 9.5 in the cobalamins is probably due to the C_1-methyl group of the corrin ring (H. A. O. Hill, personal communication, 1970) rather than the 5′-methyl group of the 5,6-dimethylbenzimidazole moiety as previously suggested (Hill *et al.*, 1968). This can be supported by contrasting the cobalamin spectra with that of methylcobinamide in which this resonance is shifted downfield to below τ 9.

Properties of Fluoromethylcobalamins. This interesting series of halomethylcobalamins offered the opportunity of comparing various physical and chemical properties as a function of the alkyl ligand. As a result, we have examined (a) the stability of the cobalt–carbon σ bond to light, (b) the stability of the Bz–cobalt interaction, and (c) the effect of electron withdrawal from cobalt on the position of the γ band.

Photolysis experiments were conducted as outlined in Materials and Methods, and the stability of the Bz–cobalt interaction was determined by obtaining the pK_A value for displacement of Bz spectrophotometrically by the method of Hogenkamp *et al.* (1965). The results from a typical pK_A determination are shown in Figure 4. A linear relationship exists between the stability of the upper and lower axial ligands

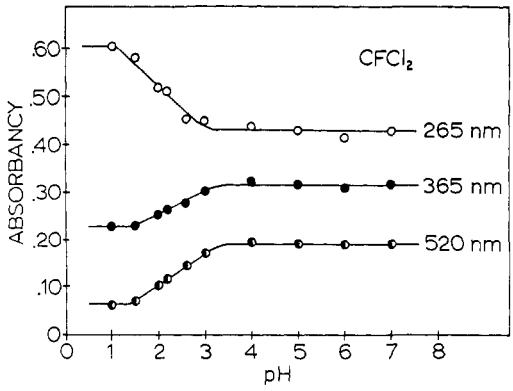


FIGURE 4: Effect of pH on the spectrum of Co-$CFCl_2$ at various wavelengths. Each cuvet contained 1.95×10^{-5} M Co-$CFCl_2$ in 1 M phosphate buffer.

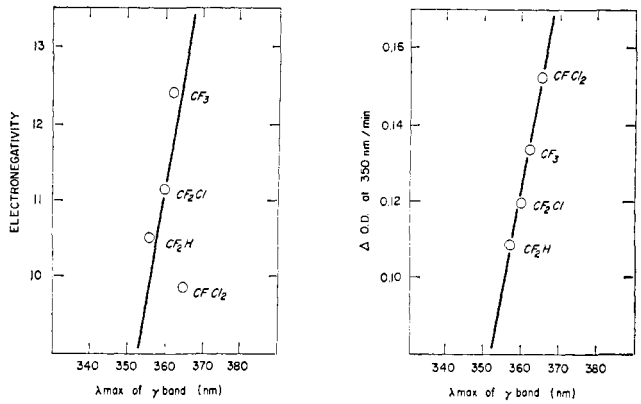


FIGURE 6: Correlation of both the stability of the upper axial ligand to light and the electronegativity of this ligand with the position of the γ band in the ultraviolet–visible spectra.

on cobalt and the electronegativity of each fluoroalkyl ligand. With an increase in the electron-withdrawing ability of the upper ligand, the electrophilic character of the cobalt will be enhanced resulting in a more strongly coordinated Bz ligand. This is reflected in the pK_A values determined for protonation and displacement of Bz (Figure 5).

The photolysis of these fluoromethyl derivatives was shown to be first order, and for these derivatives an increase in the electronegativity of the upper axial ligand promotes cleavage of the Co–C σ bond (Figure 5). The anomalous behavior of the $CFCl_2$ ligand may be attributed to two factors. First, the presence of two chlorine atoms has made the upper axial ligand bulky, introducing strain into the Co–C bond and rendering the derivative much less stable to light. Secondly, there is much evidence that gem dihalides are photolyzed to give carbenes, whereas ligands having one halogen atom (excluding fluorine) produce radicals by homolytic cleavage.

Figure 6 shows that there is a correlation between both the stability of the Co–C σ bond to light and the electronegativity of this ligand with the position of the γ band in the ultraviolet–visible spectra. The establishment of this relationship is particularly helpful for future predictions from spectral data on the electronic state of alkylcobalamins in general.

A summary of the physical and chemical properties of this homologous series of haloalkylcobalamins is presented in Table III. This summary of the various parameters of the homologous series emphasizes the differences in properties of haloalkylcobalamins which contain more than one chlorine atom.

For $CFCl_2$ cobalamin the position of the γ band does not reflect the electronegativity of the ligand, the Bz–Co interaction is weaker than would be predicted, and the Co–C σ bond is less stable to light than any of the other haloalkylcobalamins tested.

Fluoroalkylcobalamins as Substrates and Inhibitors of Methane Formation. When these four haloalkylcobalamins were tested as substrates for enzymatic methane formation in cell extracts of the methanogenic bacterium MOH, CF_2Cl_2-, CF_2Cl-, and CF_3-cobalamins were found to be competitive inhibitors when methylcobalamin was used as substrate (Figure 7). Apparent K_I values of $CFCl_2 = 2.0 \times 10^{-4}$ M, $CF_2Cl = 7.0 \times 10^{-4}$ M, and $CF_3 = 11.0 \times 10^{-4}$ M were obtained, and these data clearly show that the extent of inhibition of this enzyme reaction can be related to the number of chlorine atoms present on the ligand being tested. Apparently, steric factors are involved in the formation of the enzyme–substrate complex when methylcobalamin is substrate.

The most interesting aspect of this study is that CF_2H-cobalamin replaces methylcobalamin as a substrate in the methane system to give methane as the major product. The substrate dependency of methane formation from CF_2H-cobalamin is presented in Figure 8.

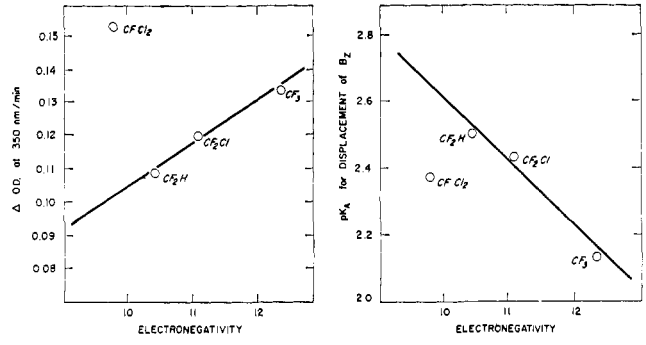


FIGURE 5: Relationship between the stability of the upper and lower axial ligands on cobalt and the electronegativity of each floroalkyl ligand.

TABLE III

Ligand	λ_{max} of γ Band[a] (nm)	Electronegativity[b]	pK_A for Displacement of Bz	Photolysis ΔOD/min at 350 nm
CF_2H	357	10.4	2.50	0.109
CF_2Cl	360	11.03	2.43	0.120
CF_3	362	12.30	2.13	0.134
$CFCl_2$	365	9.76	2.37	0.153

[a] γ band falls between CH_3 = 340 nm and CN = 368 nm. [b] Determined from Cotton and Wilkinson (1962).

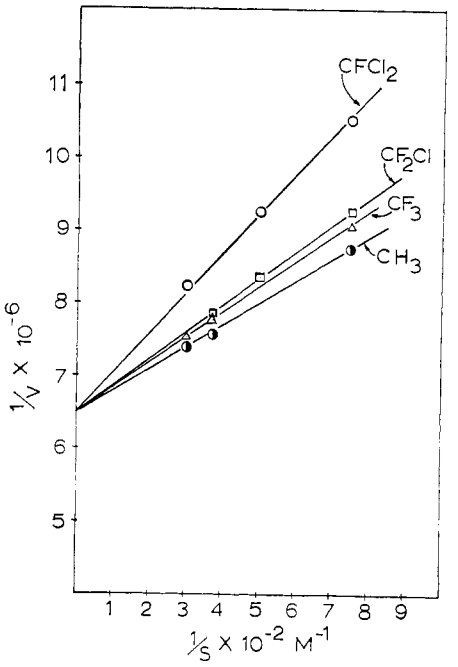


FIGURE 7: Competitive inhibition of methane formation from methylcobalamin by fluoromethylcobalamins. Reaction mixtures contained: extract, 34.5 mg of protein; dichlorofluoromethylcobalamin, difluorochloromethylcobalamin, trifluoromethylcobalamin, difluoromethylcobalamin, 0.25 μmole, respectively; ATP, 10 μmoles; potassium phosphate buffer (pH 7.0), 800 μmoles; and variable levels of methylcobalamin. Total liquid volume 1.50 ml, gas phase H_2, incubated at 40°.

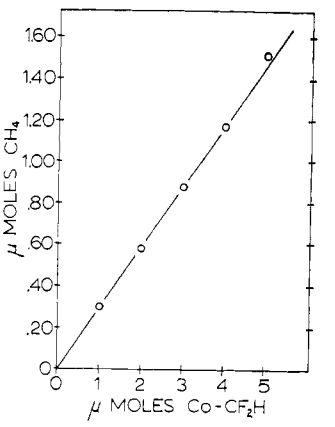


FIGURE 8: Formation of methane with difluoromethylcobalamin as substrate. Reaction mixtures contained: extract, 51.0 mg of protein; ATP, 10 μmoles; potassium phosphate buffer (pH 7.0), 800 μmoles; and variable levels of difluoromethylcobalamin. Total liquid volume 1.50 ml, gas phase H_2, incubated at 40° for 20 min.

In a separate experiment, mass spectrometry was used to examine the composition of the gaseous products formed when CH_3-cobalamin and CF_2H-cobalamin were used as substrates, respectively. In both reactions methane was the major product, but in the CF_2H cobalamin reaction a peak at *m/e* 34 corresponding to CFH_3 was found to be present. This fragment was absent in the methylcobalamin control. There was no indication of a *m/e* 52 peak corresponding to CF_2H_2.

Quantitation of Reaction Products. A standard reaction mixture containing extract, 51.0 mg of protein, 10.0 μmoles of ATP, 760 μmoles of K-PO_4 buffer, and 3.0 μmoles of difluoromethylcobalamin was allowed to incubate in a total volume of 1.5 ml at 40° for 45 min. Control flasks minus enzyme and plus difluoromethylcobalamin, and plus enzyme minus difluoromethylcobalamin yielded 0 and 0.13 μmole of endogenous methane in 45 min. However, the reaction flask yielded 2.14 μmoles of methane in 45 min. Immediately, the reaction mixture flask was placed into an ice–water bath; 0.5 ml of 10% trichloroacetic acid was added per ml of reaction mixture. The precipitated protein was removed with a clinical centrifuge, and the supernatant was phenol extracted to remove all corrinoids. The phenol-extracted material was lyophilized to dryness. The lyophilisate was dissolved in 0.05 N HOAc and applied to a phosphocellulose column (1.5 × 8.5 cm). Two bands were eluted from the column with 0.015 N NaOAc(pH 4.1). These bands were spectrally identified as unreacted Co-CF_2H- and aquocobalamin. These two solutions were diluted to an equal volume (25.0 ml), and the CF_2H-cobalamin was photolyzed 5 min at 750 W and a distance of 60 cm. The absorbancy of both solutions was compared at various wavelengths. The ratio of unreacted to reacted substrate was 38 to 62%. The amount of methane produced in 45 min was 2.01 μmoles showing 2.01/3.00 or 67% of the Co-CF_2H had reacted to form methane. Thus, within experimental error, 100% of the Co–CF_2H reacting is producing methane. The recovery of corrinoids from the reaction mixture was 1.74/3.00 μmoles or 58%.

Discussion

After the initial observation of Bauchop (1967) that Freons are inhibitors of methane formation in the rumen, it occurred to us that fluoromethylcobalamins could be synthesized and that these compounds should provide interesting analogs of methylcobalamin in methyl-transfer enzymes. With the use of ^{19}F nuclear magnetic resonance and 220-MHz nuclear magnetic resonance, we have characterized four different fluoromethylcobalamins. From this nuclear magnetic resonance study, coupled with a study of the stability of the axial ligands of these derivatives, we have obtained new information on the chemistry of alkylcobalamins, and, in addition, we are able to shed some light on the mechanism of the methane enzyme system.

Nuclear Magnetic Resonance. Fluorine chemical shifts are governed to a large extent by the local paramagnetic contributions to the shielding. Since correlations have been made between fluorine chemical shifts and the electronegativity of the group bound to fluorine (Pople *et al.*, 1959), the ^{19}F chemical shifts for the fluorinated corrinoids should tell us something about the electronic environment of the fluoroalkyl group. It is interesting to compare the ^{19}F chemical shifts for the fluoroalkyl B_{12} compounds and those for the parent-substituted methanes. As can be seen from Table IV, CF_3-B_{12} and CF_2H-B_{12} show ^{19}F chemical shifts very similar to CF_3H and CF_2H, respectively. When more fluorines are placed on the carbon, the chemical shift decreases as expected. This can be contrasted with CF_2Cl-B_{12}. Here, the ^{19}F chemical shift for $CFCl_2$-B_{12} occurs at a much higher field than might be expected. There is a reasonable explanation for this anomalous be-

TABLE IV

Compound	Chemical Shift (ppm)
$CFCl_3$	0
CF_2Cl_2	16.3
Trifluoromethylcobaloxime	30.6
CF_3Cl	39.9
CF_4	76.7
Trifluoromethylcobalamin	94.2
Difluorochloromethylcobalamin	94.2
CF_3H	94.9
CF_2H_2	157.6
Difluoromethylcobalamin	212.0
Fluorodichloromethylcobalamin	213.5
CFH_3	286.7

havior. Due to steric effects, the Co–C bond in $CFCl_2$-B_{12} is probably artificially long. If the effect of this lengthening is to make the alkyl ligand electron rich (*i.e.*, favor a resonance structure $B_{12}^+ CFCl_2^-$), the fluorine would be highly shielded and the resonance would occur upfield. Thus, the ^{19}F nuclear magnetic resonance data support the photolysis and pK_A data indicating a large amount of steric strain in the $CFCl_2$-B_{12} compound. Furthermore, the effect of this strain is to build up electron density on the alkyl ligand.

Finally, a comparison should be made between trifluoromethylcobaloxime and trifluoromethylcobalamin. If the bis(dimethylglyoximato)Co(III) species and the Co(III) corrinoid system had approximately the same electrophilicity, the ^{19}F chemical shifts should be about equal. The downfield resonance position for the dimethylglyoxime $(dmg)_2$ species compared to the cobalamin indicates that the bis(dimethylglyoximato)Co(III) group withdraws electrons to a much greater extent than the Co(III) corrinoid species. Thus, it may be questionable whether cobaloximes are good B_{12} model compounds with which to study reactions involving cleavage of the cobalt–carbon bond.

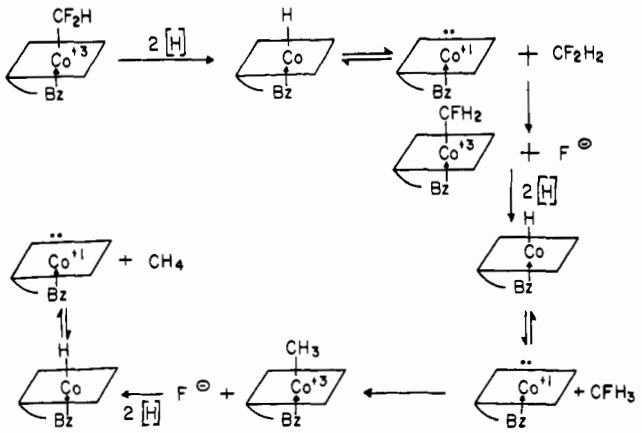
The proton nuclear magnetic resonance spectra confirm the nature of the alkyl ligands in the fluoroalkyl derivatives. The high-field resonance at about τ 10 occurs in methylcobalamin and difluoromethylcobalamin. In addition, the relative intensities of the high-field peaks of the CH_3 and CHF_2 compounds is 3:1. This peak is missing in the trifluoromethyl derivative. Thus, during the synthesis, the trifluoromethyl group remains intact. There is evidence in the synthesis of other trihaloalkylcobalamins and -cobaloximes that this is not the case (T. L. Brown and R. Stewart, personal communication, 1970). A further interesting observation from these spectra is that the resonance position of the C_1-methyl group on the corrin ring is not affected by changing the electron-withdrawing ability of the alkyl ligand. The resonance position is approximately τ 9.5 because of ring current effects of the benzimidazole group. It might be expected that the benzimidazole is bound more closely to the cobalt as the electronegativity of the alkyl group increases. If this effect caused significant geometrical changes, the resonance position for the C_1-methyl should change since ring current effects are very sensitive to the distance from the ring to the proton affected. Thus, the fact that the C_1-methyl resonance position remains constant indicates that the alkyl group does not significantly affect the Co–benzimidazole bond length. In all likelihood, the benzimidazole approaches the cobalt as closely as possible in all cases, and the distance of approach is determined by steric factors.

Photolysis. A comparison of the first-order photolysis rates of the fluoromethylcobalamins with the electronegativity of each upper axial ligand (Figure 6) reveals a lineal correlation which gives further substantiation to the suggestion by Smith *et al.* (1964) that the relative ease of photolysis depends on the characters of the groups attached to the cobalt atom. Although the primary reaction in the photodecomposition of alkylcobalamins involves the homolytic cleavage of the carbon–cobalt σ bond with the formation of vitamin $B_{12\text{-}r}$ and a free radical (Pratt, 1964; Hogenkamp *et al.*, 1963); a very fast secondary reaction is the oxidation of vitamin $B_{12\text{-}r}$ to aquocobalamin (Hogenkamp, 1966). In the presence of excess oxygen, the rate-determining step should be the rate of the initial cleavage of the carbon–cobalt σ bond rather than the very rapid oxidation of vitamin $B_{12\text{-}r}$ with either hydroxyl or alkyl peroxide radicals (Pratt, 1964). Consequently, one can evaluate the relative rates of photolysis of each of the haloalkylcobalamins by measuring the rate of formation of aquocobalamin (ΔOD at 350 nm) from each derivative.

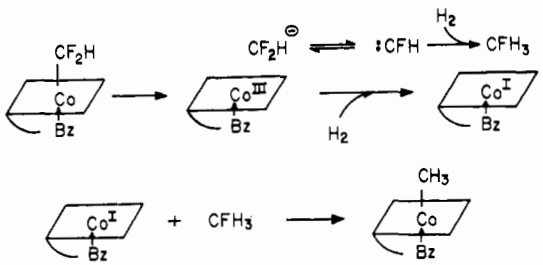
Decomposition by Alkaline Cyanide. Exposure of the haloalkylcobalamins to alkaline cyanide (1 M NaOH and 1 M KCN) caused the immediate displacement of the two most electronegative fluoromethyl ligands (CF_2Cl and CF_3), whereas there was no apparent displacement of the two least electronegative ligands ($CFCl_2$ and CF_2H) within a 1-hr time period. This is consistent with the suggestion of Johnson and Shaw (1962) that the cyanide decomposition of alkylcobalamins involves a nucleophilic displacement of the upper axial ligand by cyanide. With this mechanism an increase in the electrophilic character of the alkyl ligand should facilitate its removal; presumably because of the resulting increase in the polarization of the carbon–cobalt σ bond toward the carbon atom (Hogenkamp *et al.*, 1965). In the case of the two least electronegative ligands ($CFCl_2$ and CF_2H) the cyanide decomposition proceeds very slowly or not at all, perhaps because these alkylcarbanions are poor leaving groups (Bonnett, 1963).

Methane Formation. Of the alkylcorrinoids tested as substrates for methane formation: methylcobalamin (Blaylock and Stadtman, 1964; Wolin *et al.*, 1963), methylcobinamide, and methyl factor III (Wood *et al.*, 1966) were shown to be active. Ethylcobalamin and propylcobalamin (Wolfe *et al.*, 1966; Wood and Wolfe, 1966) and a number of haloalkylcobalamins (Wood *et al.*, 1968) were shown to be inhibitors. Recent studies by McBride *et al.* (1968) and Sibert and Schrauzer (1970) demonstrated that a variety of methycobaloximes would function as methyl donors in the methane system. However, in the case of methane formation from methylcobaloximes, this reaction would only proceed in the presence of catalytic amounts of *Cob(II)alamin*. When [^{14}C]-methylcobaloxime was used as substrate, then [^{14}C]methylcobalamin was isolated from reaction mixtures (McBride *et al.*, 1968). This experiment clearly demonstrates that [^{14}C]-methyl is transferred from [^{14}C]methylcobaloxime to reduced cobalamin under the reaction conditions employed. Differences in rates of methane formation from methylcobaloximes containing different axial bases could be attributed to the lack

SCHEME III



SCHEME IV



of solubility of some of these derivatives. Based on the conclusions which can be drawn from a comparative study of cobaloximes and cobalamins by ^{19}F nuclear magnetic resonance, it is clear that alkylation of Cob(I)alamin by alkylcobaloximes does not lead to any mechanistic difficulties as was suggested by Sibert and Schrauzer (1970). Furthermore, there is no kinetic evidence to support the conjecture that cobaloximes and cobalamins compete for the same binding site in the methane system (Sibert and Schrauzer, 1970).

One of the most interesting observations of this research is that methane is the principal product formed when difluoromethylcobalamin is used as a substrate. Furthermore, the methane appears to be derived from the carbon atom bound to the cobalt. This phenomenon offers some quite interesting mechanistic possibilities. One possible mechanism for methane formation from difluoromethylcobalamin is shown in Scheme III. In this scheme the carbon–cobalt is cleaved reductively by a hydrogenase yielding CF_2H_2 and $B_{12\text{-}s}$. The difluoromethane would function as an alkylating group giving fluoromethylcobalamin. If this group were then cleaved reductively to $B_{12\text{-}s}$ and fluoromethane, another alkylation would result in methylcobalamin which could act as the methyl donor. A difficulty with such a scheme is that two alkylations are required. This might be expected to be too slow for the rate of methane formation observed; however, if the fluoromethyl groups were enzyme bound after the reductive cleavage, realkylation might be a facile process.

Another mechanistic possibility is presented in Scheme IV. This involves a heterolytic cleavage of the Co–C bond leaving CF_2H^- and $B_{12\text{-}a}$. This carbanion might be expected to decompose to :CFH which would react with H_2 to form CFH_3. Under the conditions present, $B_{12\text{-}a}$ would be reduced to $B_{12\text{-}s}$, which could be realkylated by CFH_3 to give methylcobalamin, which in turn could act as a substrate to evolve methane. The reaction between the carbene and H_2 could proceed simultaneously with the alkylation step. The fact that CH_3F but not CF_2H_2 is detected in the mass spectrum of the atmosphere over the reaction mixture gives some support to such a scheme.

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Equine Antihapten Antibody. Studies on the Primary Structure and Conformation of Equine Immunoglobulins*

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ABSTRACT: Equine γGab, γGc, and γT anti-Lac (*p*-azophenyl β-lactoside) antibodies have been purified from the serum of a hyperimmunized horse. γGab- and γT-globulins have been isolated from equine diphtheria antitoxin, γGab-globulins from normal equine serum, and a γT-globulin paraprotein from the serum of a horse with a malignant lymphoma. γGab-, γGc-, and γT-globulin heavy and light chains have been prepared and their amino acid composition determined. The light-chain compositions were closely similar. Small differences were observed between the γGab, γGc, and γT heavy-chain data. Papain 3.5S Fab and Fc fragments and pepsin 5S $F(ab')_2$ fragments have been prepared from the equine immunoglobulins. Papain 5S $F(ab)_2$ fragments also have been obtained from equine γGab- and γT-globulins by hydrolysis with 2-mercaptoethanol-activated papain in the absence of free reducing agent. Equilibrium dialysis with *p*-(*p*-dimethylaminobenzeneazo)phenyl β-lactoside hapten established that the anti-Lac Fab and $F(ab')_2$ fragments retained in full the affinity for hapten of the parent γGab, γGc, and γT antibodies. Two-dimensional peptide maps have been prepared from tryptic hydrolysates of extensively reduced and alkylated equine γGab and γT heavy and light chains, and γGab and γT Fab and Fc fragments. The γGab and γT light-chain maps displayed a high degree of homology. Numerous peptide differences were evident between the γGab and γT heavy-chain maps. γGab and γT Fc fragment maps contained both common and distinct peptides. Common and distinct γGab and γT Fd fragment (Fab fragment less light chain) tryptic peptides also were identified. The amino-terminal residues of heavy chains from equine γGab- and γT-globulins have been studied. The heavy chains lacked free α-amino groups. After mild alkaline hydrolysis, glutamic acid was identified as the terminal amino acid of both γGab- and γT-globulin heavy chains by reaction with dimethylaminonaphthalenesulfonyl chloride, tentatively identifying pyrrolidonecarboxylic acid (PCA) as the unreactive terminal residue. Amino-terminal peptides lacking a free amino group have been isolated from subtilisin, α-chymotrypsin and pronase digests of γGab- and γT-globulin heavy chains. The amino acid sequences of the heavy-chain amino-terminal subtilisin tetrapeptides were equine γGab heavy chains, PCA-Val-Gln-Leu; and equine γT heavy chains, PCA-Val-Gln-Leu. No class-specific amino-terminal sequences were apparent. Optical rotatory dispersion curves of equine γGab-, γGc-, and γT-globulins have been examined. The optical rotatory dispersion spectra showed Cotton effect minima near 225 and 230 nm. The depth of the minima varied for different immunoglobulin classes, and between distinct immunoglobulins of a single class. A small Cotton effect centered near 240 nm, characteristic of the γG-globulin spectra, including equine γGab and γGc spectra, was absent from the γT anti-Lac antibody, γT diphtheria antitoxin, and γT paraprotein spectra. γGab and γT $F(ab')_2$ fragment optical rotatory dispersion spectra lacked the Cotton effect minimum near 230 nm and showed only a single minimum near 225 nm. The principal difference between the γGab and γT $F(ab')_2$ spectra was the presence of a Cotton effect near 240 nm in the γGab $F(ab')_2$ spectrum and the absence of the 240-nm effect from the γT $F(ab')_2$ spectrum. The experimental results are briefly discussed in terms of an expanded two-gene translocation hypothesis for generating immunoglobulin heavy-chain sequence variations and antibody specificity.

The immunoglobulins[1] of a given species are a heterogeneous population of related, but not identical, proteins, and in addition to diversities in antibody specificity, may be subdivided into distinct classes, subclasses, and allotypes on the basis of the antigenic and chemical structure of their heavy chains (Dubiski *et al.*, 1961; Natvig *et al.*, 1967; Rockey,

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