A SPECTROSCOPIC ANALYSIS OF VITAMIN B12 DERIVATIVES

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Summary

1. Several vitamin B12 derivatives including descobalt B12 show unusual inversion of their CD signs upon rapid cooling to liquid N2 temperature, although the room temperature CD signs are conserved by a slower cooling to the same temperature. As a possible explanation for this puzzling observation, a micro-environmental birefringence around the chromophore imbedded in an organic glass is proposed.

2. Absorption, fluorescence, phosphorescence, and polarization spectra of descobalt B12 can be correlated with those of porphyrin free bases, as these two molecular systems share many similarities in their electronic structure.

3. Molecular orbital calculations of polarization directions further support the analogy between the spectroscopic characteristics of corrins and porphyrins, and are generally in good agreement with the fluorescence polarization data.

Introduction

There are several papers in the literature concerning circular dichroism spectra of a number of vitamin B12 derivatives [1–3], including a report of an inversion of the circular dichroism spectra of cyanocobalamins at temperatures from 173°K to 77°K [2]. Bonnett et al. [3] report that they do not observe an inversion of CD at low temperature. This phenomenon at low temperature is not unique to corrin ring systems, but has been reported for other compounds as well [4,5]. No definitive explanation for the CD inversion has been offered in the literature to date.

Aside from the absorption spectra for a number of vitamin B12 derivatives [6–8] relatively little work has been done on the electronic excited states of vitamin B12 and its derivatives, due to the fact that they exhibit no luminescence, with the exception of emission at 303 nm when excited at 278 nm [9], this is attributed to the benzimidazole moiety [10].
Eckert and Kuhn [11] have reported linear dichroism of vitamin B12 in polyvinyl alcohol film in which a very small dichroism can be observed; positive dichroism was assigned to the longest wavelength and to the region from 300–200 nm and negative dichroism between these two regions. The first and only work on the fluorescence spectroscopy of metal-free vitamin B12 corrin [12] was reported by Thomson [13].

In this report, we further examine the cause of the sign inversion in CD of vitamin B12 derivatives upon lowering the temperature. Particular attention is paid to the relationship between the CD sign inversion and rate of cooling. In addition, we examine the high resolution fluorescence and polarization spectra of descobalt B12 in order to ascertain the chromophore structure at low temperature. Phosphorescence and phosphorescence excitation polarization of the compound will also be described.

Materials and Methods

Materials

Vitamin B12 (cyanocobalamin) and coenzyme B12 were purchased from Sigma Chemical Company and used without further purification. Descobalt B12 (a corrin) was generously supplied by Dr John I. Toohey of the University of California, Berkeley.

Descobalt B12 was shown by electrophoresis in 0.5 M acetic acid (run at 30 V • cm\(^{-1}\)) to contain a small amount of fluorescent impurity, presumably another form of descobalt B12. Spectra were run on the unpurified sample, since it was difficult to elute the band from the electrophoretogram. Dicyanocobalamin was prepared by adding cyanobalamin to 0.001 M KCN in ethanol. See Fig. 1 and Table I for structures of these compounds. Absolute ethanol and methanol were spectro grade and were further purified by fractional distillation immediately before use. Compounds were handled under reduced light in all
TABLE I
SUBSTITUENTS OF CORRIN RING
see Fig. 1 for numbering.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanocobalamin</td>
<td>-CN</td>
<td>N³ of 1</td>
<td>N¹ of 1</td>
</tr>
<tr>
<td>Dicyanocobalamin</td>
<td>-CN</td>
<td>-CN</td>
<td>N¹ of 1</td>
</tr>
<tr>
<td>Coenzyme B₁₂</td>
<td>C⁵ of 2</td>
<td>N³ of 1</td>
<td>N¹ of 1</td>
</tr>
<tr>
<td>Descobalt B₁₂</td>
<td>No cobalt</td>
<td></td>
<td>-OH</td>
</tr>
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</table>

cases and descobalt B₁₂ was handled in darkness. All solutions for low temperature spectra were degassed prior to use by the freeze-pump-thaw cycles, using a specially designed cell.

Methods
Absorption spectra were carried out on a Cary 118 C spectrophotometer. For low temperature spectra, special cell holders and optical dewars with 6 mm cylindrical quartz cells were utilized after a careful alignment.

Circular dichroism measurements were carried out on a modified JASCO J-20 ORD-CD spectropolarimeter, equipped with a photoelastic modulator (PEM-1, Morvue Electronic Systems, Portland, Oregon). For low temperature CD measurements, a liquid N₂ dewar with quartz windows was manually aligned in the optical path of the instrument. A 1 cm cuvette was used, and only organic glasses with no cracks were used for measurements. All samples were degassed by several freeze-pump-thaw cycles. Also a cryostat (Model 20/70 cryodyne cooler, Cryogenic Technology Inc., Waltham, Mass.) was used for slower cooling from 298° to 14°K.

All samples were run in ethanol with the exception of coenzyme B₁₂, which was run in methanol : ethanol (1 : 1), because of extremely low solubility in ethanol. Additionally, some samples were run in ethanol + 10% isopentane with no visible effect on the spectra. Baselines were run in the cell used with solvent only at 298°K and 77°K and no dichroic signal was observed.

Fluorescence and phosphorescence (technical) at 77°K were measured on a modified Aminco-Bowman spectrofluorometer, with a phosphoroscope attachment for the latter [14]. The phosphorescence lifetime was measured with a shutter-trigger device and a Tektronics oscilloscope described elsewhere [15]. Fluorescence and phosphorescence polarization was measured with Glan-Thompson polarizers, and was corrected by the method of Azumi and McGlynn [16].

Results
Table II presents the CD parameters for the spectra of several B₁₂ derivatives at room temperature. Rotational strength for the kth band with wavelength maximum at λₖ was approximately calculated by

\[ Rₖ = 0.406 \cdot 10^{-38} \Delta \varepsilonₖ \lambdaₖ \]
TABLE II
CD SPECTRAL DATA

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cyanocobalamin</th>
<th>Dicyanocobalamin</th>
<th>Coenzyme B12</th>
<th>Descobalt B12</th>
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<tr>
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<td>556 $^c$</td>
<td>588 $^c$</td>
<td>559</td>
<td>533</td>
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<td>3.20 $\times 10^{-40}$</td>
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<td>$-2.83 \times 10^{-39}$</td>
<td>$7.70 \times 10^{-40}$</td>
</tr>
</tbody>
</table>

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*a* Rotational strength for the $k$th band was calculated by the formula

$$R_k = 0.406 \times 10^{-38} \cdot \Delta \epsilon_k \cdot \Delta \epsilon_k / \lambda_k$$

*b* Assumed molecular weight of descobalt corrin and extinction coefficient taken from Toohey [12].

$c$ Rotational strength for this band was calculated including the area down to $\approx 470$ nm.

where usual CD notations are used; $\Delta \epsilon$ is the half-bandwidth at [$\theta$] / $e$.

The most striking observation from the CD spectra is the complete inversion of the ellipticity of each band at 77°K; this anomaly is subject to the rate of cooling. Fig. 2 shows a clear inversion of the CD spectrum of cyanocobalamin at 77°K, which was achieved by rapid cooling with liquid $N_2$. Dicyanocobalamin (Fig. 3), deoxyadenosyl cobalamin (coenzyme B12, Fig. 4) and descobalt B12 (Fig. 5) all show similar CD inversions at liquid $N_2$ temperature. The low temperature CD spectra, particularly Figs. 3 and 5, show some vibrational resolution. This is expected from the enhanced resolution seen in the corresponding absorption spectrum (Figs. 6 and 7).

In contrast to the CD inversion observed by rapid cooling with liquid $N_2$, a gradual cooling from 298°K to 14°K (including several intermediate temperatures, at an average rate of decrease in temperature of approx. 4°/min) produced no CD inversion for all compounds examined, the only apparent effect of the cooling being the enhanced vibrational resolution. Precooling to 161°K with a bromobutane/liquid $N_2$ slush and subsequent immersion of the optical
Fig. 2. CD spectra of cyanocobalamin in ethanol at 298°K (—) and 77°K (-----, liquid N₂).

Fig. 3. CD spectra of dicyanocobalamin in ethanol (0.001 M KCN) at 298°K (—) and 77°K (-----).

Fig. 4. CD spectra of coenzyme B12 in methanol/ethanol (1:1, v/v) at 298°K (—) and 77°K (-----).
cell in a liquid N\textsubscript{2} dewar also produced no CD inversion. Thus, we were repeatedly able to produce CD inversion or non-inversion by merely changing the mode of cooling, i.e., different rate of cooling. It is clear that more or less instantaneous cooling by immersing the optical cell in liquid N\textsubscript{2} produces the CD inversion, while slower cooling does not.

Fig. 6 shows fluorescence, phosphorescence and their polarization spectra of descobalt B12. The variation of the fluorescence excitation polarization is consistent with the CD spectrum (Fig. 5), thus confirming that the CD signs are determined by the directions of the electric dipole transition moments [13]. On the other hand, the phosphorescence excitation polarization remains within

![Graph showing CD spectra of descobalt B12 in ethanol at 298°K (-----) and 77°K (-----).](image)

![Graph showing absorbance, fluorescence and phosphorescence spectra of descobalt B12 (5.2 \cdot 10^{-5} M) in ethanol at 77°K. •: fluorescence excitation polarization, (\lambda_p \approx 609 \text{ nm}); ○, phosphorescence excitation polarization (with respect to \lambda_p \approx 638 \text{ nm}).](image)
0-0.1 range over the entire absorption spectrum. The phosphorescence excitation spectrum (not shown) is identical with the absorption and fluorescence excitation spectra after correction. The phosphorescence lifetime was found to be $\approx 7 \cdot 10^{-2}$ s. The enhancement of the phosphorescence in KI-saturated ethanol was not significant.

Fig. 7. Absorbance spectrum of descobalt B12 (5.2 $\cdot$ 10$^{-5}$ M) in ethanol at 298°K.

Fig. 8. A high resolution fluorescence emission spectrum of descobalt B12 at 77°K (liquid N$_2$) excited at 525 nm. The emission bandpass used was 0.22 nm.
In order to ascertain whether the CD inversion as a function of rate of cooling can be correlated with the fluorescence of the chromophore, a high resolution, single-photon counting spectrometer was used to record the fluorescence spectrum of descobalt B12 in liquid N₂ (rapid cooling, Fig. 8) and in the crycooler (slow cooling). No significant difference (within 30 cm⁻¹) in the high resolution spectra was found. The peak height ratio of the two vibronic bands of the fluorescence, \( I_{542.5 \text{nm}}^F / I_{542.5 \text{nm}}^F \), (c.f. Fig. 8) was found to be dependent upon the rate of cooling, the ratio being higher with the slow cooling method. However, this ratio was more strongly dependent on the excitation wavelength. Thus, the peak ratio increased from 0.48 to 0.72 in going from \( \lambda_{ex} \approx 500 \text{ nm} \) to 450 nm. Since the electrophoretic patterns indicates the presence of a trace amount of impurity (not enough to offset the peak ratio) and the peak ratio is apparently dependent on temperature (i.e., effect of slow cooling), tautomeric forms of the descobalt B12 exist in the sample solution. However, it is doubtful that such a tautomeric equilibrium is responsible for the CD inversion, since the CD inversion is observable only with the rapid cooling.

Fig. 9 shows polarization directions of five major bands calculated by the SCF MO P-P-P method. The first transition, designated as \( Q_y \) in analogy to the porphyrin notation, is polarized along y-axis, while the second band, \( Q_x \), is polarized along x-axis. The third and fourth transitions, \( B_x \) and \( B_y \), are also polarized along the expected polarization axes. These data are consistent with the fluorescence polarization data shown in Fig. 6. The fifth transition is predicted to be polarized along an axis between x and y axes. This prediction is consistent with the increasing degree of polarization in the region of 250–270 nm (Fig. 6).

Discussion

First, we will discuss the CD inversion data. We have noted in the previous section that the CD inversion in corrinoid complexes is observable only upon rapid cooling to low temperature (77°K). Slow cooling does not induce the CD inversion. Consequently, it is not likely that the inversion is a result of the
temperature lowering on a conformational equilibrium, since the slow cooling does not induce the CD inversion. We offer a tentative explanation of the CD inversion in terms of a microenvironmental birefringence around the corrin chromophore and/or side chain. A rapid cooling of an ethanolic solution could conceivably be accompanied by the formation of strain in the organic glass in which the solute is imbedded. To test this possibility, we have selected several carotenoids which absorb in the same wavelength region as B12 derivatives but without the chirality center within the molecule*. The major absorption bands at 450–550 nm indeed showed apparent ellipticity upon rapid cooling, while such CD signals were absent in the case of slow cooling. We have eliminated all possible optical artifacts arising from the instrumental and optical bias, except for the frozen glass itself. Consequently, it is suggested that both the induced CD of carotenoids without chirality and the CD inversion of corrinoids originate from microscopic birefringence around the chromophore imbedded in the organic glass (ethanol and EPA)**.

The possibility of using different solvents to study induced conformational changes is very difficult because the solvent must meet not only a solubility criterion, but also must be glass-forming at liquid N\textsubscript{2} temperature. We report that the addition of 10% (v/v) isopentane does not affect the spectra either at 298 or 77°K. Additionally the dicyano- and cobalt free-corrin ring systems reported as base-off examples while cyanocobalamin and coenzyme B12 are base-on examples, all showing the same trend. Thus, conformational equilibria such as base-on/base-off effects are not responsible for the observed CD inversion.

The polarization directions of major electronic bands of descobalt B12 calculated by the MO method are consistent with the fluorescence polarization data (Figs. 6 and 9). The polarization characteristics, along with the relative intensity of the visible and near UV (Soret) bands shown in Fig. 10, enable us to assign these bands in terms of the spectroscopic notations for porphyrin spectra. Although the calculated oscillator strengths are invariably greater than the experimental values (Fig. 10), as is usually produced by the P-P-P type MO calculations, the ratio of the oscillator strengths predicted is $Q_y (14.0) : Q_x (1.0) : B_{x, y} (95.4)$, which is in excellent agreement with the experimental ratio of 14.2 : 1.0 : 90.6. A similar theoretical ratio has been obtained by the P-P-P method using different approximations and parameters [17]. Furthermore, from correlation between fluorescence polarization and CD spectra (Figs. 5 and 6) and from the predicted polarization diagram (Fig. 9), we have confirmed the proposal that the CD signs of descobalt B12 are determined by polarizations of

* The amount of dichroism for carotenoids without the intrinsic chiral center that can be induced in rigid glass is considerable. For carotenoids of 9 conjugated C=C bonds with molar extinction coefficient of 10\textsuperscript{5}, we observe $\Delta c \approx 80$ (20-benzal-x, x-carotene) and $\approx 100$ (8,8'-diapocarotene-8,8', 20 trial) subject to geometric alignment of the Dewar which affects the signal intensity slightly. Temperature dependent CD measurements indicate no optical activity at any temperature on slow cooling, consistent with our B12 data.

** We ran another experiment with the optical dewar, cell and solvent placed between the photoelastic modulator and the room temperature B12 solution, both at 298 and 77°K. $\Delta c$ decreased by 15%. Thus, the birefringence induced in the glass is microscopic in nature as a result of the imbedded chromophore.
Fig. 10. Comparison between theoretical and experimental absorbance spectra (energy and oscillator strength) for descobalt-B12.

the electronic transition moments [13]. A theoretical analysis also supports an essentially similar correlation between the electronic transition moments and CD signs [18].

Effects of the metal ion (Co) on the corrin spectrum have also been studied using an analogy between the orbital models for porphyrins and corrins [19]. However, correlation between the electronic structure and observed CD spectra was not described.

Phosphorescence of corrins has not been reported previously. The phosphorescence polarization (Fig. 6) indicates that the emission is neither exclusively in-plane nor out-of-plane polarized. This mixed polarization characteristic is somewhat similar to porphyrins which show nearly $\lambda_c x$-independent polarization degrees [20,21] and phosphorescence is in-plane polarized [20–22]. Recently, phosphorescence of porphyrin free bases has been unambiguously established using time resolved excitation spectroscopy [23]. The descobalt B12 phosphorescence appears to be more intense and longer lived than the free base porphyrin phosphorescence. The singlet-triplet split is 3088 cm$^{-1}$ which is of the same order as for porphyrin free bases. Thus, the singlet and triplet excited states of descobalt B12 possess similarities in their electronic structure to those of porphyrin free bases. We tentatively assign the phosphorescent state of descobalt B12 to $^3Q_y$ in analogy to chlorin, monohydrogenated porphyrin.

Acknowledgements

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References