mens (Subject 1) were obtained prior to drug administration and at 0.5, 1, 2, 4, 6, 8, 12, 24, and 48 hr postadministration. Urine specimens (Subjects 2 and 3) were collected prior to drug administration from −24 to 0 hr and at the following intervals: 0–2, 2–4, 6–8, 8–12, 12–24, and 24–48 hr after drug administration.

The plasma concentrations for Subject 1 were determined for both free and total I (Table I). The free plasma levels of I are plotted in Fig. 5. The levels of conjugated I (total I − free I) measured were higher than or equal to the corresponding free I levels at each specimen time in this subject.

The urine specimens were pooled for Subject 2 and analyzed for free and total I (Table II). The rate of excretion of total I is plotted in Fig. 6 for this subject. The urine specimens of Subject 3 were analyzed only for total I, because the recovery of the dose as total I in the urines of Subjects 2 and 3 during the 48-hr interval were similar, 43.7 and 48.6%, respectively. The rate of excretion of total I for Subject 3 is plotted in Fig. 7. The levels of free I were only 5% of the dose in Subject 2, and 39% was recovered as conjugated I. The data obtained from these studies indicate that the flame-ionization GLC assay can be successfully used in the pharmacokinetic evaluation of I in humans based on both plasma level and urinary excretion data.

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# *NOTES*

# Determination of Cyanocobalamin by Thermal Decomposition of the Cyano Group Using an Ion-Selective Electrode


**SANDU GOLDSTEIN [x] and ALEXANDRU DUCA**



**Abstract** □ A method for the simultaneous determination of cyanocobalamin, cobinamide (Factor B), and hydroxocobalamin in the solid state is described. The method is based on heating at 120° for cobinamide and at 140–145° for cyanocobalamin (15–20 min). The cyano content in the sample is distilled as hydrocyanic acid, trapped in 0.1 *M* potassium nitrate at pH 12–13, and determined by means of the cyanide ion-selective electrode. The error of this method, statistically established, does not exceed ±3%.



**Keyphrases** □ Cyanocobalamin—analysis by thermal decomposition of the cyano group, solid pharmaceutical preparations □ Cobinamide—analysis by thermal decomposition of the cyano group, solid pharmaceutical preparations □ Hydroxocobalamin—analysis by thermal decomposition of the cyano group, solid pharmaceutical preparations □ Thermal decomposition—simultaneous analysis of cyanocobalamin, cobinamide, and hydroxocobalamin, solid pharmaceutical preparations □ Vitamins—cyanocobalamin, cobinamide, and hydroxocobalamin, simultaneous analysis by thermal decomposition of the cyano group, solid pharmaceutical preparations


For some time, this laboratory has been investigating methods for extracting and purifying vitamin $B_{12}$ group substances (1–6). In recent years, these studies have been extended to the coordination chemistry of the cobalt corrinoids, the analytical chemistry of cyanocobalamin, and the thermal stability of cyanocobalamin and some of its analogs (7–11). The methods of determining vitamin $B_{12}$ group substances have been reviewed (12–14), but no procedure was found that gives satisfactory results for the cyano group determination of cyanocobalamin in the solid state.

Therefore, it was decided to investigate the decomposition of cyanocobalamin and some analogs [cobi-

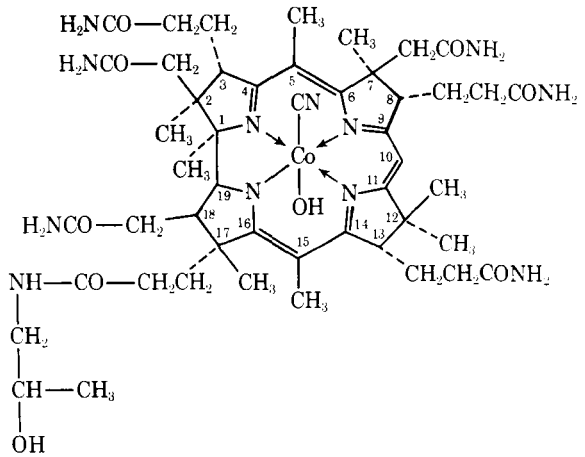

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Table I—Determination of Cyanocobalamin in Presence of Cobinamide and Hydroxocobalamin

| Amount Taken, mg | | | Amount Found | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Cyanocobalamin | | Cobinamide | | Hydroxocobalamin | |
| Cyano-cobalamin | Cobinamide | Hydroxo-cobalamin | mg | % | mg | % | mg | % |
| 1.35 | — | — | 1.31 | 97 | — | — | — | — |
| 2.70 | — | — | 2.75 | 102 | — | — | — | — |
| 6.00 | — | — | 5.82 | 92 | — | — | — | — |
| 8.50 | — | — | 8.30 | 98 | — | — | — | — |
| 10.80 | — | — | 11.20 | 103 | — | — | — | — |
| — | 1.00 | — | — | — | 0.96 | 96 | — | — |
| — | 3.90 | — | — | — | 3.80 | 98 | — | — |
| — | 6.50 | — | — | — | 6.71 | 102 | — | — |
| 6.00 | 6.20 | — | 5.79 | 95 | 6.00 | 97 | — | — |
| 7.92 | 4.10 | — | 8.08 | 102 | 3.90 | 95 | — | — |
| 6.00 | 6.20 | 2.40 | 5.76 | 96 | 6.30 | 101 | 2.31 | 95 |
| 6.00 | 6.20 | 4.10 | 6.06 | 101 | 5.83 | 95 | 4.07 | 98 |

namide (I) and hydroxocobalamin] in a flow of nitrogen at an adequate temperature. This idea was developed into an analytical procedure for the quantitative determination of cyanide in cyanocobalamin, cobinamide, and hydroxocobalamin. By heating at 120° (for cobinamide) or at 140–145° (for cyanocobalamin) in sodium hydroxide solution (pH 12–13) while bubbling nitrogen through the mixture, the cyano group can be converted into cyanide and quantitatively determined by a cyanide ion-selective electrode.

The proposed method has the advantage of permitting a rapid determination of the cyano group in a cyanocobalamin molecule. Moreover, it is selective and accurate. This method is the first that demonstrates the possible direct quantitation of cyanocobalamin together with cobinamide and hydroxocobalamin in the solid state.

## EXPERIMENTAL

**Apparatus**—The apparatus consisted of an electric oven with an iron–constantan thermocouple standardized with a mercury thermometer, a purified nitrogen source, and glass equipment. The glass equipment included a quartz test tube (containing a vial where the sample was introduced) and common test tube (receiver) to receive the hydrocyanic acid from the quartz test tube where it is transformed to cyanide. The two test tubes are connected by a completely leakproof glass tube, and the quartz test tube is connected to the nitrogen system.

A cyanide electrode[1], a saturated calomel electrode[1], an agar–agar bridge, and a scale-expansion pH meter[2] were used for potentiometry. Before measurements were made, the cyanide electrode was kept for 5 hr in a 0.1 *M* potassium nitrate buffer solution at pH 11, adjusted with sodium hydroxide, and then was carefully washed with distilled water and standardized.

**Reagents and Standards**[3]—All reagents were prepared from analytical grade substances and double-distilled water.

Standard cyanide solutions of known pH and ionic strength were prepared by several dilutions of a 0.1 *M* stock solution in 0.1 *M* potassium nitrate. The pH was adjusted with sodium hydroxide. The stock 0.1 *M* cyanide solution was standardized conductometrically with silver nitrate and potentiometrically with a cyanide ion-selective electrode.

**Analytical Procedure**—About 1–10 mg of the sample was weighed directly in the vial, avoiding moisture contact, and the vial was carefully introduced into the quartz test tube. Then the quartz test tube was placed in the described installation. After nitrogen was removed from the quartz test tube, the tube was placed in the electric oven. The nitrogen flow was adjusted to 45–50 bubbles/min in the receiving test tube containing 10 ml of 0.1 *M* potassium nitrate (pH adjustment to 12–13 with 0.1 *N* sodium hydroxide). By heating the oven to about 120° for cobinamide or 140–145° for cyanocobalamin (15–20 min), the cyanide content in the sample of the quartz tube was distilled as hydrocyanic acid and trapped in 0.1 *M* potassium nitrate at pH 12–13, where it was determined by means of the ion-selective electrode.

[1] Model OP-CN-7111, Radelkis, Hungary.
[2] Pye model 78.
[3] Cyanocobalamin, cobinamide, and hydroxocobalamin were obtained from the Antibiotics Plant of Jassy, Romania.

## RESULTS AND DISCUSSION

For cyanide measurement, a standard curve was prepared in the same manner used for the samples, using known solutions of cyanocobalamin, cobinamide, or potassium cyanide (0.001–0.00001 *M*).

The values for the cyanide solution are in agreement with the results calculated from the Nernst equation, which indicates that the curve should have a slope of −59.2 mv/p(CN) at constant pH and ionic strength. The experimental values for the slope were −59.8 mv/p(CN) for cyanide alone, −59.6 mv/p(cyanocobalamin) for the curve derived from cyanocobalamin solutions, and −59.9 mv/p (cobinamide) for the curve derived from cobinamide solutions, which were evaluated statistically (15).

Results of recovery experiments on cyanide from cyanocobalamin in the presence of cobinamide and hydroxocobalamin are shown in Table I and were satisfactory. By statistical analyses, the relative error was found to be ±3%.

Surprisingly, at higher temperatures than 120° for cobinamide or 140–145° for cyanocobalamin, the potential value of the ion-selective electrode was higher than the value corresponding to the theoretical cyano content of cyanocobalamin or cobinamide. Average values of the potential at different temperatures are shown in Table II. This discrepancy may result because degradation products arising at temperatures higher than 120 or 140–145° interfere with the cyanide ion-selective electrode. Moreover, an undegraded substance possibly could act as a catalyst, facilitating formation of hydrocyanic acid from the carbon and nitrogen atoms of cyanocobalamin, cobinamide, or hydroxocobalamin.

Other aspects of this method are under investigation.

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Table II—Potential Values at Different Temperatures by Cyanocobalamin and Cobinamide Determination

| Compound | Temperature | Amount Taken, mg | Potential, mv |
|---|---|---|---|
| Cyanocobalamin | 140° | 6.00 | 206 |
| Cyanocobalamin | 180° | 6.00 | 246 |
| Cyanocobalamin | 200° | 6.00 | 260 |
| Cyanocobalamin | 260° | 6.00 | 280 |
| Cobinamide | 120° | 6.00 | 214 |
| Cobinamide | 180° | 6.00 | 230 |

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# Steric Inhibition of Conjugation in Lowest Excited Singlet State of 9-Anthramide by Hydrogen Bond Donor Solvents: Role of Solvent in Chemical Structure

**ROY J. STURGEON and STEPHEN G. SCHULMAN** [x]

**Abstract** □ 9-Anthramide has electronic absorption and fluorescence spectra that, in water, are similar to those of anthracene. This result is attributed to steric hindrance of the 9-carboxamido group with the *peri*-hydrogen atoms in the 1- and 8-positions of the anthracene ring. However, in aprotic solvents, although the absorption spectrum of 9-anthramide is anthracene-like, its fluorescence spectrum is red shifted and structureless. This finding is attributed to excited-state rotation of the 9-carboxamido group into coplanarity with the anthracene ring and indicates that, in water, the hydrogen-bonded solvent cage affects the steric inhibition of conjugation in excited 9-anthramide. These findings suggest that studies of structure and reactivity of drugs in nonaqueous or solid matrixes are probably of only limited value, since in the strongly interacting aqueous media the aqueous solvent cage plays a substantial role in determining molecular structure and reactivity.

**Keyphrases** □ 9-Anthramide—electronic absorption and fluorescence spectral study, effect of solvent on molecular structure □ Solvent—effect on molecular structure of 9-anthramide, electronic absorption and fluorescence spectral study □ Molecular structure—9-anthramide, effect of solvent, electronic absorption and fluorescence spectral study □ Conjugation—steric inhibition in 9-anthramide, effect of solvent, electronic absorption and fluorescence spectral study

9-Anthroic acid and many of its derivatives have absorption spectra that are very similar to those of anthracene in vibrational structure and position in the electromagnetic spectrum. However, in various solvents, these compounds demonstrate a fluorescence band that is unstructured and at considerably longer wavelengths than that of anthracene. This band has been explained in terms of rotation of the carboxyl group from a configuration perpendicular to that of the anthracene ring in the ground state to one coplanar with the anthracene ring in the thermally equilibrated lowest excited singlet state (1).

It has been suggested that the steric hindrance to coplanarity, exerted by the *peri*-hydrogen atoms in the 1- and 8-positions of the anthracene ring in the ground state, is circumvented by reduction of the O—C—O bond angle of the carboxyl group as a result of electronic excitation. In the 9-anthroate anion, however, the greater electron density at the carboxylate group has been suggested to prevent sufficient alteration of the structure of the latter to permit coplanarity, even in the excited state, because both the absorption and fluorescence spectra of the anion are anthracene-like in appearance (2).

Based on spectra of the ethyl esters of 1- and 2-naphthoic acids (3), it was thought that the inability of the 9-anthroate anion to conjugate in the lowest excited singlet state may not be purely a function of structure but may be due to environmental effects such as a tightly bound solvent cage producing the steric interference. This hypothesis is, however, rather difficult to test on the 9-anthroate anion, because the ion-pairs, upon which spectra would be taken in low dielectric media, would include cations; these cations would introduce further complications in the interpretation of the spectra.

The amide of the 9-anthroic acid affords at least a partial solution of this problem. The carboxamido group is intermediate between the carboxyl group and the carboxylate anion in its electronic distribution and, therefore, in its geometrical structure. Moreover, 9-