Technical Papers

Vitamin B_{12}: The Identification of Vitamin B_{12} as a Cyano-Cobalt Coordination Complex

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Vitamin B_{12} contains one cyano group bound coordinatively to the cobalt atom. Vitamin B_{12a}, however, does not contain this group.

During an oxidation of vitamin B_{12} in dilute sulfuric acid solution at 0° C with potassium permanganate, the characteristic odor of hydrogen cyanide was noted. The solution was distilled, and the distillate examined for the presence of cyanide, using the copper-benzidine (*2*) and the ferrous sulfate tests (*8*). Both were positive. In another oxidation, carried out in a closed system, a solution of 49.1 mg of vitamin B_{12} in 5 ml of 0.75 N sulfuric acid was stirred in an ice bath during the dropwise addition of 3.5 ml of 2% potassium permanganate solution. The reaction mixture was warmed to 75°, and nitrogen was bubbled through the solution. The exit gases were passed into dilute sodium hydroxide solution. A volumetric determination of the hydrogen cyanide evolved (*5*) gave a value of 0.66 mg, corresponding to 0.67 mole/mole of vitamin B_{12}.

Hydrogen cyanide was also liberated readily upon heating solutions of vitamin B_{12} in hydrochloric or aqueous oxalic acid. In the presence of saturated aqueous oxalic acid, 0.96 mole of hydrogen cyanide was liberated per mole of the vitamin when the solution was boiled under reflux for 5 hr. Due to simultaneous hydrolysis of the cyanide, its yield was somewhat lower when hydrochloric acid was employed. No cyanide could be detected when vitamin B_{12} was warmed with dilute sulfuric acid under the same conditions used for its liberation by hydrochloric or oxalic acid.

Since chloride and oxalate are known to coordinate readily with cobalt, while sulfate shows only a slight tendency to enter in coordination complexes with this metal (*7*), it seems probable that the cyanide group in vitamin B_{12} is a member of the cobalt coordination complex, and that it may be displaced from the coordination sphere by anions with strong coordination tendencies.

No cyanide could be detected when pure vitamin B_{12a} was treated with oxalic acid solution. Vitamin B_{12} possesses an absorption band at 4.69 μ in the infrared region, which may be ascribed to the presence of a carbon-nitrogen triple bond; but this band does not appear in the absorption spectrum of vitamin B_{12a}. Vitamin B_{12a} and vitamin B_{12b} are apparently identical (*4*); and the absence in the infrared spectrum of vitamin B_{12b} of a band that is present at 2,140 cm^{-1} in the spectrum of vitamin B_{12} has been observed by others (*1*). It has been reported (*3*) that vitamin B_{12a} does not differ greatly in its composition from vitamin B_{12}. One significant difference is the presence of a coordinatively bound cyano group only in the latter compound.

The extreme lack of toxicity of vitamin B_{12} (*6*) indicates that the cyano group is tightly bound within the coordination complex. Dose levels of 1,600 mg/kg, both intraperitoneally and intravenously in mice, produced no deaths or toxic manifestations. This dose level corresponds to 112,000,000 times the daily human dose of 1 μg of vitamin B_{12} listed in the *U. S. Pharmacopeia.*

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Vitamin B_{12}: Reactions of Cyano-Cobalamin and Related Compounds

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It has been found that a cyano group (*1*) is present in the vitamin B_{12} cobalt coordination complex, but not in

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vitamin B_{12a}. Further investigation has led to the discovery of some new reactions of structural interest.

Addition of cyanide ions to a solution of vitamin B_{12a} yielded vitamin B_{12}, which was obtained crystalline and identified as described herein. Since an aqueous solution of vitamin B_{12a} has a pH of about 9 and behaves on titration as a weak base, it appears that a hydroxo group has replaced the cyano group. This conversion of vitamin B_{12a} to vitamin B_{12} shows that, in the formation of vitamin B_{12a}, the only structural change is a replacement of the cyano group.

TABLE 1

REFRACTIVE INDICES*

	α	β	γ
Chloro-cobalamin	1.620 ± 0.002	1.628 ± 0.002	1.634 ± 0.002
Product from $H_2S + B_{12}$	1.600 ± 0.002	1.648 ± 0.002	1.674 ± 0.004
Hydroxo-cobalamin	1.580 ± 0.002	1.640 ± 0.002	1.656 ± 0.002
Sulfato-cobalamin	ε 1.606 ± 0.002 ;	ω 1.640 ± 0.002	

* Samples dried *in vacuo* at *ca* 25° C.

New nomenclature is needed to describe the large number of modifications of vitamin B_{12} that result from similar replacement reactions. The name cobalamin designates all the vitamin B_{12} molecule except the cyano group, and by convention (*7*) vitamin B_{12} becomes cyano-cobalamin, and vitamin B_{12a}, hydroxo-cobalamin.

Three and seven-tenths mg of hydroxo-cobalamin (vitamin B_{12a}) was dissolved in 2 ml of water. To this solution was added 0.5 ml of a solution containing 6.6 mg of potassium cyanide. The mixture was adjusted to pH 5–6 with dilute hydrochloric acid, and the vitamin B_{12} was extracted with two 1.5-ml portions of a solution of phenol-carbon tetrachloride (1–7 by volume), which was equilibrated with water. The extracts were combined and then extracted four times with 1-ml portions of water that had been equilibrated previously with the phenol-carbon tetrachloride solution. The phenol-carbon tetrachloride solution was then diluted with *ca* 12 ml of carbon tetrachloride, and the vitamin B_{12} was extracted with two 1-ml portions of water. The combined aqueous extracts were washed with ether to remove traces of solvent and then evaporated to dryness *in vacuo* at 25°–30° C. The residue was dissolved in *ca* 0.5 ml of water, and the solution was diluted with *ca* 12 ml of acetone. After the solution stood for about 30 min, slender red needles of cyano-cobalamin (vitamin B_{12}) began to crystallize. The crystals were separated by centrifuging, washed with acetone and dried; yield, 2.3 mg. The absorption spectrum showed maxima at 2,780A, 3,610A, and 5,500A, which is characteristic of vitamin B_{12}.

Reaction of vitamin B_{12} with sulfurous acid gave crystalline sulfato-cobalamin (K, 2.5).[2] Reaction of vitamin B_{12} with hydrogen sulfide does not remove the cobalt, but introduces a sulfur-containing group that does not appear to be sulfido, sulfito, or sulfato; this product is crystalline (K, 0.36. Anal. Found: S, 2.2). Vitamin B_{12a} reacted with chloride ions to give chloro-cobalamin (K, 2.0. Anal. Found: Cl, 2.6). Chloro-cobalamin reacts in water with potassium cyanate to give cyanato-cobalamin (K, 0.8).

Other information characterizing these new products are refractive indices and microbiological assays, which are summarized in Tables 1 and 2. Cyano-cobalamin has activity as a growth-promoting factor for *Lactobacillus lactis* equivalent to 11,000 LLD units per μg (*6*). The microbiological activity values of vitamin B_{12a} appear to be dependent on the conditions of assay and vary from 30–100% of that of vitamin B_{12}. The titrimetric method values that are given were obtained by autoclaving the sample with the assay medium and, under these conditions, possess about 70% of the activity of vitamin B_{12} (*4*).

TABLE 2

MICROBIOLOGICAL DATA

	L. lactis (units/μg)	
	titrimetric (*2*)	cup (*3*)
Chloro-cobalamin	7,000	10,400
Product from $H_2S + B_{12}$	6,600	12,300
Hydroxo-cobalamin	7,700	11,000
Sulfato-cobalamin	6,300	10,900
Cyano-cobalamin	11,000	11,000

These analogues—for example, chloro-cobalamin, cyanato-cobalamin, sulfato-cobalamin, and the crystalline sulfur-containing complex from hydrogen sulfide—have also been converted to crystalline vitamin B_{12} by reaction with cyanide ions.

Comparative data show that vitamin B_{12a} and vitamin B_{12b} appear to be identical (*5*). When 0.3 mg of vitamin B_{12b} (Lederle NP-92-58-4)[3] in water was treated with cyanide ions, 0.2 mg of crystalline vitamin B_{12} resulted.

The essential differences in the spectra of vitamin B_{12a} and related compounds appear to be only those brought about by the pH conferred by the ion associated with the particular analogues. They are exemplified (*5*) by the change in the spectrum of vitamin B_{12a} with pH change.

Further details on the preparation of these and other members of the vitamin B_{12} group that have been made from vitamins B_{12} and B_{12a} will be submitted for publication.

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[2] Distribution coefficient, C_w/C_s, determined in carbon tetrachloride-phenol (7–1 by volume)-water.

[3] Furnished through the courtesy of T. H. Jukes, of the Lederle Laboratories Division of the American Cyanamid Company.