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Vitamin B_{12}. XVI.[1,2] Modifications of Cyano-cobalamin

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Vitamin B_{12} (cyano-cobalamin) reacts with sulfurous acid to yield sulfato-cobalamin and with hydrogen sulfide to give a sulfur-containing cobalamin. Vitamin B_{12a} (hydroxo-cobalamin) reacts with chloride, bromide, sulfate, cyanate and nitrite ions to give chloro-, and bromo-, sulfato-, cyanato- and nitro-cobalamin. The cobalamin derivatives are converted to vitamin B_{12} (cyano-cobalamin) by reaction with cyanide ions. An interpretation of the formation of vitamin B_{12}-like compounds from vitamin B_{12} is discussed.

Vitamin B_{12} has been isolated from liver and microbiological sources. Vitamin B_{12a} has been prepared[3] from vitamin B_{12} and also isolated[4,5] from *Streptomyces griseus*. A compound originally designated vitamin B_{12b},[6,7] which later could not be distinguished[5] from vitamin B_{12a}, was isolated from *Streptomyces aureofaciens* and *Streptomyces griseus*. The isolation of vitamins B_{12c}[8] and B_{12d}[9] has been reported. An interpretation of the mode of formation of the vitamin B_{12} group of compounds and a nomenclature for them has been reported,[2] which was based on the finding[1] that a cyano group is present in the vitamin B_{12} cobalt coördination complex and has been replaced by other groups in vitamin B_{12}-like compounds. The vitamin B_{12}-like compounds may be converted[2] to vitamin B_{12} by reaction with cyanide ions. It has been reported[10] that when a mildly acid solution of vitamin B_{12} is exposed to light the spectrum changes to that of vitamin B_{12b}, and that addition of potassium cyanide to vitamin B_{12b} yields either vitamin B_{12} itself or a very closely related compound with the same spectrum.

A large variety of vitamin B_{12}-like compounds may be formed by replacement of the cyano group of vitamin B_{12} by other groups. A nomenclature for these compounds is based on designating all of the vitamin B_{12} molecule except the cyano group with the name cobalamin, and then applying Werner's nomenclature of metal complexes for the modifications. Accordingly, vitamin B_{12} is cyano-cobalamin.

It has been concluded that the vitamin B_{12} cobalt coördination complex is neutral, and that the cobalt is trivalent and has a coördination number of six, and all of the molecule is involved in the coördination. The vitamin B_{12} molecule may be represented as

$$[R\bar{o}\bar{o}o\ Co^{+++}\ CN^-]$$

The cyano group contributes one negative charge, and satisfies one coördination position, and the two minus signs and the ciphers represent another group or groups which contribute two negative charges and three electron pairs for satisfying the remaining five coördination bonds.

Vitamin B_{12a} was obtained initially by treatment of cyano-cobalamin in aqueous solution with hydrogen in the presence of a catalyst and exposure to air of the resulting solution. Two possible mechanisms for removal of the cyano group in this reaction are: (1) The reduction of Co^{+++} to Co^{++} to give an acid cobaltous complex, which could be unstable and lose the coördinating cyano group. The removal of cyanide ions from solution might take place by hydrogenation or by other means. (2) Hydrogenation of the cyano group in the complex followed by dissociation of the reduced group from either the Co^{+++} or Co^{++} complex. One of the steps in the formation of vitamin B_{12a} is the addition of a group to fill the position of the cyano group, before or after Co^{++} is oxidized by air to Co^{+++}. When the reduction is carried out in water, there is no opportunity (unless impurities are present) for an ion or molecule to enter the complex except those composed of hydrogen and/or oxygen. Since vitamin B_{12a} titrates with hydrochloric acid as a weak base,[11] it appears that a hydroxo-group has replaced the cyano group. Accordingly, vitamin B_{12a} is designated hydroxo-cobalamin, which in aqueous solution probably exists in equilibrium with the aquo compound, as

$$[R\bar{o}\bar{o}oCo^{+++}OH^-] \overset{H_2O}{\rightleftharpoons} [R\bar{o}\bar{o}oCo^{+++}H_2O]^+ + OH^-$$

Various analogs of cyano-cobalamin are possible. When vitamin B_{12} is present in an environment containing a variety of ions or groups, and the cyano group is removed, the analog that is formed will depend on such factors as relative concentrations of the ions or groups and affinity of the ions or groups to coördinate with the cobalt. The separation of mixtures of cobalamins may be accomplished through the differences in the solubility properties.

In the course of our investigation of the reaction of vitamin B_{12} with various reagents besides hydrogen, it was found that hydrogen sulfide and sulfurous acid react with vitamin B_{12}. After oxidation of the resulting solutions with air, crystalline products similar to vitamin B_{12a} could be obtained. The reaction with hydrogen sulfide was particularly interesting in connection with an attempt to remove the cobalt atom from vitamin B_{12}. The treatment of vitamin B_{12} with hydrogen sulfide did not remove cobalt, but it did cause

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(10) W. L. C. Veer, J. H. Edelhausen, Wijemga and Lens, *J. Biochim. Biophys. Acta*, **6**, 225 (1940).
(11) Buhs, Newstead and Trenner, *Science*, in press.

oxidation of sulfide ion to sulfur, and did cause changes in the absorption spectrum. Although the properties of the products of reactions of vitamin B_{12} with hydrogen sulfide and sulfurous acid are very similar to the properties of vitamin B_{12a}, these three compounds were shown by detailed purification and comparison studies to be different. Purity was established by solubility analyses. Each product exhibits a very similar absorption spectrum and microbiological activity for *L. lactis* and *L. leichmannii*. The compounds have been differentiated by partition coefficients, refractive indices and analytical determinations.

The reaction product, from vitamin B_{12} and hydrogen sulfide, was prepared by the addition of hydrogen sulfide to a slightly alkaline alcoholic solution of vitamin B_{12}. The dark red solution turns dark brown, and after oxidation, brought about by contact with air, the solution changes back to a dark red color. The residue from such a solution, by fractional crystallization from water by the addition of acetone, yields dark red needle-like crystals, very similar in appearance to vitamin B_{12a}. This crystalline product contains sulfur in a form which did not give a positive test for sulfide, sulfite or sulfate. The failure to detect the presence of these groups, indicates that the sulfur atom might be in an intermediate state of oxidation or that the sulfide group might be so firmly bound in the complex that it did not readily respond to the usual tests for sulfide.

The reaction product, sulfato-cobalamin, from vitamin B_{12} and sulfurous acid, was prepared by bubbling sulfur dioxide into an aqueous solution of vitamin B_{12}. The dark red solution becomes brown in color and after standing for a short time in contact with air, the color of the solution changes back to dark red. Fractional crystallization of the residue from water by the addition of acetone yields dark red needle-like crystals, which in appearance are very similar to vitamin B_{12a} and the reaction product of vitamin B_{12} and hydrogen sulfide. This product appears to contain a sulfate group in place of the cyano group. The presence of sulfate was established by treating an aqueous solution of the compound with barium acetate whereby an acid-insoluble, white precipitate was obtained. Analyses showed 2.24 and 2.50% sulfur and 6.24 and 6.67% sulfate.

Investigation of the reactions of vitamin B_{12a} showed that the hydroxyl group can be replaced with other ions. Thus, hydroxo-cobalamin reacts in aqueous solution with hydrochloric acid to give chloro-cobalamin and with hydrobromic acid to give bromo-cobalamin. Vitamin B_{12a} was also converted to chloro-cobalamin under some conditions where chloride ions were present as an impurity. This latter conversion was noted when a purification of vitamin B_{12a} was attempted by countercurrent distribution between a water–*o*-cresol–carbon tetrachloride mixture. A sample of vitamin B_{12a}, which was carried through a 9-plate countercurrent distribution using the above mixture, yielded the chloro compound which was shown by solubility analysis to be higher than 98% pure. This conversion was apparently due to a small amount of hydrogen chloride in the carbon tetrachloride. Chloro-cobalamin and hydroxo-cobalamin react with cyanate ions to give cyanato-cobalamin which can be differentiated from the starting compounds by the differences in their partition coefficient.

Hydroxo-cobalamin reacts with sulfuric acid in aqueous solution in a manner similar to the reaction with hydrochloric acid. The sulfato-cobalamin which is obtained has the same partition coefficient and composition as does the product from cyano-cobalamin and sulfurous acid.

Hydroxo-cobalamin reacts with nitrite ions in aqueous solution to give nitro-cobalamin.

The ease of removal of the cyano group from vitamin B_{12} accounts for the observation[12] that paper chromatography of vitamin B_{12} leads to doublet zones,[13] one of which has the R_F value of vitamin B_{12a}. The result can now be explained as due to chemical reaction of vitamin B_{12} during paper chromatography and not to separation of components in the starting material.

It is well known that the cyano group coördinates easily and is firmly bound in metal complexes, and it was found[2] that this group reacts in aqueous solution with the various cobalamins to give cyano-cobalamin. Thus, hydroxo-, chloro-, cyanato-, sulfato-cobalamin, the hydrogen sulfide-reaction product of vitamin B_{12}, and a sample of vitamin B_{12b} (Lederle NP-92-58-4) reacted with cyanide ions to give cyano-cobalamin.

One method used for distinguishing the various cobalamins has been the determination of partition coefficients in the system phenol–carbon tetrachloride (1–7 by volume)–water. Although this system allowed a differentiation of the compounds, the exact values of the coefficients varied when different batches of this solvent system were used, or when the system was allowed to stand for several days. Temperature effects on this system were also significant. Therefore, the constants reported herein have only relative value.

Some of these modifications of vitamin B_{12} have been tested clinically. Dr. Tom Spies[14] has tested parenterally sulfato-cobalamin, cyanato-cobalamin, and the hydrogen sulfide-reaction product of vitamin B_{12} and has found that they are potent in Addison's pernicious anemia, nutritional macrocytic anemia and sprue. Whether these modifications are as potent per unit of weight as vitamin B_{12} *per se* has not been determined.

Experimental

Reaction of Vitamin B_{12} with Hydrogen Sulfide.—Sixty-six milligrams of vitamin B_{12} was dissolved in 25 ml. of ethyl alcohol. One drop of ammonium hydroxide was added to this solution and then a slow stream of hydrogen sulfide was bubbled in for about five minutes. The solution was allowed to stand at about 25° for three hours and then evaporated to dryness *in vacuo*. The residue was dissolved in water and the solution was filtered in order to remove sulfur. The aqueous solution was diluted with acetone. After about one-half hour, dark red needles were removed (49.5

(12) Hendlin and Woodruff, Am. Chem. Soc. Meeting, Atlantic City, N. J., September, 1949, Abstracts of papers, p. 33a. Foster and Woodruff, in press.

(13) Winsten, Eigen and Oser, Am. Chem. Soc. Meeting, Atlantic City, N. J., September, 1949, Abstracts of papers, p. 32a.

(14) Personal communications.

mg.) and recrystallized from water by dilution with acetone; yield 43 mg. The absorption spectrum of this compound in water showed maxima at 2750, 3520 and 5300 Å. This material was further purified by a 9-plate countercurrent distribution in the system *o*-cresol–carbon tetrachloride (two parts to five parts by volume) and water. The residues from tubes 2, 3 and 4 were crystallized from water by dilution with acetone. Since the absorption spectra corresponding to the crystals from the above tubes were the same, the crystals were combined and recrystallized from water by dilution with acetone. The yield of this sulfur-containing cobalamin was 25.6 mg. Solubility analysis showed that the crystalline product was higher than 98% pure. The distribution coefficient (C_w/C_s) in phenol–carbon tetrachloride (1–7 by volume)–water is about 0.36.

Anal. Found: S, 2.2.

Reaction of Vitamin B_{12} with Sulfurous Acid.—Seventy-three and eight-tenths milligrams of vitamin B_{12} was dissolved in 30 ml. of water. Sulfur dioxide was bubbled into this solution for about one minute and then the solution was heated on a steam-bath for ten minutes. During this time, the color of the solution changed from red to brown. The brown solution was aerated with a slow stream of air for one hour and then evaporated to dryness *in vacuo*. The residue was dissolved in about 4 ml. of water and then the solution was diluted with 5 to 6 volumes of acetone. After about one hour, dark and red needles of sulfato-cobalamin began to form in the solution and after 20 hours, 61.1 mg. of product was obtained. The absorption spectrum of the crystalline compound showed maxima at 2750, 3510–3520 and 5300 Å. The compound was further purified by a 9-plate countercurrent distribution in the system *o*-cresol–carbon tetrachloride (two parts to five parts by volume)–water. The residues obtained from tubes 5, 6 and 7 were crystallized and each showed the same absorption spectrum. The residue from tube 8 was redistributed through nine plates in the system *o*-cresol–carbon tetrachloride (one part to two parts by volume)–water. The residues from tubes 5, 6, 7 and 8 were crystallized from water by dilution with acetone, and each showed the same absorption spectrum. The crystalline fractions from tubes 5, 6 and 7 of the first distribution and tubes 5, 6, 7 and 8 of the second distribution were combined and solubility analysis showed that the purity was 98% or higher. The distribution coefficient (C_w/C_s) in phenol–carbon tetrachloride (1–7 by volume)–water is about 2.5.

Twenty and eight-tenths milligrams of vitamin B_{12} was dissolved in 2 ml. of a sulfurous acid solution prepared by bubbling sulfur dioxide into water until the solution was strongly acid. The solution was allowed to stand at 25–30° for 24 hours and then evaporated to dryness *in vacuo*. The residue was crystallized in the form of dark red slender needles from water by the addition of acetone.

Reaction of Hydroxo-cobalamin with Hydrochloric Acid.—Thirty-eight milligrams of hydroxo-cobalamin was dissolved in 5 ml. of water and the solution was adjusted to *p*H 4 with 0.1 *N* hydrochloric acid. This solution was then diluted with *ca.* 30 ml. of acetone and after a short time chloro-cobalamin began to crystallize in the form of dark red slender needles. The crystals were separated by centrifuging, washed with acetone, and dried; yield 35 mg.

A solution containing 150 mg. of vitamin B_{12} in 50 ml. of water was shaken with *ca.* 100 mg. of platinum catalyst and hydrogen gas under substantially atmospheric pressure at 25° for 20 minutes. During the absorption of hydrogen the color of the solution changed from red to brown. The solution was separated from the catalyst and evaporated to dryness *in vacuo*. The residue was dissolved in about 10 ml. of water and then diluted with 25 ml. of acetone. The solution on standing deposited a small amount of amorphous precipitate. The precipitate was removed and the solution was diluted with an additional 5 ml. of acetone. This solution on standing gave small amounts of amorphous and crystalline precipitates. After removal of these precipitates the solution was evaporated to dryness *in vacuo* at *ca.* 25°. The residue was combined with the precipitates and this mixture was subjected to a 9-plate countercurrent distribution in the system *o*-cresol–carbon tetrachloride (two parts to five parts by volume)–water. The total yield of residues obtained from the distribution was 132 mg. The residues from plates 3, 4, 5, 6 and 7 were crystallized, combined (yield 63 mg.) and distributed through eight plates using the same solvent system. The residues of plates 2, 3, 4 and 5 were crystallized from water by dilution with acetone. Since the absorption spectra of the crystalline products from the above plates were identical, the products were combined (yield 43.3 mg.). Purification of vitamin B_{12a} by the above procedure yielded chloro-cobalamin since chloride ions from a small amount of hydrogen chloride present in the carbon tetrachloride, reacted with vitamin B_{12a}.

Anal. Found: Cl, 2.6.

The distribution coefficient of chloro-cobalamin (C_w/C_s) in the system phenol–carbon tetrachloride (1–7 by volume)–water is about 2.0.

Solubility analysis showed that the crystalline chloro-cobalamin was higher than 98% pure.

Reaction of Hydroxo-cobalamin with Hydrobromic Acid.—Forty milligrams of hydroxo-cobalamin (vitamin B_{12a}) was dissolved in 5 ml. of water and two drops of 48% hydrobromic acid was added. After a few minutes, this solution was diluted with acetone to a volume of *ca.* 60 ml. Crystallization began after a short time, giving dense crystals of bromo-cobalamin. The compound was twice recrystallized, from water by the addition of acetone, in the form of slender dark reddish-purple needles. The absorption spectrum of an aqueous solution of bromo-cobalamin showed maxima at about 2740 Å. ($E^{1\%}_{1\,cm.}$ 140); 3520 Å. ($E^{1\%}_{1\,cm.}$ 168); and 5200–5300 Å. ($E^{1\%}_{1\,cm.}$ 59). The distribution coefficient (C_w/C_s) in phenol-carbon tetrachloride (1–7 by volume)–water is about 2.

Reaction of Hydroxo-cobalamin with Nitrite Ions.—Forty-six milligrams of hydroxo-cobalamin (vitamin B_{12a}) was dissolved in 5 ml. of water. To this solution, there was added 33 mg. of sodium nitrite and two drops of acetic acid. After a few minutes, this solution was diluted with acetone to a volume of *ca.* 25–30 ml. Crystallization began after a short time, giving slender dark red needles of nitro-cobalamin. The compound was twice recrystallized from water by the addition of acetone. The absorption spectrum of an aqueous solution of nitro-cobalamin showed maxima at 3540 Å. ($E^{1\%}_{1\,cm.}$ 185); and 5300 Å. ($E^{1\%}_{1\,cm.}$ 75) and a shoulder at about 2550–2750 Å. ($E^{1\%}_{1\,cm.}$ 187–180).

The distribution coefficient (C_w/C_s) in phenol–carbon tetrachloride (1–7 by volume)–water is about 0.15.

Reaction of Chloro-cobalamin with Potassium Cyanate.—Twenty-nine milligrams of chloro-cobalamin was dissolved in 5 ml. of water and *ca.* 30 mg. of potassium cyanate was added to this solution. After the solution stood for a few minutes, it was diluted with *ca.* eight volumes of acetone. A dark red compound crystallized; it was separated, washed with acetone, and dried. This product was further purified by a 10-plate countercurrent distribution in the system carbon tetrachloride–phenol (seven parts to one part by volume)–water. In this system, chloro-cobalamin has a distribution coefficient (C_w/C_s) of about 2. Cyanato-cobalamin has a distribution coefficient of about 0.8 in this system but was found essentially in the first two tubes. The residues of these two tubes were crystallized from water by the addition of acetone.

Reaction of Hydroxo-cobalamin with Potassium Cyanate.—Twenty-five milligrams of hydroxo-cobalamin was dissolved in 5 ml. of water and 10 mg. of potassium cyanate was added to this solution. After the solution stood for *ca.* five minutes, it was diluted with *ca.* eight volumes of acetone. Crystallization began after a short time, giving dark red needles of cyanato-cobalamin. The compound was recrystallized from water by the addition of acetone; it separated in the form of slender dark red needles. The distribution coefficient (C_w/C_s) in phenol–carbon tetrachloride (1–7 by volume)–water is about 0.8.

Reaction of Hydroxo-cobalamin with Sulfuric Acid.—Thirty milligrams of hydroxo-cobalamin was dissolved in 5 ml. of water and three drops of 2 *N* sulfuric acid was added to this solution. After a few minutes, this solution was diluted with *ca.* 6 volumes of acetone. Sulfato-cobalamin began to crystallize from the solution after a short time. The compound was recrystallized twice from water by the addition of acetone. The distribution coefficient (C_w/C_s) in phenol–carbon tetrachloride (1–7 by volume)–water is about 2.5.

Conversion of Hydroxo-cobalamin to Cyano-cobalamin.—Three and seven-tenths milligrams of vitamin B_{12a} was dis-

solved in *ca.* 1 ml. of water, and to this solution there was added 0.5 ml. of a solution containing 6.6 mg. of potassium cyanide. The solution was adjusted to *p*H 5–6 with dilute hydrochloric acid and then extracted twice with 1.5-ml. portions of a solution of phenol–carbon tetrachloride (1–7 by volume, equilibrated with water) for removal of vitamin B_{12}. This phenol–carbon tetrachloride solution was extracted four times with 1-ml. portions of water which had been equilibrated with the original phenol–carbon tetrachloride solution, in order to remove any vitamin B_{12a}, and then diluted with *ca.* 12 ml. of carbon tetrachloride. This solution was then extracted three times with 1-ml. portions of water. The aqueous extract of vitamin B_{12} was washed with ether to remove phenol and carbon tetrachloride and it was evaporated to dryness *in vacuo*. The residue was dissolved in *ca.* 0.5 ml. of water and the solution diluted with *ca.* 12 ml. of acetone. After allowing the solution to stand for a short time, slender red needles of vitamin B_{12} began to form. After *ca.* 16 hours, crystallization was complete; the crystals were removed by centrifuging, and were washed with acetone and dried; yield 2.3 mg.

Three and seven-tenths milligrams of vitamin B_{12a} was dissolved in *ca.* 1 ml. of water and to this solution was added *ca.* 1 ml. of a solution of ammonium cyanide (prepared by reacting a solution of potassium cyanide with sulfuric acid and allowing the hydrogen cyanide to collect in a dilute solution of ammonium hydroxide). After allowing this solution to stand for a short time, it was evaporated to dryness *in vacuo*. The residue was dissolved in *ca.* 0.5 ml. of water and this solution was diluted with *ca.* 12 ml. of acetone. After allowing the solution to stand for a short time, slender red needles of vitamin B_{12} began to form; yield 3.3 mg.

Five milligrams of vitamin B_{12a} was dissolved in 3 ml. of water. To this solution, was added a solution of hydrogen cyanide which was prepared from 50 mg. of sodium cyanide and 20 ml. of a 5% sulfuric acid by distillation. The solution was allowed to stand at *ca.* 25° for a few minutes, heated for 3.5 hours on the steam-bath, and then evaporated to dryness *in vacuo*. The residue was dissolved in 0.5 ml. of water and this solution was diluted with 5 ml. of acetone. After allowing the solution to stand for a short time, needle-like crystals of vitamin B_{12} were obtained; yield 3.4 mg. The absorption spectrum of a solution of these crystals showed maxima at 2780, 3610 and 5500 Å.

Conversion of Chloro-cobalamin to Cyano-cobalamin (Vitamin B_{12}).—Four milligrams of chloro-cobalamin and 42 mg. of potassium cyanide were dissolved in 1 ml. of water and then after about one minute the solution was adjusted to *p*H 4–5 with dilute hydrochloric acid. Following the procedure described for the conversion of vitamin B_{12a} to vitamin B_{12}, but using a mixture of *o*-cresol–carbon tetrachloride saturated with water instead of the phenol–carbon tetrachloride solution, 2.4 mg. of crystalline vitamin B_{12} was obtained.

The absorption spectrum of these crystals of vitamin B_{12} showed maxima at 2780 Å. ($E^{1\%}_{1\,cm.}$ 112); 3620 Å. ($E^{1\%}_{1\,cm.}$ 193); and 5500 Å. ($E^{1\%}_{1\,cm.}$ 60).

Five milligrams of chloro-cobalamin was dissolved in 1 ml. of water and to this solution there was added *ca.* 10 mg. of yellowish-green cuprous cyanide. This solution was allowed to stand for 12 hours at *ca.* 25–30° with frequent shaking and then heated in a water-bath at *ca.* 75° for one-half hour. The absorption spectrum of this solution showed maxima at 2780, 3610 and 5400–5500 Å. which is characteristic of a solution of vitamin B_{12}. After separating a small amount of insoluble material by centrifuging, the solution was evaporated to dryness *in vacuo*. The residue was dissolved in *ca.* 0.5 ml. of water and this solution was diluted with *ca.* 12 ml. of acetone. After allowing the solution to stand for a short time, dense red crystals of vitamin B_{12} began to form. After 18 hours the crystals were separated by centrifuging, washed with acetone and dried; yield 3.1 mg.

The absorption spectrum of the crystals showed maxima at 2780, 3610 and 5500 Å.

Conversion of the Product of Reaction of Vitamin B_{12} with Hydrogen Sulfide to Cyano-cobalamin (Vitamin B_{12}).—Three and four-tenths milligrams of the reaction product of vitamin B_{12} and hydrogen sulfide and 38 mg. of potassium cyanide were dissolved in 1 ml. of water, and by the procedure described for the conversion of chloro-cobalamin to vitamin B_{12}, 2.3 mg. of crystalline vitamin B_{12} was obtained.

Conversion of Sulfato-cobalamin to Cyano-cobalamin (Vitamin B_{12}).—Two milligrams of sulfato-cobalamin and 18 mg. of potassium cyanide were dissolved in 1 ml. of water, and by the procedure described for the conversion of chloro-cobalamin to vitamin B_{12}, 1.1 mg. of crystalline vitamin B_{12} was obtained.

Conversion of Cyanato-cobalamin to Cyano-cobalamin (Vitamin B_{12}).—Three milligrams of cyanato-cobalamin and *ca.* 10 mg. of potassium cyanide were dissolved in 1 ml. of water, and by the procedure described above, 0.5 mg. of crystalline vitamin B_{12} was obtained.

Conversion of Vitamin B_{12b} (Lederle NP-92-58-4) to Vitamin B_{12}.—Three-tenths milligram of vitamin B_{12b} (Lederle NP-92-58-4) and 10 mg. of potassium cyanide were dissolved in 1 ml. of water and by the procedure described for conversion of chloro-cobalamin to vitamin B_{12}, 0.2 mg. of crystalline vitamin B_{12} was obtained.

Determination of Distribution Coefficients.—One distribution system was composed as follows: 11.2 g. of phenol (Merck, Reagent grade) was dissolved in 73 ml. of carbon tetrachloride and this solution was equilibrated with 83 ml. of distilled water. The second distribution system was composed as follows: 20 ml. of redistilled *o*-cresol (Paragon) and 50 ml. of redistilled carbon tetrachloride equilibrated with 70 ml. of distilled water.

Three to four hundred micrograms of the sample, whose distribution constant was to be determined was dissolved in one drop of water and then 7 ml. of the aqueous phase of the distribution system was added. Three and one-half milliliters of this solution was then equilibrated with 3.5 ml. of the solvent phase of the distribution system in a 15-ml. centrifuge tube using a small glass pipet, equipped with a rubber bulb, for mixing the two liquid layers. The two layers easily separated on centrifuging. The top aqueous phase was transferred with a pipet to a 1-cm. quartz absorption cell and the optical density of this solution was determined at ten different wave lengths between 3200 and 5600 Å. using a Beckman quartz spectrophotometer. The optical density of the unequilibrated half of the original solution was determined at the same time at the same wave lengths. The ratio of the optical density of the solution after equilibration to the difference in optical densities of the unequilibrated and equilibrated solution is defined as C_w/C_s.

TABLE I

ABSORPTION SPECTRA

	Compound	Absorption spectrum in H_2O–maxima Wave length (Å.)	$E^{1\%}_{1\,cm.}$
1	Hydroxo-cobalamin (Vitamin B_{12a})	2700–2770	137
		3525	150
		5300	56
2	Chloro-cobalamin	2740–2750	142
		3520–2530	174
		5250–5300	59
3	Bromo-cobalamin	2740–2750	140
		3520	168
		5200–5300	59
4	Reaction product of vitamin B_{12} and hydrogen sulfide	2730–2750	140
		3520	170
		5250–5300	58
5	Sulfato-cobalamin	2720–2750	135
		3520	163
		5200–5300	55
6	Nitro-cobalamin	2550–2750	187–180
		3540	185
		5300	75
7	Cyanato-cobalamin	2720–2780	139
		3530	140
		5200–5300	54
8	Vitamin B_{12}	2780	115
		3610	204
		5500	63

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[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF IOWA STATE COLLEGE]

A Microbiological Method for the Determination of Sequences of Amino Acid Residues[1]

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A subtractive microbiological method for the determination of terminal amino acid residues in peptides is presented. Results are given for glycyl-DL-valine, L-prolyl-L-leucine, leucylphenylalanine, valylleucine, leucylvaline, L-valyl-D-valine, D-valyl-L-valine and valylglycylphenylalanine. By employing selective hydrolysis, the sequence of residues in the tripeptide could be assigned. The general procedure is uniquely applicable to quantitative determinations of terminal amino acid residues on a micro scale. When employed comparatively with paper-strip procedures, this method serves to indicate D-residues. Other advantages and recognized current limitations are discussed.

Elucidation of the structure of proteins and of peptides requires methods which will assign positions of amino acid residues in the chains. This is theoretically possible from determination of sequence in smaller peptides obtained on hydrolysis,[2] advances in this direction have been made.[3-5] Improvements in methodology are needed, as well as independent methods which will provide confirmation for those already available. In the determination of the structure of Gramicidin S,[3] for example, it was deemed desirable to synthesize the peptides indicated by a simpler method, and to compare the R_f values of the synthetic peptides with those of the isolated materials. Both of these procedures relied, however, on chromatography; an independent principle for a micro method is desirable.

The use of microbiological techniques was suggested earlier[2] and has now been applied to some synthetic peptides by proceeding subtractively as follows: (a) All residues are first determined on a complete hydrolysate by setting up tubes each of which is nutritionally complete except for one amino acid. (b) The terminal residue is blocked chemically, in a second sample. (c) Treated sample (b) is hydrolyzed, and the microbiologically recoverable amino acids are again evaluated. (d) The missing residue is the one which occupied the terminal position in the original peptide.

The microbiological procedure, in contrast to chromatography, is applicable for relatively convenient quantitative determinations, and this in turn for peptides with recurrent residues. In conjunction with chromatography, on the other hand, it is possible to locate D-residues readily, since the paper strip does not distinguish between L- and D-amino acids, whereas the microörganisms usually do. The occasional utilization of D-residues by bacteria (*cf.* ref. 6) may be avoided.

A number of blocking agents have been used in the present study in a comparative survey of the applicability of various reagents[2] in a microbiological approach. The dinitrophenyl residue[7] has been found to offer difficulties in quantitative applications, an observation which agrees with the report of Consden, Gordon, Martin and Synge.[3] Nitrous acid is not a practical reagent, since some of the resultant hydroxyacids can be utilized microanabolically.[8] Nitrous acid also reacts abnormally with a number of amino acids.[9] Phenyl isocyanate[10] and the thio analog[11] have proved to be useful and they are the type of reagent described here although others are, in principle, applicable. In chemical procedures these agents have also been used in a stepwise manner on peptides containing more than two residues, and this is a principal reason for studying the microbiological applicability of this kind of blocking group.

Model studies were performed on glycyl-DL-valine, L-prolyl-L-leucine, L-valyl-D-valine and D-valyl-L-valine, leucylphenylalanine, leucylvaline and valylleucine, and valylglycylphenylalanine (Table I).

The operating conditions first arrived at, and presented in the experimental section, proved adequate for conclusive results with all of the above dipeptides except glycyl-DL-valine. After treatment, recovery of 19% glycine indicated, however, sufficiently large quantitative loss in this residue that the correct sequence could be deduced from microbiological results even though the paper-strip picture was equivocal. The 19% recovery was smaller than a 41% value obtained when the isothiocyanate/peptide ratio was half of that given in the experimental section. When the blocking reaction was carried out with added alkali, the glycine recovery was eliminated entirely. The pertinent reaction conditions are being studied further; this one instance with glycine however suggests that a generally standard procedure may be described only after peptides of all amino acids in the free amino

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