value of x_α in Table III is 2.56, so that the mole ratio of the two components is alpha:beta = 2.56:4.68.

It was suggested that the results might be due to the accumulation of hydrogen ion in the reaction medium. Conceivably, such an accumulation could repress a simultaneous bimolecular elimination reaction by either ethoxide or hydroxide ion. However, the addition of sufficient hydrogen chloride to the reaction medium to make it 0.05 *N* in hydrogen ion (equivalent to the acidity of a completely hydrolyzed solution of the tertiary chloride) did not noticeably alter the results. Nor did decreasing the initial concentration of the tertiary chloride to one-half the usual value affect the results. In both experiments the data could be analyzed into two first order rate constants in good agreement with the data in Table III.

Rate Data for "Methyldiisopropylcarbinyl Chloride."—The tertiary chloride obtained from methyldiisopropylcarbinol also hydrolyzes at a rate which appears to decrease with time. The calculated first order rate constant decreases from the value 0.143 at 0.50 hour, to 0.90 at 5.00 hours, to 0.045 at 25.00 hours. These data were analyzed in a manner identical with that described for "diethyl-*t*-butylcarbinyl chloride" into $k_1^\alpha = 0.46$ and $k_1^\beta = 0.032$.

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Summary

1. It is suggested that the peculiar behavior of the highly branched carbon compounds is to be attributed in part to a steric effect. The attachment of three or four alkyl groups to a single carbon atom introduces a center of strain. This strain facilitates reactions which involve the formation of less strained carbonium ions, the rearrangement of atoms or groups of atoms located at the strained center, or the rupture of carbon-to-carbon bonds at this center.

2. The need for quantitative data to test the proposal led to an investigation of the rate of hydrolysis of twelve tertiary alkyl chlorides in 80% ethanol at 25°. The effect of structural changes on the rate of hydrolysis cannot be explained on the basis of polar effects alone. With the aid of the steric strain hypothesis, a satisfactory interpretation of the observed facts is possible.

3. Certain peculiarities in the experimental data obtained with chlorides prepared from diethyl-*t*-butylcarbinol, methyldiisopropylcarbinol, and diethylisopropylcarbinol are explained in terms of a facile rearrangement accompanying the reaction of these carbinols with hydrochloric acid. It is suggested that no tertiary chloride containing a branch in the beta position has ever been prepared in pure form from the corresponding alcohol, with the exception of those few cases where the rearranged product is identical with the original structure.

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[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF MERCK & CO., INC.]

Vitamin B_{12}. IV.[1] Further Characterization of Vitamin B_{12}

BY NORMAN G. BRINK, DONALD E. WOLF, EDWARD KACZKA, EDWARD L. RICKES, FRANK R. KONIUSZY, THOMAS R. WOOD AND KARL FOLKERS

Some chemical, physical and biological properties of crystalline vitamine B_{12} from liver[1a] and from a culture broth[1c] of a grisein-producing strain of *Streptomyces griseus* have been reported. The finding that vitamin B_{12} is a cobalt coördination complex which also contains nitrogen and phosphorus was described.[1b] Further observations on the physical and chemical characterization of this extremely biologically active vitamin are described herein.

Samples of crystalline vitamin B_{12} have had a cobalt content of about 4.5%, corresponding to an approximate minimum molecular weight of 1300. An ebullioscopic determination of the molecular weight carried out in methanol solution gave a value of 1490 ± 150, showing that the molecule contains one atom of cobalt.

The analytical data indicate that vitamin B_{12} has a composition typified by but not necessarily limited to $C_{61-64}H_{86-92}N_{14}O_{13}PCo$. Numerically, the formulas $C_{62}H_{86-90}N_{14}O_{13}PCo$ and $C_{63}H_{88-92}N_{14}O_{13}PCo$ agree excellently with the analytical results.

Vitamin B_{12} is optically active. The intense red color of its aqueous solutions made observations difficult, but working in dilute solution at the red end of the spectrum, a specific rotation of $[\alpha]^{23}_{6563} = -59 \pm 9°$ was observed. Vitamin B_{12} is a polyacidic base, as revealed by potentiometric titration in glacial acetic acid solution. The basic groups are quite weak, however, and were not detected when the compound was titrated in aqueous solution.

It is of considerable initial interest to determine the type or class of organic structure to which vitamin B_{12} belongs. Prior to its isolation in crystalline form, some investigators have reported the presence of amino acids in acid hydrolyzates of concentrates of the antipernicious anemia factor at progressing stages in the purification,[2] and the association of activity with a polypeptide was

(1) Three previous papers have contained the initial information on the properties of vitamin B_{12}: (a) I, Rickes, Brink, Koniuszy, Wood and Folkers, *Science*, **107**, 396 (1948); (b) II, *ibid.*, **108**, 134 (1948); (c) III, *ibid.*, **108**, 634 (1948).

(2) For a review by SubbaRow, Hastings and Elkin on earlier investigations, see "Vitamins and Hormones," Vol. III, 257, Academic Press, Inc., New York, N. Y., 1945.

considered. More recently, the clinical activity of pteroylglutamic acid in treating patients with pernicious anemia led to the question of a chemical structural relationship between vitamin B_{12} and the pterins. *A priori*, the activity of vitamin B_{12} in blood regeneration might also suggest a possible relationship to the nucleic acids or to the porphyrins. Observations bearing on the nature of vitamin B_{12} are as follows.

The absorption spectrum of vitamin B_{12} in aqueous solution is characterized by maxima at 2780 A. ($E^{1\%}_{1\,cm.}$ = 115), at 3610 Å. ($E^{1\%}_{1\,cm.}$ = 204), and at 5500 Å. ($E^{1\%}_{1\,cm.}$ = 63). The spectrum does not change markedly with change of *p*H; in acid solution the intensity of the 3610 Å. band decreases by about 10%, and in alkaline solution there are other small changes and the fine structure becomes less marked. The ultraviolet absorption spectrum of pteroylglutamic acid in solution at *p*H 7 shows bands at 2800 Å. ($E^{1\%}_{1\,cm.}$ = 679) and 3475 Å. ($E^{1\%}_{1\,cm.}$ 186), and this spectrum changes considerably with a change to either acidic or alkaline solution. Comparison of the spectra of vitamin B_{12} and pteroylglutamic acid fails to indicate the presence in vitamin B_{12} of a pterin-type structure.

Crystalline vitamin B_{12} has been examined after acid hydrolysis for amino acids by paper partition chromatography[3] and by microbiological assay. From hydrolyzates of vitamin B_{12}, no amino acids could be detected. Therefore, vitamin B_{12} is not a polypeptide and does not appear to contain even one α-amino acid moiety.[4]

The vapors from the pyrolysis of a small sample of vitamin B_{12} give a positive pine splinter test. A pyrrole or porphyrin-like structure would be expected to give a positive result in this classical test, although the test is non-specific for such structures. Partly because of this result, an alkaline fusion of vitamin B_{12} was carried out. Hematoporphyrin was degraded by Piloty and Merzbacher[5] to a mixture of pyrroles by an alkali fusion reaction. A mixture of vitamin B_{12} and sodium hydroxide was heated with a free flame, and the distillate was collected and treated with Ehrlich reagent (*p*-dimethylaminobenzaldehyde) and concentrated hydrochloric acid. The typical red color indicative of pyrroles and certain cyclic five-membered nitrogen-containing nuclei was observed. Treatment of such distillates with aqueous mercuric chloride gave an immediate precipitation of nearly white solid. Control experiments on known pyrroles showed similar behavior. Further examination of the products in such distillates is being made.

Isolation of a crystalline antipernicious anemia factor from liver has been reported by Smith and Parker.[6] It was also found[7] to contain cobalt. Since this product showed 4.0% cobalt and a molecular weight of about 1600, it is closely related to or identical with vitamin B_{12}.

More recently, Ellis, Petrow and Snook[8] have reported a crystalline antipernicious anemia factor from liver, and which contains 4.0% cobalt. Their reported ultraviolet absorption spectrum is essentially identical with that of vitamin B_{12} except for small differences in the intensities of the bands. They found that their product upon acid hydrolysis and paper chromatography consistently revealed the presence of only one substance, unidentified, which gave a purple color with ninhydrin. A sample of their crystalline factor has been received through the courtesy of Dr. Frank Hartley of the British Drug Houses, Ltd., and we have compared this sample with vitamin B_{12}. By our techniques, the crystals darkened to black at 190–215° on the hot stage and did not melt below 300°. The addition of this crystalline factor to a saturated solution of vitamin B_{12} in 80% acetone did not result in a significant change in the concentration of the supernatant solution, although the factor itself was soluble in the solvent mixture alone. The material showed an activity of about 10×10^6 u./mg. (average value) for the growth of *L. lactis*. Thus, the product of Ellis, Petrow and Snook appears to be identical with our vitamin B_{12}. Their product[8] of acid hydrolysis which reacted with ninhydrin appears to be due to residual impurities. They examined a hydrolysis mixture and observed no evidence for the presence of purines indicative of a nucleic acid-like structure.

Experimental

Analyses of Vitamin B_{12}.—Samples of material were dried for analysis in a weighing pig *in vacuo* for two hours. For a sample dried at 100°, found: C, 56.35, 56.11; H, 6.72, 6.72; N, 14.51, 14.76; P, 2.24, 2.27; Co, 4.42, 4.58. For a sample dried at 140°, found: C, 56.21; H, 6.70; N, 14.53; Co, 4.45. Calcd. for $C_{62}H_{88}N_{14}O_{13}PCo$: C, 56.10; H, 6.68; N, 14.77; P, 2.34; Co, 4.44. Calcd. for $C_{63}H_{90}N_{14}O_{13}PCo$: C, 56.40; H, 6.76; N, 14.62; P, 2.31; Co, 4.39.

Examination of Vitamin B_{12} Hydrolyzates for Amino Acids.—A 6.3-mg. sample of vitamin B_{12} was hydrolyzed in 6 *N* hydrochloric acid for sixteen hours at 150°. The solution was filtered and evaporated to dryness. The residue was dissolved in water, the *p*H adjusted to 6.5, and the solution was diluted to a volume of 8 ml. No leucine, isoleucine or valine could be detected by microbiological assay. In a second experiment, the hydrolyzate from 5.1 mg. of another sample of vitamin B_{12} was subjected to paper chromatography in one dimension, using phenol as the developer. After the paper strip had been dried, no

(3) Consden, Gorden and Martin, *Biochem. J.*, **38**, 224 (1944).

(4) From hydrolysis of certain early samples of crystalline vitamin B_{12} containing small amounts of impurities, aspartic acid, glutamic acid, alanine, proline, valine, and either leucine, norleucine or isoleucine were identified by the paper chromatographic technique, and at least one other substance giving a purple color with ninhydrin was observed. The yields, however, were extremely low and variable, being of the order of 0.5 to 2%. Therefore, it is evident that a peptide can be carried along with certain samples of crystalline vitamin B_{12} but that such contaminants can be eliminated by recrystallization.

(5) Piloty and Merzbacher, *Ber.*, **42**, 3258 (1909).

(6) Smith and Parker, *Biochem. J.*, **43**, Proc. VIII (1948).

(7) Smith, *Nature*, **162**, 144 (1948).

(8) Ellis, Petrow and Snook, *J. Pharm. and Pharmacol.*, **1**, 6 (1949).

substances giving colored spots with ninhydrin were observed. Since 1 μg. or less of an amino acid could have been detected by this method, it would appear that no more than 0.2% (theoretical yield) of any amino acid was present.

Alkaline Fusion of Vitamin B_{12}.—Ten milligrams of vitamin B_{12} was ground in a mortar with 100 mg. of sodium hydroxide. The powdered mixture was placed in a tube, moistened with water, and the tube was heated slowly to 250°. The aqueous distillate gave a negative test with Ehrlich reagent. The bottom of the tube containing the alkali melt was then gently heated with a free flame. A small amount of distillate collected on the cool upper walls of the tube. The distillate was washed from the tube with a few drops of methanol and the solution treated with a drop of Ehrlich reagent (*p*-dimethylaminobenzaldehyde in ethanol) and a drop of concentrated hydrochloric acid. A deep red color developed immediately.

In another experiment, the fusion was carried out at reduced pressure and the distillate was collected in a dry-ice trap. The distillate gave a strong positive test with Ehrlich reagent, and when added dropwise to a 4% aqueous solution of mercuric chloride, precipitation took place immediately.

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Summary

The vitamin B_{12} molecule possesses one cobalt and one phosphorus atom. An ebullioscopic molecular weight determination gave a value of 1490 ± 150. Its composition is typified by $C_{61-64}H_{86-92}N_{14}O_{13}PCo$, with $C_{62}H_{86-90}N_{14}O_{13}PCo$ and $C_{63}H_{88-92}N_{14}O_{13}PCo$ agreeing very well with the analytical data.

Vitamin B_{12} is levorotatory, and shows absorption maxima at 2780, 3610 and 5500 Å. which do not shift markedly with a change in *p*H.

Hydrolysis of vitamin B_{12} does not liberate α-amino acids; thus, the molecule is not a peptide. Alkali fusion of vitamin B_{12} forms products which react with *p*-dimethylaminobenzaldehyde, characteristic of certain cyclic five-membered nitrogen-containing compounds including pyrroles.

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[CONTRIBUTION FROM THE MASSACHUSETTS INSTITUTE OF TECHNOLOGY, DEPARTMENT OF CHEMISTRY]

A New Synthetic Route to Peptides

BY JOHN C. SHEEHAN AND VICTOR S. FRANK[1]

Knowledge of protein structure gained through the synthesis and study of relatively simple peptides has contributed enormously to a better understanding of the chemical nature of toxins, antibodies, hormones, enzymes and viruses. With the recent discoveries of the peptide nature of certain antibiotic substances, including gramicidin,[2] tyrocidin[3] and diplococcin,[4] there is renewed interest in practical methods for the synthesis of peptides.

Excellent reviews of the early work on the chemistry of the peptides may be found in the collected papers of Fischer[5] and of Abderhalden.[6] Bergmann has edited a volume[7] in which the field was reviewed to 1923, and Greenstein has written a more recent survey[8] of peptide syntheses.

Most of the methods for peptide synthesis involve the protection of the amino group while the carboxyl function is converted to an acid chloride, anhydride, azide or ester for coupling with a second amino acid or peptide. Removal of the masking group completes the synthesis. The procedures of Fischer include the use of diketopiperazines,[9] α-haloacyl halides,[10] amino acid chloride hydrochlorides[11] and esters of peptides.[12] N-Carboxyamino acid anhydrides[13] were also employed. Bergmann's azlactone methods[14] permitted the synthesis of peptides containing tyrosine, arginine, histidine, glutamic acid and other amino acids more complex than Fischer was able to use. Blocking groups which could not be removed without destruction of the peptide include the benzoyl,[15] carbomethoxy[16] and carboethoxy.[10]

The introduction, in 1932, by Bergmann and Zervas[17] of the use of the carbobenzoxy group (removable by hydrogenolysis) in peptide synthesis made practical the preparation of a wide variety of peptides. Bergmann's procedure has been used very extensively by other workers, including Dunn, Fruton, Greenstein, Harington, du Vig-

(1) Present address: Research Laboratories, Merck and Company, Incorporated, Rahway, New Jersey.

(2) Hotchkiss and Dubos, *J. Biol. Chem.*, **132**, 793 (1940).

(3) Hotchkiss, *ibid.*, **141**, 171 (1941); Cristensen, Edwards and Piersma, *ibid.*, **141**, 187 (1941).

(4) Oxford, *Biochem. J.*, **38**, 178 (1944).

(5) Fischer, "Untersuchungen über Aminosäuren, Polypeptide und Proteine," Vol. I, Springer, Berlin, 1909.

(6) Abderhalden, "Neuere Ergebnisse auf dem Gebiete der Speziellen Eiweisschemie," Fischer, Jena, 1909.

(7) Bergmann, "Untersuchungen über Aminosäuren, Polypeptide und Proteine," Vol. II, Springer, Berlin, 1923.

(8) Schmidt, "The Chemistry of Amino Acids and Proteins," C. C. Thomas, Springfield, Ill., 1945, pp. 252-333.

(9) Fischer and Fourneau, *Ber.*, **34**, 2868 (1901).

(10) Fischer and Otto, *ibid.*, **36**, 2106, 2982 (1903).

(11) Fischer, *ibid.*, **38**, 2914 (1905).

(12) Fischer, *ibid.*, **39**, 453, 3893 (1906).

(13) Sigmund and Wessely, *Z. physiol. Chem.*, **157**, 91 (1926).

(14) Bergmann, Stern and Witte, *Ann.*, **449**, 277 (1926).

(15) Curtius, *J. prakt. Chem.*, **26**, 175 (1882).

(16) Fischer, *Ber.*, **41**, 2860 (1908).

(17) Bergmann and Zervas, *ibid.*, **65**, 1192 (1932).