787. *Chemistry of the Vitamin B_{12} Group. Part III.* The Course of Hydrolytic Degradations.*

By J. B. Armitage, J. R. Cannon, A. W. Johnson, L. F. J. Parker, E. Lester Smith, W. H. Stafford, and A. R. Todd.

The hydrolysis of vitamin B_{12} under a wide variety of experimental conditions has been studied. Electrophoresis on paper has proved most useful for analysing the complex mixtures of basic, neutral, and acidic products so formed. Vitamin B_{12} contains three primary amide groups, which can be hydrolysed stepwise, under comparatively mild conditions, to the corresponding acids. The acids so obtained, three mono-, three di-, and one tri-basic, have all been reconverted into the parent vitamin. Conditions for the hydrolytic removal of the 5 : 6-dimethylbenziminazole nucleotide, both before and after hydrolysis of the amide groups, have been defined and the crystalline free nucleotide has been isolated. 1-Aminopropan-2-ol is believed to be attached by ester linkage to the nucleotide and by amide linkage to the rest of the molecule. The moderately stable cobalt-containing end products from vigorous acid hydrolysis contain five, six, and seven acidic groupings and, from alkaline hydrolysis, five and six acidic groupings.

The combined results of the degradative work so far reported on vitamin B_{12}, the anti-pernicious anaemia factor, have led to the partial formula (I) for the vitamin. Its precise molecular formula is not known with certainty, but $C_{61-64}H_{84-92}O_{13-14}N_{14}PCo$ covers the

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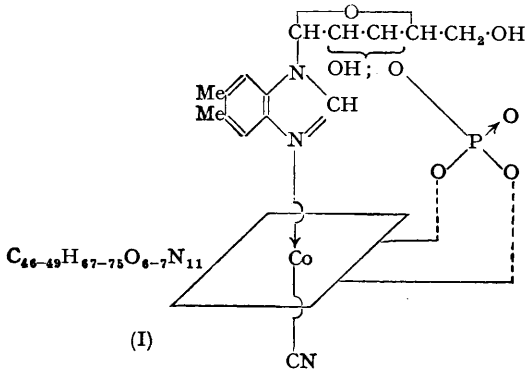
figures so far published (Brink, Wolf, Kaczka, Rickes, Koniuszy, Wood, and Folkers, *J. Amer. Chem. Soc.*, 1949, **71**, 1854; Alicino, *ibid.*, 1951, **73**, 4051).



(I)

A feature of the structure (I) is the 5 : 6-dimethyl-1-(α-D-ribofuranosyl)benziminazole-2′(or 3′) phosphate residue, which must be fully esterified as the vitamin itself contains no free acidic groups. The benziminazole nucleotide has been isolated from the products of acid hydrolysis of vitamin B_{12}, both as the barium salt (Buchanan, Johnson, Mills, and Todd, *J.*, 1950, 2845) and as the free acid (Kaczka, Heyl, Jones, and Folkers, *J. Amer. Chem. Soc.*, 1952, **74**, 5549; see also Experimental section), and a synthesis has been reported (Kaczka *et al.*, *loc. cit.*), although the point of attachment of the phosphate to the sugar side-chain (*i.e.*, whether 2′ or 3′) is still uncertain. The corresponding nucleoside has also been isolated from acid hydrolysates (Brink and Folkers, *J. Amer. Chem. Soc.*, 1952, **74**, 2856) and its structure, 5 : 6-dimethyl-1-(α-D-ribofuranosyl)benziminazole, has been confirmed by synthesis (Holly, Shunk, Peel, Cahill, Lavigne, and Folkers, *ibid.*, p. 4521); 5 : 6-dimethylbenziminazole itself has been isolated from the products of vigorous acid hydrolysis of the vitamin (Brink and Folkers, *ibid.*, 1950, **72**, 4442; Beaven, Holiday, Johnson, Ellis, Mamalis, Petrow, and Sturgeon, *J. Pharm. Pharmacol.*, 1949, **1**, 957).

Ammonia and D$_g$-1-aminopropan-2-ol (Wolf, Jones, Valiant, and Folkers, *J. Amer. Chem. Soc.*, 1950, **72**, 2820) are also produced on acid hydrolysis, and it has been claimed by Chargaff, Levine, Green, and Kream (*Experientia*, 1950, **6**, 229) that two moles of the alkanolamine and four—five moles of ammonia are formed from each mole of the vitamin after hydrolysis with 6N-hydrochloric acid for 6 hr. at 100° in a sealed tube. On the other hand Cooley, Davies, Ellis, Petrow, and Sturgeon (*J. Pharm. Pharmacol.*, 1953, **5**, 257) found only one mole of aminopropanol and approximately six moles of ammonia. Under the conditions of hydrolysis specified by Cooley *et al.* we have obtained results similar to theirs, but using stronger acid (11N) we have at times obtained values approximating to 2 moles of aminopropanol per mole. Although at present we have no satisfactory explanation of the anomalous results with stronger acid, we believe that the balance of evidence favours the conclusion of Cooley *et al.* that only one aminopropanol residue is present. Hydrolysis of the vitamin with hot saturated barium hydroxide yielded *ca.* 5 moles of ammonia per mole. There is as yet no precise information on how these bases are combined within the vitamin B_{12} molecule.

Of the other features in the partial structure (I), the presence of the cyanide group was deduced from the results of permanganate oxidation (Brink, Kuehl, and Folkers, *Science*, 1950, **112**, 354), and the co-ordinate linkage between $N_{(3)}$ of the benziminazole nucleus and the cobalt atom was postulated on spectroscopic evidence (Beaven, Holiday, Johnson, Ellis, and Petrow, *J. Pharm. Pharmacol.*, 1950, **2**, 733, 944). Vitamin B_{12}, now officially designated cyanocobalamin (Kaczka, Wolf, Kuehl, and Folkers, *Science*, 1950, **112**, 354; I.U.P.A.C., *J.*, 1951, 3526), may be converted into other members of the cobalamin series, *e.g.* vitamin B_{12a} (or B_{12b}) (hydroxocobalamin) or B_{12c} (nitritocobalamin), by replacement of the cyano-group with one of several other residues (Kaczka, Wolf, Kuehl, and Folkers, *J. Amer. Chem. Soc.*, 1951, **73**, 3569; Lester Smith, Ball, and Ireland, *Biochem. J.*, 1952,

52, 395). The other cobalamins can be reconverted into vitamin B_{12} itself by treatment with cyanide ions.

The remainder of the vitamin B_{12} molecule is obtained in the acid hydrolytic products as a red cobalt-containing gum, which is acidic and from its physical nature and from the results of simple partition experiments is obviously a mixture. The acidic groups are esterified when alcoholic solutions of acids are used for the hydrolysis of the vitamin. Partition distribution has been employed by Schmid, Ebnöther, and Karrer (*Helv. Chim. Acta*, 1953, **36**, 65) in an attempt to separate the components of the esterified pigment mixture obtained from hydrolysis of vitamin B_{12} with hydrogen chloride. This method of hydrolysis has also been examined in the present investigation and the product shown to be a complex mixture. Magnetic evidence (Diehl, Vander Haar, and Sealock, *J. Amer. Chem. Soc.*, 1950, **72**, 5312; *Record Chem. Progr.*, 1952, 9; Grün and Menassé, *Experientia*, 1950, **6**, 263; Wallmann, Cunningham, and Calvin, *Science*, 1951, **113**, 55) and polarographic studies (Diehl, Morrison, and Sealock, *Experientia*, 1951, **7**, 60; Lester Smith, Fantes, Ball, Waller, Emery, Anslow, and Walker, *Biochem. J.*, 1952, **52**, 389) have shown that the cobalt in vitamin B_{12} is in the tervalent form, and the stability of the complex, *e.g.*, to precipitating agents such as hydrogen sulphide (Kaczka, Wolf, Kuehl, and Folkers, *loc. cit.*), to acid hydrolysis, and to exchange with radioactive cobalt (Fantes, Page, Parker, and Lester Smith, *Proc. Roy. Soc.*, 1949, *B*, **136**, 592; Baldwin, Lowry, and Harrington, *J. Amer. Chem. Soc.*, 1951, **73**, 4968; Boos, Rosenblum, and Woodbury, *ibid.*, p. 5446), indicates that the cobalt is bound very tightly. The possibility that vitamin B_{12} might be a cobalt porphyrin has been considered (cf. also McConnel, Overell, Petrow, and Sturgeon, *J. Pharm. Pharmacol.*, 1953, **5**, 179), but the ultra-violet absorption spectrum of the vitamin does not contain the usual Soret band characteristic of the porphyrins (cf. Lemberg and Legge, " Haematin Compounds and Bile Pigments," Interscience Publ., New York, 1949, p. 72). Controlled chromic acid oxidations of porphyrins are known to yield maleinimides (Muir and Neuberger, *Biochem. J.*, 1949, **45**, 163; 1950, **47**, 97; Wittenburg and Shemin, *J. Biol. Chem.*, 1950, **185**, 103; 1951, **192**, 315), but none was obtained from similar oxidations of the esterified resinous cobalt-containing hydrolysate. Dr. J. G. Buchanan, working at Cambridge, has shown that maleinimides can be detected conveniently on paper chromatograms by exposing the paper to chlorine or by spraying it with saturated aqueous bromine water, removing the excess halogen and then treating it with a potassium iodide–starch solution. A similar method for the detection of peptides and related compounds has been described by Rydon and Smith (*Nature*, 1952, **169**, 922).

Experiments carried out in these laboratories have for some time been centred on a study of the cobalt-containing hydrolytic products from vitamin B_{12}. Hydrolyses with acid have been carried out under divers conditions, but a complex mixture of cobalt-containing compounds has always been obtained. Hydrolyses of the vitamin with aqueous sodium or barium hydroxide have also been examined in detail; the products formed under various conditions are closely similar to those obtained from acid hydrolyses, *viz.*, ammonia, 1-aminopropan-2-ol, the 5 : 6-dimethylbenziminazole nucleotide, and mixed red cobalt-containing pigments (Tables 1 and 2, pp. 3857, 3858).

Electrophoretic experiments have furnished an overall picture of the various steps involved in the hydrolysis of vitamin B_{12}, under either acid or alkaline conditions. Except in hydrolyses with concentrated acids, which will be considered later, treatment with acid or alkali brings about several changes, including the hydrolysis of the cyanide group and the evolution of some ammonia, before the nucleotide is liberated, and this in turn occurs before the aminopropanol is set free. Finally, other transformations, not yet fully understood, are brought about under more vigorous conditions before relatively stable compounds are produced. Ionic cobalt has not been liberated under any of the hydrolytic conditions so far investigated, and the red colour of the pigments persists throughout.

The course of hydrolysis is extremely complex and in the following account it may be of assistance to indicate first of all the general methods employed, and the basic features of our interpretation of results, and to follow this by discussion of the products obtained by hydrolysis with and without removal of the nucleotide portion of the molecule.

Methods.—Several methods have been devised for separating both the nucleotide and

the mixed pigments from such a mixture of products, the most successful being those depending on the application of electrophoretic techniques or of ion-exchange resins. Thus a column of the basic resin Amberlite IR-4B retains the nucleotide and the red pigments can sometimes be eluted without difficulty. When applicable this represents a most convenient method for isolating both nucleotide and pigments.

On electrophoresis in N-acetic acid on filter paper strips, the 5 : 6-dimethylbenziminazole nucleotide travels towards the cathode with low mobility, and can be effectively separated in this way from the corresponding nucleoside as well as from free 5 : 6-dimethylbenziminazole and the cobalt-containing pigments. Electrophoresis on paper at pH 6·5 or 10 is invaluable for separating the cobalt-containing pigments into groups differing in the number of ionic charges on the molecules. For the electrophoretic separation of the components of the pigment mixture, the most convenient solvents have been aqueous cyanide solutions, *e.g.*, 0·1N-potassium cyanide (pH *ca.* 10) or 0·05N-phosphate buffer at pH 6·5 containing 0·01% of potassium cyanide. The separations were carried out in the first place using Whatman No. 4 or No. 31 extra thick paper with a voltage gradient of between 3 and 13 v/cm. In attempts to apply this technique to the preparative separation of the various groups of acids, columns of cellulose powder have been used, but for smaller-scale work (50 mg. or less) Whatman seed-testing paper (thickness 1·5 mm.) has been most satisfactory. Durrum's method (*J. Amer. Chem. Soc.*, 1951, **73**, 4875) of continuous electrophoresis on a vertical sheet of filter paper has also been used with some success.

Electrophoretic Behaviour of Compounds retaining the Nucleotide.—Suitable hydrolysates, on electrophoresis at pH 6·5, display five uniformly spaced red zones. The first of these (unchanged vitamin B_{12}) has zero mobility, while the others move one, two, three, etc., units of distance towards the anode. This is interpreted as indicating a series of compounds having roughly similar molecular weights and containing 0—4 acidic groupings.

Vitamin B_{12}, on treatment with aqueous potassium cyanide, forms a purple complex, which contains two cyanide groups (Wijmenga, Veer, and Lens, *Biochem. Biophys. Acta*, 1950, **6**, 229; Ellis, Petrow, Beaven, Holiday, and Johnson, *J. Pharm. Pharmacol.*, 1950, **2**, 735; Lester Smith, Ball, and Ireland, *Biochem. J.*, 1952, **52**, 395). Thus on electrophoresis in cyanide solution at pH 10 it forms the dicyanide complex, which is weakly acidic and therefore moves towards the anode as a purple zone.

The acidic vitamin B_{12} degradation products that still retain the nucleotide behave in a precisely analogous manner; at pH 6·5 the electrophoresis zones are red, whereas at pH 10 they are purple and display an additional negative charge due to the second cyanide group.

Electrophoretic Behaviour of Compounds lacking the Nucleotide.—The dicyanide complexes of vitamin B_{12} and the related series of acids are unstable at even slightly acid pH. In contrast, those of the nucleotide-free compounds are more stable. Consequently on electrophoresis in buffer solutions containing cyanide, either at pH 6·5 or at pH 10, compounds in this series travel as purple zones. They form another series of uniformly spaced zones, eight in all, containing 0—7 acidic groupings. On account of their lower molecular weight, these products without combined nucleotide have a mobility about 10% greater than the corresponding nucleotide-containing ones.

Hydrolysates containing products in both series separate into alternate red and purple zones on electrophoresis at pH 6·5. At pH 10 both travel as purple zones, but distinction is still possible because on exposure to the atmosphere the nucleotide-containing products change to red, whereas the nucleotide-free products remain purple for several weeks in the dark.

The purple compound of zero mobility that arises on removal of the nucleotide from vitamin B_{12} was at first presumed to contain a basic group which neutralised the acidity due to the second cyanide group. Since, however, it behaves as a neutral substance on electrophoresis between pH 6·5 and 13, it appears more likely that some group directly attached to the cobalt in vitamin B_{12} has been changed from acidic to neutral (*i.e.*, change from a covalent to a co-ordinate bond), thus compensating for the extra cyanide group and maintaining the neutrality of the co-ordination complex. In acid solutions the colour changes from purple to orange with loss of cyanide, and the molecule displays weakly

basic properties. On electrophoresis in N-acetic acid at pH *ca.* 2, for example, the compound moves towards the cathode, to about the same distance as vitamin B_{12b} (which is also basic because a neutral water molecule is attached to the cobalt in place of the CN^- group in vitamin B_{12}).

The series of acids lacking nucleotide appear to have the same co-ordination state as the parent substance; they also move as orange zones to the cathode on electrophoresis at pH 2, when the ionisation of the carboxyl groups is suppressed. This leads to the phenomenon that in the pH 6·5 electrophoresis pattern of a suitable hydrolysate, adjacent red and purple zones contain compounds with the same number of carboxyl groups; at pH 10, on the contrary, when all the zones are purple, a compound without the nucleotide contains one more carboxyl group than the adjacent zone from a compound retaining the nucleotide, because only in the latter series does the second cyanide group confer effective acidity on the molecule. Vitamin B_{12}, for example, as dicyanocobalamin, travels nearly as far towards the anode as the monocarboxylic acid lacking the nucleotide.

Hydrolysis with Retention of Nucleotide. Polyamide Character of Vitamin B_{12}.—When vitamin B_{12} was hydrolysed with N/50-hydrochloric acid at room temperature and the course of the reaction was followed by electrophoresis, it was found that after a few days the main products were the unchanged vitamin and a monocarboxylic acid fraction; on further treatment, up to several months, the amounts of these products decreased, and there was a progressive increase in the amounts of di- and tri-carboxylic acids (see Table 3). Only a trace of a tetracarboxylic acid was obtained under these conditions and the nucleotide was not removed. With N/5-hydrochloric acid the rate of hydrolysis was increased some fifteen times, although the hydrolytic products were apparently identical. Even after nearly all the vitamin B_{12} had disappeared, the colour intensity of these red solutions was diminished. Thus direct colorimetry or spectrophotometry, and also all the indirect colorimetric assay methods for vitamin B_{12} (Fantes, Ireland, and Green, *Biochem. J.*, 1950, **46**, xxxiv; Boxer and Rickards, *Arch. Biochem.*, 1950, **29**, 75; 1951, **30**, 382, 392; Rudkin and Taylor, *Analyt. Chem.*, 1952, **24**, 1155), would give false results for preparations in which partial hydrolysis had occurred. The products from cold alkaline hydrolysis of vitamin B_{12} were also similar.

Each of the zones obtained from the electrophoretic separations at neutral or alkaline pH could consist of a mixture of isomeric carboxylic acids, and sometimes did. Provided that the number of carboxyl groups was not too great, these isomers could be separated by chromatography on paper with, for example, butan-2-ol, containing a trace of acetic acid, as the developing solvent.

On paper chromatography the monocarboxylic acid fraction from electrophoresis of an acid hydrolysate separated into two main zones. The faster-moving zone was always the stronger; under favourable conditions small amounts of a third component of slightly higher R_F values separated from this faster zone. Each of the three zones yielded a crystalline acid. Column partition chromatography with water-saturated butan-1- or -2-ol was ineffective for separating these isomeric acids, even with columns 2—3 feet in length, having damp kieselguhr or cellulose powder as supporting solids. The dicarboxylic acid fraction has also been separated by chromatography on paper into two main zones, and both have yielded crystalline acids. A third minor dicarboxylic acid sometimes appeared on prolonged chromatography when the amount of starting material was small enough to give narrow zones. The tricarboxylic and tetracarboxylic acid fractions each appeared homogeneous on chromatography; each has yielded a crystalline acid.

The absorption spectra of all of these acids were closely similar to that of vitamin B_{12}. The infra-red spectrum of the main monocarboxylic acid is very similar to that of vitamin B_{12}, but the absorption at 3300 $cm.^{-1}$ is stronger and an extra band at 1715 $cm.^{-1}$ is present in the acid. These features are consistent with the formation of a carboxylic acid group during the hydrolysis. Electrometric titrations have been carried out on some of the acids. The mixed isomeric monocarboxylic acids and the most abundant individual monocarboxylic acid (pK_a 4·67) had equivalent weights of the right order for monocarboxylic acids of about the same molecular weight as vitamin B_{12}. The most abundant of the

dicarboxylic acids similarly had an equivalent weight of the right order for two acidic groups in a molecule of about the same size as vitamin B_{12}. Separate steps from the two acidic groups could not be distinguished (pK_a values 4·25 and 5·57).

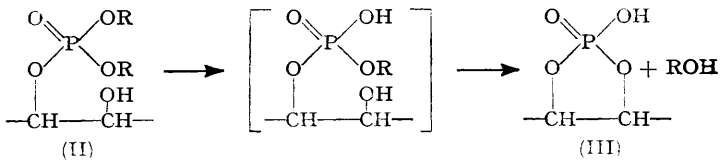
All of these acids contained the combined nucleotide and their formation is explained by postulating the existence of three primary amide groups in vitamin B_{12}, labile to both acids and alkalis, although not to the same degree. Random hydrolysis of these three labile groups should give rise to three isomeric monocarboxylic acids, three dicarboxylic acids, and one tricarboxylic acid. The complete hydrolysis of these amide groups will give rise to three mols. of ammonia, and a further mol. will arise from hydrolysis of the cyano-group. The infra-red absorption spectrum of the vitamin (Lester Smith, *et al.*, *Biochem. J.*, 1952, **52**, 389) shows two broad peaks between 1600 and 1700 cm.$^{-1}$, which would be appropriate for unsubstituted and substituted amide groups respectively, but in such a complex molecule this can be regarded only as supporting evidence at the present stage. However, strong chemical evidence has been obtained by reconverting the mixed isomers of the monobasic acid and also each of the two main crystalline isomers into the corresponding amide, *i.e.*, into vitamin B_{12}. The mixed isomers of the dicarboxylic acid, and the tricarboxylic acid, have also been reconverted into vitamin B_{12}. Several methods tried gave low conversions, *e.g.*, heating the ammonium salts and treatment of the methyl esters with ammonia, but the best method has been the action of ammonia on the mixed anhydride of the acid, obtained by reaction with ethyl chloroformate and triethylamine. Excess of the reagent causes further changes in the molecule, and in fact it was found advisable to treat the final product with 0·1N-sodium hydroxide at room temperature for $\frac{1}{2}$—2 hr. in order to hydrolyse ester groups that were also introduced by the reagent. The pure crystalline neutral product was identified as vitamin B_{12} by microbiological assay, by ultra-violet, visible, and infra-red absorption spectroscopy and by its chromatographic behaviour. In control experiments evidence of reaction was obtained with vitamin B_{12} itself under the conditions used to reconvert the acids into the parent vitamin. By treating vitamin B_{12} with triethylamine and ethyl chloroformate, decomposing the mixture with either water or dilute aqueous ammonia after various periods of reaction, and analysing the products by electrophoresis, it was shown that the amount of the neutral product decreased with increasing time of reaction and that this was more apparent in the samples treated with water than with those treated with ammonia. These side reactions diminish the yields of vitamin B_{12} obtained in the partial syntheses described above.

The rate of production of the acidic degradation products of vitamin B_{12} by treatment with cold or warm 2N-hydrochloric acid is markedly increased by nitrous acid (cf. Plimmer, *J.*, 1925, **127**, 2651). This again confirms the presence of amide groups.

Further evidence for the presence of unsubstituted amide groups in vitamin B_{12} was afforded by reaction with xanthhydrol, which is known to react with amides and urethanes. Reaction evidently occurred under reflux conditions in glacial acetic acid, for the red product was insoluble in water but soluble in acetone and chloroform. Heating vitamin B_{12} in acetic acid alone left the greater part of it unchanged.

Schindler (*Helv. Chim. Acta*, 1951, **34**, 101) has described the hydrolysis of vitamin B_{12} with dilute alkali and 30% hydrogen peroxide at room temperature for 16 hr. His product was fractionated between phenol–chloroform and potassium carbonate or potassium hydrogen carbonate buffers and was separated into " neutral," " weakly acidic," and " strongly acidic " fractions, of which the first two were obtained crystalline, were microbiologically active, and had absorption spectra similar to that of vitamin B_{12}. We have now repeated this work and have found by electrophoresis that these crystalline products are mixtures, emphasising that crystallinity is itself no guarantee of homogeneity in this series. By electrophoresis at pH 6·5 the " neutral " fraction was shown to contain approximately equal amounts of neutral products and monobasic acids with a trace of dibasic acids, and the neutral portion was found by microbiological assay to be substantially unchanged vitamin B_{12}. Chromatography on paper confirmed that this fraction was a mixture. The crystalline " weakly acidic " fraction consisted mainly of monobasic acids with a trace of neutral material, about 20% of dibasic acids, and small

amounts of two orange acidic cobalt-containing compounds. The non-crystalline " strongly acidic " material was also a mixture and consisted mainly of dibasic acids together with some mono- and tri-basic acids. A control experiment carried out in the absence of hydrogen peroxide gave a product showing an electrophoretic pattern similar to the unfractionated preparation made by following Schindler's description, but the hydrolysis was less extensive and the major product was unchanged vitamin B_{12}. This supports Schindler's suggestion that the peroxide might be acting as a hydrolysis catalyst rather than as an oxidising agent.



Except with cold dilute acid or alkali, hydrolysis of the labile amide groups is accompanied or preceded by liberation of the nucleotide. Vitamin B_{12} is an essentially neutral compound and the phosphoric acid grouping is therefore fully esterified. The hydrolysis of such a grouping must follow the same course as that of ribonucleotide esters discussed by Brown and Todd (*J.*, 1952, 52). Triesters of phosphoric acid readily lose one ester group with acids or bases; the rupture of one of the ester linkages in the grouping (II) (unless it were that between the phosphorus and the sugar residue) would yield a diester, which would in the basic or acidic medium at once cyclise with the vicinal sugar-hydroxyl group to yield a cyclic phosphate with elimination of the remaining esterifying residue; subsequent hydrolysis of the cyclic phosphate (III) would yield a mixture of the 2'- and 3'-phosphates of the benziminazole nucleoside.

Hydrolysis with Removal of Nucleotide.—Conditions have been found under which fission of the nucleotide occurs with little attack on the amide linkages. When vitamin B_{12} is treated with concentrated hydrochloric acid at 65° for 5 min., or at room temperature overnight, the main product contains no acidic groups. Removal of the nucleotide was shown by its isolation from the reaction mixture, but the aminopropanol was retained in the compound. Unlike vitamin B_{12} this product loses co-ordinated cyanide readily in solutions more acid than about pH 3. After concentration of an acidified solution to remove liberated hydrocyanic acid, and extraction of the compound from an alkaline solution, it titrates as a weak base (pK_b 6·8). The purple dicyanide complex is, however, neutral, whereas that of vitamin B_{12} is acidic, as revealed by the electrophoretic behaviour described earlier.

The nature of this hydrolytic product is of particular interest, as it is probably identical with one of the microbiologically active cow-manure factors, namely, " Factor B " (Ford and Porter, *Biochem. J.*, 1952, **51**, v). A description of these and other features of the chemistry of this degradation product is being prepared for separate publication.

The compound occurs only in small amounts in the products from dilute acid hydrolyses, because it is very easily hydrolysed with decomposition of the labile amide groups to carboxyl groups as with vitamin B_{12}. The same series of carboxylic acids lacking nucleotide can alternatively be prepared from the mono-, di-, and tri-carboxylic acids arising from mild hydrolysis of vitamin B_{12}, by treatment with concentrated hydrochloric acid for 5 min. at 65°.

None of the compounds without nucleotide has been obtained crystalline. The monocarboxylic acid without nucleotide, *i.e.*, the material recovered from the electrophoresis zone next to that of the neutral compound, titrated as a monocarboxylic acid (pK_a 4·5): moreover, it could be reconverted into the neutral compound (" Factor B ") *via* ethyl chloroformate and ammonia, in the same way as vitamin B_{12} can be resynthesised from the corresponding acid containing the nucleotide residue.

In an endeavour to obtain the relatively stable end products from these hydrolytic degradations, the action of 30% sodium hydroxide solution at 150° on vitamin B_{12} has been studied. After one hour the product was a mixture of compounds having five and

six acidic groupings, nucleotide and aminopropanol fission having occurred. However, after 12 hr. the same compounds were obtained in approximately the same ratio, so that it would appear that the former acid is not an intermediate in the formation of the latter or *vice versa*. Experiments designed to prepare these acids in quantity are at present in hand. Heating with 6N-hydrochloric acid under reflux conditions for an hour longer gave similar mixtures of acids containing five, six and, under certain conditions, seven acidic groupings. The ultra-violet and visible spectra of these acids were still much like those of the parent vitamin and they showed the same kind of shift after treatment with excess of cyanide, suggesting that the chromophoric system had undergone little change.

Conclusion.—The results obtained in the present work permit an expansion of the partial structure (I) to include at least three primary amide groups and probably one $-CO\cdot NH\cdot CH_2\cdot CHMe\cdot O-$ grouping. In the hydrolysis of the vitamin with hot alkali or acid the fission of these groups would account for at least four of the carboxyls observed in the products. The retention of the aminopropanol until the nucleotide has been released, and the absence of basic groups from the main hydrolytic products containing cobalt, support our earlier suggestions that the aminopropanol is esterified by the phosphoric acid and joined by amide linkage to the rest of the molecule (Buchanan, Johnson, Mills, and Todd, *Chem. and Ind.*, 1950, 426).

Experimental

Filter-paper Electrophoretic Analysis of Cobalt-containing Pigment Mixtures from Hydrolysis of Vitamin B_{12}.—(a) *General procedure after hydrolysis.* Cobalt-containing pigment mixtures have been prepared from vitamin B_{12} under a wide variety of acid and alkaline hydrolytic conditions, and also by the action of aqueous solutions of certain salts, *e.g.*, potassium cyanide. In general, the reaction product was acidified, when necessary, with hydrochloric acid and the pigment mixture was extracted into phenol. The phenol layer was separated, washed free from salts and excess of acid with water, and diluted with ether; the pigment was re-extracted into water. The aqueous solution was then evaporated and the residue redissolved in water (or for the more extensively degraded pigments, N-ammonia, as they are not very soluble in cold water) so that a solution of convenient concentration (*ca.* 10 mg./c.c.) was obtained for electrophoretic analysis. In practice a spot of this solution containing 0·1—0·3 mg. of pigment was used. If free 5 : 6-dimethylbenziminazole nucleotide was present it was detected by paper chromatography (Buchanan *et al.*, *loc. cit.*) or by electrophoresis in dilute acetic acid, but it was not necessary to remove it before the analysis of the pigment mixture.

After hydrolyses with volatile reagents (*e.g.*, HCl and NH_3) excess of the reagent could be conveniently removed by evaporation, either under reduced pressure on the water-bath or in a desiccator.

(b) *Apparatus.* The paper strip was supported in the middle by a Perspex frame suspended in a glass tank, and the ends of the paper were immersed in the electrolyte contained in the electrode compartments at the base of the frame. More of the electrolyte was contained in the bottom of the tank itself; the whole apparatus could be closed by a Perspex lid. The solution of the pigment mixture was applied on the apex of the paper as a spot that was allowed to dry, and then the electrode compartments were filled. The solution of the electrolyte moved up each side of the paper by capillary action as two fronts, which finally met at the top and caused the spots to contract to narrow bands on the starting line. After equilibration of the contents of the tank for about 10 min., the voltage was applied. Apparatus of rather similar design has been described by Flynn and de Mayo (*Lancet*, 1951, **261**, 235). Those described by Latner (*Biochem. J.*, 1952, **51**, xii) and by Consden and Stainer (*Nature*, 1952, **170**, 1069) have also been used for the work described here.

Several types of paper have been used. Whatman No. 2 or No. 4, and occasionally No. 54, were satisfactory for analytical work; Whatman No. 3 or No. 31 (as sheets 18 cm. in width) was used for separating up to 10 mg. of the pigment mixture, and for larger quantities (up to 50 mg.) Whatman "seed-test paper" (as sheets 27 cm. in width) was a convenient support. With the last the pigment mixture was applied as a continuous line across the paper; separations achieved by this method were much better than those obtained on columns of cellulose powder or kieselguhr. The column technique was satisfactory only when the components of a mixture differed greatly in electrophoretic mobility, *e.g.*, in separating the nucleotide from the pigment mixture in N-acetic acid electrolyte.

TABLE 1. *Electrophoretic analysis of the cobalt-containing hydrolytic products from vitamin B_{12}.*

Electrolyte: 0·1M-KCN (*i.e.*, an extra negative charge is acquired by compounds retaining nucleotide; thus vitamin B_{12} behaves as a monobasic acid). 4·6 v/cm. for 5½—6 hr.

\+ Weak, ++ medium, +++ strong pink spot after drying paper, *i.e.*, nucleotide present in pigment.

× Weak, ×× medium, ××× strong purple spot after drying paper, *i.e.*, nucleotide absent.

Conditions of hydrolysis	Effective negative charges on pigment spot: 0	1	2	3	4	5	6	7
Vitamin B_{12}, B_{12b}, B_{12c} (standards) ...		+++						
Aqueous ammonia (*d* 0·880):								
room temp.; overnight		+++	+					
100°; 30 min. on water-bath		+++	+					
100°; 12 hr. on water-bath		++	++	+				
100°; sealed tube; 8 hr.			++	++	++			
125°; sealed tube; 48 hr.			××	××	××	×		
Saturated methanolic ammonia:								
room temp.; overnight		+++	+					
,, 6 days		++	++					
,, 6 days, then refluxed 1 hr.		++	++					
5% Methanolic sodium methoxide:								
room temp.; overnight		+++	+					
,, 6 days		++	++					
,, 6 days, then refluxed 1 hr.		++	++					
Saturated (at 17°) aqueous $Ba(OH)_2$:								
room temp.; overnight		++	++	+				
,, 2 days		+	++	++	+			
,, 6 days				+	++×			
,, 6 days, then heated—								
at 100° for 1 hr.				×	××	×		
,, 100° for 3 hr.				×	××	×		
,, 100° for 12 hr.					××	××	×	
,, 100° for 30 hr.					××	××	××	
30% Aqueous sodium hydroxide:								
room temp.; overnight		++	++	++×	++×			
at 150°; 1 hr. (similar results with vitamin B_{12b} or B_{12c})						××	×××	
at 150°; 12 hr.....						××	×××	
,, 150°; 1 hr. (product isolated then treated with 11N-HCl for 1—5 days at room temp.)					××	×××	××	
Lithium chloride in 2-ethoxyethanol (cf. Clark and Todd, *J.*, 1950, 2031):								
at 100°; 4 hr.		+++	+					
,, 100°; 5 hr. then 150° for 3 hr.		Pink streak						
30% Aqueous potassium cyanide:								
room temp.; overnight	×	++	++	+				
at 150°; 1 hr.	×	××	××	××	×			
,, 150°; 3 hr.				××	××	×		
N-Hydrochloric acid at 100°:								
30 min.				×	××	××		
1 hr.					××	××	×	
2 hr.					×	××	××	×
3 hr.					×	××	××	×
6½ hr.						××	××	××
12 hr.						×	××	××
Sealed tube at 100°; 12 hr.							××	××
Further treatment of product from last experiment:								
11N-HCl at 65° for 5 min.							××	××
11N-HCl in sealed tube at 100° for 4 hr.							××	××
30% NaOH at 150° for 1 hr.							××	××

TABLE 1. (*Continued.*)

Conditions of hydrolysis	Effective negative charges on pigment spot: 0	1	2	3	4	5	6	7
2N-Hydrochloric acid at 100° :								
30 min.					××	×××	×	×
1 hr.					×	××	×××	×
2 hr.						×	××	×××
4 hr.							××	×××
8 hr.							×××	××
16 hr.							×××	×
11N-Hydrochloric acid :								
room temp.; overnight	×××	×××	××	×				
,, 3 days	×	××	××	××	×			
at 65° for 5 min.	×××	××	×					
,, 100° for 4 hr. (sealed tube)							××	××
Product from last experiment heated with 30% sodium hydroxide at 150° for 1 hr.							××	××
Saturated methanolic HCl at 20° for 3 days			×	××	××	××		
Acetic acid saturated with HCl :								
at 20° for 18 hr.	×××	++						
,, 100° for 5 min.						××	××	××
Acetic acid at 100° for 4 hr.		+++ (*i.e.*, unchanged)						

TABLE 2. *Electrophoretic analysis of the hydrolytic products from vitamin B_{12}.*

Electrolyte : 0·05M-phosphate buffer (pH 6·5) containing 0·01% of potassium cyanide. B_{12} and pigments containing the nucleotide travel as the monocyanide (*i.e.*, B_{12} co-ordination state), the dicyanide of these pigments being unstable at pH 6·5. The pigments from which the nucleotide has been removed form a dicyanide which is stable at pH 6·5. 12 v/cm. for 4 hr.

+ and × as in Table 1.

Conditions of hydrolysis	Effective negative charges on pigment spot: 0	1	2	3	4	5	6	7
Vitamin B_{12}	+++							
0·02N-Hydrochloric acid :								
room temp.; 6 days	+++							
,, 13 days	+++	+						
,, 20 days	++	++						
,, 35 days	++	+++	+					
0·05N-Sulphurous acid :								
room temp.; 4 days	+++	+						
,, 11 days	++	++						
,, 26 days	+	+++	++					
2N-Sodium hydroxide–30% H_2O_2 (conditions and method of Schindler, *Helv. Chim. Acta*, 1951, **34**, 101) :								
neutral fraction	++	++	+					
weakly acidic fraction	+	++	++					
strongly acidic fraction		++	+++	++				

Different electrolyte solutions were employed at various times. The most useful were 0·1M-potassium cyanide (pH *ca.* 10); 0·05M-phosphate buffer (pH 6·5) containing 0·01% of potassium cyanide; N-acetic acid; and 0·5—2N-acetic acid containing 0·01% of potassium cyanide. The voltage gradient and also the time of running differed with the nature of the paper and also with the electrolyte. With Whatman No. 4 paper voltage gradients of between 4·2 and 13 v/cm. were used, but with the seed-testing paper it was inadvisable to exceed 3·5 v/cm. if considerable distortion of the pigment bands was not to occur. For satisfactory separations at 4·6 v/cm. in 0·1M-potassium cyanide on Whatman No. 4 paper, the time of running was $5\frac{1}{2}$—$6\frac{1}{2}$ hr., but with the seed-testing paper at 2·8 v/cm. 12 hr. were required for

good separation of the bands. At 4·2 v/cm. in N-acetic acid on Whatman No. 4, 15 hr. were usually required for complete separation.

Chromatography on Filter Paper.—Descending development was employed and, on account of the very low R_F values, the solvent was allowed to drip off the bottom edge of the paper; to promote even flow this edge was serrated (with pinking shears). Whatman No. 4 paper was used at first, but later it became clear that dense slow-running paper gave better separations. Whatman paper No. 3MM was the best of those tried for analytical or preparative purposes. For larger amounts, the ascending technique was used on seed-test paper with the top of the paper protruding through a slit in the cover of the tank so that evaporation could proceed. This paper gave good separations but required 5 days or longer for development. Butan-1-ol and a number of solvent mixtures were tried but butan-2-ol proved the best. The developing solvent was prepared by mixing butan-2-ol (100 c.c.) with glacial acetic acid (0·2—1 c.c.), 4% aqueous hydrocyanic acid (1 c.c.), and just sufficient water to saturate the mixture. Some of this solvent and a little water were placed in the bottom of the chromatography tank. The development was usually continued for 16—48 hr. at a controlled temperature (37° or 25°).

Results for the chromatography of cold dilute-acid hydrolysis mixtures are shown in Table 3. Traces of three slow-moving monocarboxylic acids *A*, *B*, and *C* appeared, and after them the unchanged vitamin B_{12}, the main monocarboxylic acids E_1 and E_2, the dicarboxylic acids F_1 and F_2, and finally the tricarboxylic acid *G*. With these complex mixtures the third monocarboxylic acid E_3 overlapped the slowest dicarboxylic acid F_1; similarly the third dicarboxylic acid F_3 and the tricarboxylic acid did not yield distinct zones.

Preparation of Monocarboxylic Acids.—Vitamin B_{12} (air-dried; 250 mg.) was dissolved in 0·1N-hydrochloric acid (50 c.c.), and the solution was kept in the dark at room temperature for 65 hr. The solution was then adjusted to pH 4 and put on to a column of acid-washed alumina (30 × 2 cm.). The greater part of the unchanged vitamin B_{12} was eluted with water and the aqueous eluate was found by colorimetric assay to contain 155 mg. of vitamin B_{12} (anhydrous). The alumina column was then washed with aqueous ammonia (0·1N) until the red acidic pigments were removed. This eluate was concentrated under reduced pressure (to about 2 c.c.) and transferred quantitatively to a sheet of Whatman seed-test paper. Electrophoresis was carried out in 0·05M-phosphate buffer containing 0·01% of potassium cyanide at 4 v/cm. for 15 hr., by which time the intensely coloured monobasic acid zone had moved 4·2 cm. towards the anode. There also appeared a small neutral zone, due to a small proportion

TABLE 3. *Chromatography of the hydrolytic products from vitamin B_{12}.*

Descending development at 25° with Whatman No. 3MM filter paper, and butan-2-ol (1 l.) mixed with glacial acetic acid (10 c.c.), aqueous hydrocyanic acid (4%; 10 c.c.), and just sufficient water to saturate the mixture.

+ as in Table 1.

Chromatogram zones	*A*	*B*	*C*	*D* (vitamin B_{12})	E_1	E_2	F_1	F_2	*G*
R_F (relative to that of vitamin B_{12})	*ca.* 0·1	0·3—0·4	0·65—0·75	[1·0]	1·2—1·35	1·45—1·65	1·8—2·2	2·0—2·5	2·3
No. of acidic groups:	1	1	1	0	1	1	2	2	3
Conditions of hydrolysis:									
0·02N-HCl—									
room temp.; 6 days			Trace	+++		+			
,, 13 days			Trace	+++	Trace	+	Trace		
,, 20 days			Trace	+++	+	++	Trace		
,, 35 days		Trace	Trace	+++	+	+++	+	+	
0·05N-H_2SO_3—									
room temp.; 4 days		Trace	Trace	+++	Trace	+	Trace		
,, 11 days	Trace	Trace	Trace	+++	+	++	Trace	Trace	
,, 26 days	Trace	Trace	Trace	++	+	+++	++	+	+

of unchanged vitamin B_{12}, as well as some dibasic acid and a trace of tribasic acid. The monobasic acid zone was cut out, eluted with water, and freed from buffer salts by extraction with phenol as described above. Crystallisation from aqueous acetone occurred slowly during a few days and gave the mixed monobasic acids (40 mg.).

Separation of Isomeric Acids by Paper Chromatography.—The mixture of crystalline isomeric monobasic acids was dissolved in warm water (1 c.c.), and the solution was streaked across 4 sheets

of Whatman No. 3MM filter paper (30 × 38 cm.). The chromatograms were developed for 30 hr. at 25° with butan-2-ol (1 l.), glacial acetic acid (2 c.c.), 4% aqueous hydrocyanic acid (10 c.c.), and just sufficient water to saturate the mixture. Several faint slow-moving zones appeared and 3 major red zones. The two fast-moving ones separated poorly and each was eluted and chromatographed again under the same conditions. Corresponding zones were cut from the paper sheets and eluted with water; the solutions were concentrated (to about 0·5 c.c.) and acetone was added until a faint turbidity was produced. The slowest-moving acid, E_1, crystallised after a few days, as did the more abundant acid, E_2. The fastest-moving and least abundant acid E_3 crystallised only after a long time.

Properties of the Monobasic Acids.—On being dried *in vacuo* at 100° to constant weight, the *monocarboxylic acid* E_2 lost 14·4% of moisture (Found, on the dried sample: C, 54·7; H, 6·6; N, 13·5. $C_{61}H_{89}O_{15}N_{13}PCo$ requires C, 54·9; H, 6·7; N, 13·65%).

The principal maxima in the absorption spectrum of an aqueous solution of the acid E_2 occur at 277, 360, and 545 mμ. The infra-red absorption spectrum of this monocarboxylic acid in Nujol mull is similar to that of vitamin B_{12}, but there are small differences at the following wave numbers: 3300, 1715, 1610, 1210, 1055, 1030, 970, 840, and 800 cm.$^{-1}$. The band at 1715 cm.$^{-1}$, characteristic of carboxyl and some other carbonyl groups, is present in the acid but absent from vitamin B_{12}. Absorption at 3300 cm.$^{-1}$, characteristic of bonded hydroxyl groups, is stronger in the acid than in vitamin B_{12}.

Preparation of Dicarboxylic, Tricarboxylic, and Tetracarboxylic Acids.—Vitamin B_{12} (air-dried, 200 mg.) was dissolved in 0·2N-hydrochloric acid (40 c.c.), and the solution was kept in the dark at 37° for 6 days. The solution was then extracted through phenol, and the final concentrated aqueous solution was applied to 4 sheets of seed-test paper for electrophoresis, as described above. The di- and tri-carboxylic acid zones were about equally intense. Some much less intense zones also present were due to unchanged vitamin B_{12} and mono- and tetra-basic acids. The leading edge of each zone was purple, owing to loss of nucleotide from a small proportion of each acid. Corresponding zones were cut out, the purple portions being rejected, and eluted with water, and the extracts were purified from salts *via* phenol extraction.

The dicarboxylic acids separated on chromatography into three zones. The acids F_1 and F_2 from the slow-moving zones crystallised slowly from aqueous acetone, but the fastest-moving acid F_3 could not be crystallised. Chromatography of the tribasic acid gave only one rather broad fast-running zone that showed no tendency to divide; this acid crystallised very slowly from aqueous acetone. The tetrabasic acid also gave a rather broad homogeneous zone on chromatography, but it ran more slowly than even the monobasic acids; like the di- and tri-carboxylic acids, it crystallised very slowly from aqueous acetone. The absorption spectrum of an aqueous solution of the dibasic acids (mixture of isomers) showed maxima at 277·5, 361, and 550 mμ, with additional subsidiary maxima at 273, 305, 322, and 520 mμ.

Preparation of Vitamin B_{12} from the Monocarboxylic Acid Hydrolytic Products.—(i) The crystalline mixture of the monocarboxylic acids (10·1 mg. after drying *in vacuo* at 120°) was dissolved in dimethylformamide (0·25 c.c.), and triethylamine (0·005 c.c.) was added, causing formation of a red precipitate. After cooling to −5°, ethyl chloroformate (0·03 c.c. of 10% v/v solution in dimethylformamide) was added, the precipitate rapidly redissolving and a colourless solid slowly separating. The mixture was kept at −5° for 10 min. and was then treated with an excess of dry ammonia. After 30 min. at room temperature the mixture was held at 50° for 5 min. Ether (5 c.c.) was added, the red precipitate was dissolved in water, and the solution was applied to a sheet of Whatman No. 31 paper for electrophoresis at pH 6·5. The greater part of the colour appeared in the neutral zone, which was cut out and eluted with water. The solution so obtained contained 5·9 mg. of vitamin B_{12} by microbiological assay (Harrison, Lees, and Wood, *Analyst*, 1951, **76**, 696), or 5·4 mg. by colorimetric assay. Salts were removed by extraction through phenol, and the product was crystallised from aqueous acetone and characterised as vitamin B_{12} by its infra-red absorption spectrum and paper chromatography.

(ii) Two similar preparations were carried out, but with a greater excess of reagents, namely, triethylamine (0·05 c.c.) and ethyl chloroformate (0·03 c.c.) with the mixed monobasic acids (6·9 mg.). To the first was added aqueous 2N-ammonia (0·2 c.c.) and to the second water (0·2 c.c.), as a control. After 90 min. at room temperature 0·1N-sodium hydroxide (1 c.c.) was added to each and the solutions were left at room temperature for 30 min. Both solutions were then acidified, extracted through phenol, and submitted to electrophoresis at pH 6·5. The first preparation that had been treated with aqueous ammonia showed a strong neutral zone and also zones corresponding to mono- and di-carboxylic acids, which from their colours

appeared in part to have lost nucleotide. Comparison of colorimetric and microbiological assays indicated about 25% conversion into vitamin B_{12} in this preparation. The other preparation that had been treated with water showed on electrophoresis only a faint trace of neutral material and negligible microbiological activity.

(iii) The mixed monobasic acids (4 mg.; previously dried at 130° under reduced pressure for 10 min.) were dissolved in dimethylformamide (0·25 c.c.), and benzoyl chloride (0·02 c.c.) was added. The mixture was kept at −5° for 1 hr., the solid that had separated slowly dissolving. The solution was treated with excess of dry ammonia after 30 min. at room temperature, water (5 c.c.) was added to the mixture, and the coloured material was purified by extraction with phenol as before. Dilution of the phenol with ether enabled the material to be transferred to the aqueous layer, which was subjected to electrophoresis on paper at pH 6·5. The neutral zone was eluted from the paper with water, purified by phenol extraction, and crystallised from aqueous acetone. The product and authentic vitamin B_{12} were identical in microbiological activity when assayed with either the *E. coli* mutant (Harrison *et al.*, *loc. cit.*) or the more specific *L. leichmannii* (Emery, Lees, and Tootill, *Analyst*, 1951, **76**, 141). They also showed identical behaviour when chromatographed side by side on paper with the butan-2-ol solvent system.

Preparation of Vitamin B_{12} from the Dicarboxylic Acid Hydrolytic Products.—The dibasic acids (6·0 mg.; previously dried at 120° for 20 min. under reduced pressure), separated by electrophoresis from the product of hydrolysis of vitamin B_{12} with cold dilute hydrochloric acid, were dissolved in dimethylformamide (0·25 c.c.), and triethylamine (0·005 c.c.) was added. After cooling to −5°, ethyl chloroformate (0·007 c.c.) was added, the red precipitate slowly dissolving. After 15 min. at −5°, the product was treated with excess of dry ammonia and kept for a further hour at room temperature. Acetone–ether (1 : 1; 5 c.c.) was then added to the mixture, and the red precipitate separated on the centrifuge and fractionated by electrophoresis at pH 6·5. The neutral zone was eluted with water and found to contain vitamin B_{12} (1·58 mg. by both colorimetric and microbiological assay). Salts were removed by extraction through phenol, and the product crystallised readily from aqueous acetone. Its infra-red absorption spectrum was identical with that of authentic vitamin B_{12}.

Preparation of Vitamin B_{12} from Tricarboxylic Acid Hydrolytic Products.—The tribasic acid (8 mg.) separated by electrophoresis from the product of hydrolysis of vitamin B_{12} with cold dilute hydrochloric acid at 37° was dried at 120° for 10 min. under reduced pressure. It was dissolved in dimethylformamide (0·2 c.c.), and triethylamine (0·04 c.c. of a 20% v/v solution in dimethylformamide) was added. After cooling to −5°, ethyl chloroformate (0·02 c.c. of a 20% v/v solution in benzene) was added. After 10 min. at −5° the product was treated with excess of dry ammonia and kept for 3½ hr. at room temperature. Acetone–ether (1 : 1; 5 c.c.) was added and the red precipitate separated on the centrifuge. The product was fractionated by electrophoresis at pH 6·5, most of the colour appearing in the neutral zone, which was eluted with water and fractionated further by chromatography on paper. The chromatogram showed a complex pattern, but the strongest band was in the same position as the vitamin B_{12} marker. This band was eluted with water, and the product crystallised from aqueous acetone. It had nearly the full microbiological activity of vitamin B_{12}.

Hydrolysis of Vitamin B_{12} with Concentrated Hydrochloric Acid.—Vitamin B_{12} (100 mg.; hydrated) was dissolved in concentrated hydrochloric acid (10 c.c.). The solution was heated quickly to 65° and held at this temperature for 5 min. The solution was distilled to small bulk under reduced pressure to remove hydrochloric acid; water was added and the distillation repeated (twice). The colour of the reaction mixture, measured (Spekker absorptiometer with No. 604 filter) at a suitable dilution, was practically unchanged by the treatment with acid. Microbiological assay with *Lactobacillus leichmannii* (Emery, Lees, and Tootill, *loc. cit.*) showed that less than 1% of unchanged vitamin B_{12} remained, but the reaction mixture showed high activity with the *E. coli* mutant (Harrison *et al.*, *loc. cit.*). The entire preparation was submitted to electrophoresis at pH 6·5 on seed-test paper. The yield from the "neutral" zone was approx. 55% (assessed colorimetrically). Approx. 20% was recovered from the "monobasic acid" zone and 3% from the "dibasic acid" zone.

Smaller portions of vitamin B_{12} were heated with concentrated hydrochloric acid at 65° for 3 min. and 7 min. respectively, instead of 5 min. as for the main preparation. The appearance of the zones on electrophoresis and chromatography indicated that a small proportion of unchanged vitamin B_{12} remained in the 3-min. preparation, although less extensive conversion into acidic products had occurred. The 7-min. product was free from vitamin B_{12}, but contained higher proportions of mono- and di-basic acids.

The electrophoresis zones from the main experiment were cut out and eluted with water; the pigments were freed from salts by extraction through phenol, and the solutions were freeze-dried. On concentration of a solution of the neutral compound ("Factor B") at pH 3·5 under reduced pressure, the colour changed to yellow-orange, apparently with loss of cyanide (see below). The solution showed principal maxima in the absorption spectrum at 354, 498, and 527 mμ.

On addition of aqueous potassium cyanide (1 mol.) the colour changed to orange-red, but the absorption spectrum was little altered except for a broadening of the main band in the visible region. The principal maxima were at 355, 500, and 530 mμ. Addition of excess of aqueous sodium nitrite caused similar changes. Excess of cyanide, however, at pH 8 caused a colour change to purple, the absorption maxima being shifted to 367, 540, and 580 mμ; in addition, inflexions and subsidiary maxima in the ultra-violet became much more intense (at 277 and 309 mμ). The curve resembled that given by dicyanocobalamin.

A sample of the freeze-dried material (from a solution previously evaporated with dilute acetic acid) was made into a mull with Nujol and sodium chloride for infra-red spectroscopy. The curve showed a general similarity with that of vitamin B_{12} except in the "finger-print" region. Notable differences, however, were the absence of the bands at 860 and 842 cm.$^{-1}$ due to the aromatic ring in the nucleotide part of vitamin B_{12}, and the $-CN$ band at 2130 cm.$^{-1}$. By chromatography on paper this substance showed an R_F value of 1·5 (relative to that of vitamin B_{12}). It appears to be unstable, because solutions that had been kept for some days showed sometimes one, sometimes two, additional zones on chromatography.

The monocarboxylic acid without nucleotide obtained from the above hydrolysis showed three (occasionally four) zones on chromatography, presumably owing to isomerism as with the analogous nucleotide-containing acids; the R_F values relative to that of vitamin B_{12} were [1·15]; 1·3; 1·65; and 1·95.

Preparation of "Factor B" from the Nucleotide-free Monocarboxylic Acid Hydrolysis Products.—The monocarboxylic acids separated by electrophoresis from the products of hydrolysis of vitamin B_{12} with concentrated hydrochloric acid at 65° (freeze-dried material from previous experiment; 9·5 mg.) were dried at 120° for 10 min. under reduced pressure. They were dissolved in dimethylformamide (0·2 c.c.), and triethylamine (0·025 c.c. of a 20% v/v solution in dimethylformamide) was added. After cooling to −5° ethyl chloroformate (0·013 c.c. of a 20% v/v solution in benzene) was added. After 10 min. at −5° the product was treated with excess of gaseous ammonia and kept for 3 hr. at room temperature. Acetone–ether (1 : 1; 5 c.c.) was added and the precipitate separated on the centrifuge. The product was fractionated by electrophoresis at pH 6·5, most of the colour appearing in the neutral zone, which was eluted with water and chromatographed on paper. The main band appeared in the same position as the "Factor B" marker. It was eluted with water and found to have the same microbiological activity as "Factor B" from natural sources.

Butyl Esters of Mixed Nucleotide-free Cobalt-containing Pigments.—Vitamin B_{12} (133 mg. of air-dried material) was heated with a mixture of N-hydrochloric acid (15 c.c.) and butan-1-ol (15 c.c.) for 1½ hr. on the water-bath (cf. Buchanan, Johnson, Mills, and Todd, *loc. cit.*). The mixture was cooled and the orange aqueous layer separated from the dark red butanol layer, this being washed with water (3 × 1 c.c.) to remove excess of acid. Paper chromatography (Part I) revealed that most of the free nucleotide and aminopropanol was contained in the aqueous layer, but analysis of the butanol layer in the same manner showed that free 5 : 6-dimethylbenziminazole nucleotide was also present in this fraction. From the top layer of a butan-1-ol–acetic acid–water mixture (4 : 1 : 5; henceforward described as the butanol–acetic acid–water mixture) the pigment travelled as a red streak, R_F *ca.* 0·7 to 1·0, the colour of which changed to orange-yellow when the paper was dried in air at room temperature. The red colour was restored when the paper was treated with hydrogen chloride. More of the nucleotide was removed from the butanol layer by further washing with water, but this process was not practicable for the complete separation of the nucleotide. The butanol was removed from the solution, to yield a dark red glass (116 mg.), which was taken up in acetone leaving a pink amorphous residue (12 mg.) consisting mainly of nucleotide. The acetone solution was brought on to a column of "AnalaR" calcium carbonate (10 g.; 16 × 1 cm.). When the column was washed with more acetone, the main diffuse red band passed rapidly through the column, leaving a thin red band of strongly adsorbed material at the top. This was eluted with methanol and after removal of the solvent gave an orange residue (7 mg.), which again consisted mainly of nucleotide (paper chromatography). Evaporation of the main eluate gave a red glass (100 mg.), which was shown to be free from nucleotide. The ultra-violet absorption

of this material in methanol showed maxima at 320, 353, 404—406, and 486—492 mμ. No further nucleotide and only a trace of aminopropanol were liberated after hydrolysis of the above crude pigment (38 mg.) with a mixture of concentrated hydrochloric acid (" AnalaR "; 1·5 c.c.) and water (1 c.c.) in a sealed tube at 100° for 48 hr.

Acid Hydrolysis of Vitamin B_{12}; Isolation of the 5 : 6-*Dimethylbenziminazole Nucleotide.*—Vitamin B_{12} (1·015 g.) was heated at 70° with concentrated hydrochloric acid (10 c.c.) for 5 min., and the deep red solution was evaporated rapidly to dryness under reduced pressure. Excess of hydrogen chloride was removed from the dark red residue by drying it for 18 hr. *in vacuo* over potassium hydroxide, and the product was then dissolved in water (20 c.c.), brought on to a column of Amberlite IR-4B (12 × 2·5 cm.), and eluted with water (250 c.c.). Evaporation of the eluate gave a dark red gum (610 mg.; see above). The nucleotide was removed from the column by elution with N-hydrochloric acid (500 c.c.), and the eluate on evaporation to dryness gave a pink amorphous solid (250 mg.), which on repeated crystallisation from aqueous acetone gave 5 : 6-dimethyl-1-(α-D-ribofuranosyl)benziminazole-2′(or -3′) phosphate as colourless crystals m. p. 240—241° (decomp.) (Kaczka, Heyl, Jones, and Folkers, *J. Amer. Chem. Soc.*, 1952, **74**, 5549, give m. p. 240—241°). Light absorption in water at pH 2 : maxima at 277 and 285 mμ, log ε 3·86 and 3·82 respectively.

The ion-exchange resin Dowex 2-X10 has also been used successfully for this separation.

Alkaline Hydrolysis of Vitamin B_{12}; Electrophoretic Separation of 5 : 6-*Dimethylbenziminazole Nucleotide from the Cobalt-containing Pigments.*—Vitamin B_{12} (1·1 g.; dried at 80° for 3 hr. *in vacuo*) was heated with aqueous barium hydroxide (150 c.c.; saturated at 17°) for 36 hr. at 100°. Excess of aqueous ammonium carbonate was added to the hot solution, the barium carbonate separated, the filtrate evaporated to dryness, and the red residue dissolved in N-acetic acid (20 c.c.). Meanwhile cellulose powder (800 g.; passed through a 90-mesh sieve) was slurried with N-acetic acid and packed as tightly as possible to form a column (39 × 6 cm.). Half of the pigment solution (10 c.c.) was brought on to the column; after it had been adsorbed, it was washed down the column to the extent of *ca.* 2 cm. by a further quantity of N-acetic acid. The column was then placed inside a concentric jar containing N-acetic acid and a potential of 220 v was applied, the anode being placed in the top of the column and the cathode in the solution in the outside jar, *i.e.*, effectively at the bottom of the column. After 21 hr. the orange-red pigment band had moved 24 cm. towards the cathode. The front of the band was diffuse and a faintly pink band remained at the top of the column. The cellulose was extruded and the main pigment band was separated and eluted with water. The remaining half of the pigment solution was treated in the same manner, and evaporation of the combined pigment fractions from both columns yielded a red residue (660 mg.), which was shown to be nucleotide-free by

TABLE 4. *Electrophoresis of nitrous acid hydrolysis products of vitamin B_{12} in phosphate buffer at* pH 6·5 *containing* 0·01% *of* KCN (cf. *preceding experiment*).

+ and × as in Table 1.

Conditions of hydrolysis	Effective negative charges on pigment spot: 0	1	2	3	4	5	6	7
Vitamin B_{12}	+++							
2N-HCl at 65° for 15 min.:								
HNO_2 absent ...	+×	++××××	++××××	+××				
HNO_2 present ...			+×	+××	++××××	++××××	+××	×
2N-HCl at room temp. for 2 hr.:								
HNO_2 absent ...	+++	++	+					
HNO_2 present ...	+	+++×	+++×	++×	+			
2N-HCl with HNO_2 at room temp.:								
for 5½ hr.	+	+×	++××	++++××	+×	+		
„ 16 hr.				+×	+××	++××××	+××	×
0·5N-HCl with HNO_2 at room temp. for 16 hr.	+++	+++	+					
N-HCl with HNO_2 at room temp. for 16 hr.	+	+++	+++	+				

paper chromatography with the butanol–acetic acid–water system. The faintly pink bands at the top of each column were eluted with water, and evaporation of the combined extracts yielded a brownish gum (415 mg.), which contained the nucleotide (R_F 0·48 in the butanol–acetic acid–water).

Hydrolysis of Vitamin B_{12} with Nitrous Acid.—Vitamin B_{12} (10 mg.; anhydrous) was dissolved in 2N-hydrochloric acid (10 c.c.), and the solution was divided into two equal fractions. Each was heated at 65° for 15 min., but to one fraction sodium nitrite (3 × 17 mg.) was added after 0, 5, and 10 min. Each of the fractions was adjusted to pH 3, a small amount of potassium cyanide (*ca.* 1 mg.) was added, and the red pigments were concentrated by extraction with phenol as described above. After electrophoresis in 0·05N-phosphate buffer at pH 6·5 containing 0·01% of potassium cyanide, the fraction to which the nitrite had been added showed much the more extensive hydrolysis, the principal products being tetra- and penta-basic acids, whereas without nitrous acid the principal product was the monobasic acid fraction. The colours of the electrophoresis zones indicated that nucleotide fission had occurred to an appreciable extent. Nitrite had little effect on the hydrolysis in hydrochloric acid solutions more dilute than 2N. The results of this and similar experiments are summarised in Table 4.

Estimation of Ammonia evolved on Hydrolysis of Vitamin B_{12} with Barium Hydroxide.—Vitamin B_{12} (989 mg.; N, 13·1%) was heated with aqueous barium hydroxide (150 c.c.; saturated at 17°) at 85° for 36 hr. in an atmosphere of acid-washed, oxygen-free nitrogen, and the volatile bases evolved were bubbled into a solution of chloroplatinic acid. The stream of nitrogen through the solution was continued for a further 24 hr. at room temperature and the precipitated ammonium chloroplatinate was then separated and dried (729 mg. corresponding to 46 mg. of nitrogen, *i.e.*, 5·0 mols. of ammonia per mol. of vitamin B_{12}, on a N_{14}-structure). An *X*-ray powder photograph of the product was identical with that of authentic ammonium chloroplatinate.

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University Chemical Laboratory, Cambridge.
Glaxo Laboratories Limited, Greenford, Middlesex. [*Received, July 7th,* 1953.]