226. *Chemistry of the Vitamin B_{12} Group. Part IV.*[1] *The Isolation of Crystalline Nucleotide-free Degradation Products.*

By R. Bonnett, J. R. Cannon, A. W. Johnson, and Sir Alexander Todd.

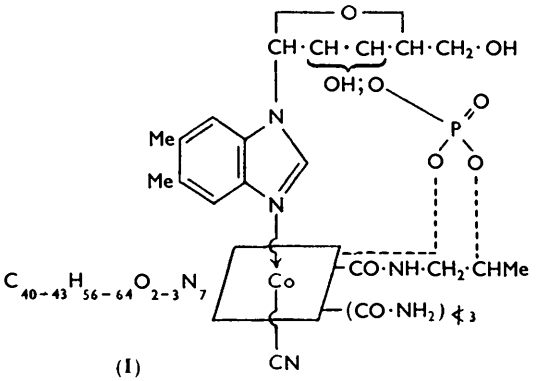
The mixture of tetra-, penta-, and hexa-carboxylic acids formed on vigorous alkaline hydrolysis of vitamin B_{12} has been fractionated on an ion-exchange column. The pure pentacarboxylic acid so obtained has been crystallised in the form of its dicyanide, and the hexacarboxylic acid both as the dicyanide and as the monochloride monocyanide, the latter proving particularly suitable for *X*-ray studies. The combination of *X*-ray and chemical evidence has enabled structural formulæ to be advanced both for the hexacarboxylic acid (IV) and for vitamin B_{12} itself (VII). Separation of the mixture of tetra-, penta-, hexa-, and hepta-carboxylic acids obtained on vigorous acid hydrolysis of the vitamin has also been effected, but the individual acids have not yet been obtained crystalline.

In Part III [1] of this series (where earlier work is cited) the behaviour of vitamin B_{12} under varying conditions of hydrolysis was reported and its polyamide character was recognised. These and earlier findings by us and others being taken into consideration the partial formula (I) was advanced for the vitamin. Hydrolysis of the vitamin gave a complex mixture of red cobalt-containing acids and by paper electrophoresis of hydrolysates it was found that according to the conditions of hydrolysis two series of acids could be recognised. The first series (mono- to hepta-carboxylic acids) lacked the benziminazole nucleotide while the second (mono- to hexa-carboxylic acids) still retained it in combination. The close similarity between the visible spectra of all these acids and of vitamin B_{12} itself suggested that the chromophore had been little altered by hydrolysis and hence that these acids would be of direct value in endeavouring to elucidate the structure of the cobalt-containing core of the vitamin. For structural study it seemed most desirable to obtain at least one of the nucleotide-free acids in a pure crystalline condition. It is the

[1] Part III, Armitage, Cannon, Johnson, Parker, Smith, Stafford, and Todd, *J.*, 1953, 3849.

purpose of the paper to describe in detail how this object was attained, and to discuss the main structural features of one of the acids.

Although no conditions of hydrolysis have so far been discovered which yield a single product, treatment of vitamin B_{12} with 30% aqueous sodium hydroxide for one hour at 150° was found to yield a mixture containing mainly the nucleotide-free penta- and hexa-carboxylic acids with only a small amount of tetracarboxylic acid, and this method of



(I)

hydrolysis was adopted as the basis for isolation work. Electrophoresis on paper strips was clearly impracticable for preparative separation of the mixture of acids; it was indeed possible, by using Whatman " seed-testing " paper, to handle up to 50 mg. of material at one time but this represented the practical limit. Countercurrent distribution experiments were discouraging, and ion-exchange chromatography was therefore studied with Dowex 1 × 2 resin in which the degree of cross-linkage is small (*ca.* 2%) so that the cobalt-containing polycarboxylic acids can be readily eluted. The first success came from experiments in which the resin was used in the acetate form and dilute aqueous ammonium acetate was the eluting agent.

The alkaline hydrolysate was subjected in portions to preliminary electrophoresis on Whatman " seed-testing " paper, and the nucleotide separated from the tetra-, penta, and hexa-carboxylic acids; the crude hexacarboxylic acid fraction was then fractionated further on the ion-exchange column. Several minor fractions were eluted first, followed by the major band which was collected separately. After removal of inorganic salts an aqueous solution of the product was evaporated and the gummy residue dissolved in aqueous acetone, diluted with ether, and set aside. A small quantity of red prisms slowly separated; this sample of the hexacarboxylic acid was submitted to Dr. D. C. Hodgkin and the results of her elegant *X*-ray crystallographic studies on it have already been outlined.[2,3] Further dilution of the mother-liquor with ether yielded fine red needles; these were unsuitable for *X*-ray studies but were evidently a polymorphic form of the same hexacarboxylic acid since, on one occasion, the needle form was converted when kept in contact with the aqueous-acetone–ether mother-liquor into the prism form (identified by *X*-ray powder photography). Recrystallisation of the hexacarboxylic acid was best effected by cautious addition of ether to its aqueous-acetone solution although the process was rather wasteful. Analysis of a sample of the acid dried at 50° gave values which, if the degree of hydration is assumed to be that observed in the *X*-ray examination, correspond to a formula $C_{46}H_{60}O_{13}N_6CoCl,2H_2O$. This formula differs slightly from that mentioned in a preliminary communication [4] but it is clear that with molecules of this size precise definition of molecular formula by elementary analysis alone is hardly possible since a number of formulæ lie within the tolerance of the carbon and hydrogen values; the

[2] Brink, Hodgkin, Lindsey, Pickworth, Robertson, and White, *Nature*, 1954, **174**, 1169.
[3] Hodgkin, Pickworth, Robertson, Trueblood, Prosen, and White, *ibid.*, 1955, **176**, 325.
[4] Cannon, Johnson, and Todd, *ibid.*, 1954, **174**, 1168.

combined analytical and *X*-ray determinations, however, justify the formula quoted here. The infrared spectrum of the crystalline acid showed a band at 2141 cm.$^{-1}$ characteristic of the cyanide group and a solution of the acid in dilute nitric acid contained chloride ion. The absence of strong " aromatic bands " in the 690—870 cm.$^{-1}$ region of the infrared spectrum was also noteworthy. The ultraviolet and visible spectra of the acid (Figs. 1 and 2) both in water and in 0·1M-potassium cyanide strongly resemble those of vitamin B_{12}, showing the absence of any marked change in chromophore; differences in the

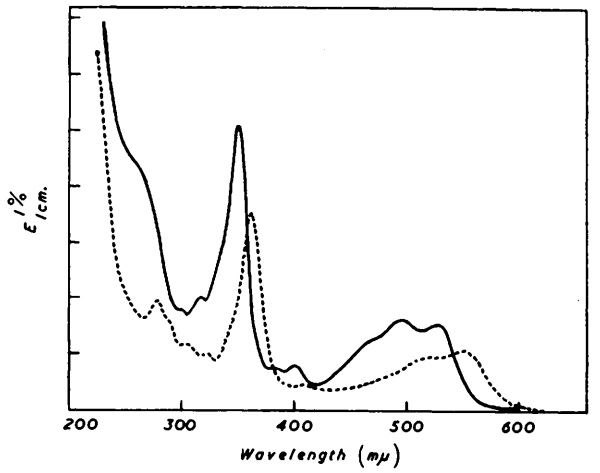


FIG. 1. *Ultraviolet absorption spectra of vitamin B_{12} (air-dried, – – – –) and the hexacarboxylic acid degradation product (——) in water.*

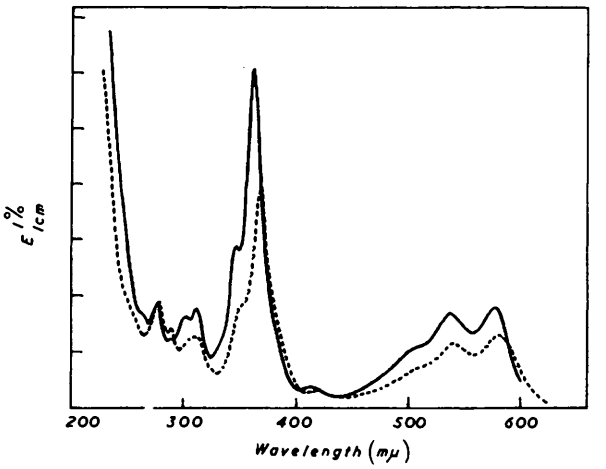


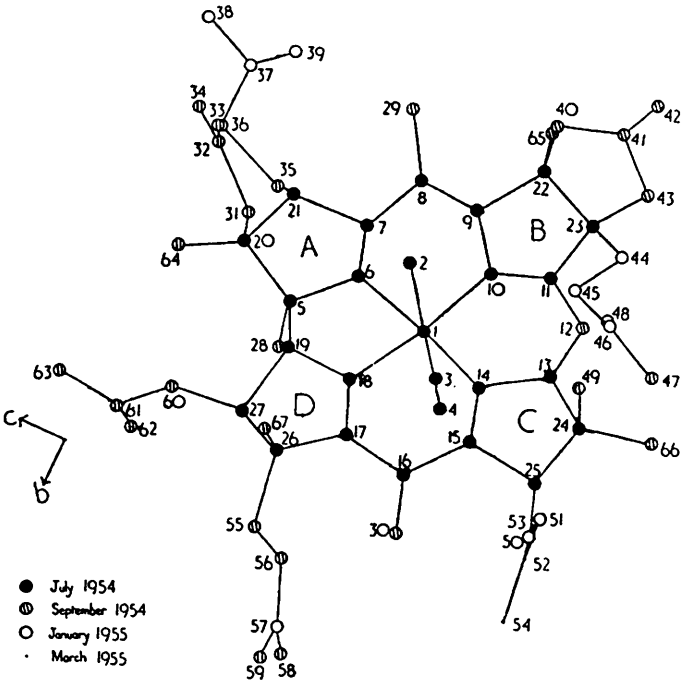
FIG. 2. *Ultraviolet absorption spectra of vitamin B_{12} (air-dried, – – – –) and the hexacarboxylic acid degradation product (——) in 0·1M-potassium cyanide.*

280 mμ region are attributable to the absence of the benziminazole nucleus in the hexacarboxylic acid.

Later studies suggested that the multiplicity of bands obtained by eluting electrophoretically homogeneous acids from ion-exchange columns with solutions of salts in the absence of excess of cyanide, as well as the reluctance of the acids to crystallise, is due, in part at least, to the ease of exchange of anions other than cyanide attached to the central cobalt atom. To overcome this difficulty and to establish a better standardised procedure for isolating hydrolysis products the above method leading to a chloro-cyanide was abandoned. Instead the ion-exchange resin was used in the chloride form and the chromatography carried out in presence of excess of cyanide. The mixed alkaline hydrolysate

was added to the column without preliminary electrophoresis, and the nucleotide was eluted with dilute acetic acid. The penta- and hexa-carboxylic acids in the dicyanide form were then successively eluted with dilute aqueous sodium chloride, again in the presence of cyanide. Aqueous-acetone solutions of the products, when kept at 0° in presence of hydrogen cyanide, yielded the dicyanides of the pentacarboxylic and hexacarboxylic acid in crystalline form. The hexacarboxylic acid derivative gave analytical values corresponding to $C_{47}H_{60}O_{13}N_7Co$ in agreement with the formula above deduced for the same acid in the monochloride-monocyanide form, while the pentacarboxylic acid dicyanide analysed as expected for $C_{47}H_{59-61}O_{12}N_8Co$. The ultraviolet and visible absorption spectra of both products again closely resembled those of vitamin B_{12}.

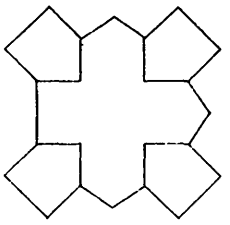
FIG. 3. *Atomic positions found in the hexacarboxylic acid derived from vitamin B_{12} projected on the* a *plane. The shading illustrates the course of the crystallographic study; the dates show approximately when each group of atoms was selected.*



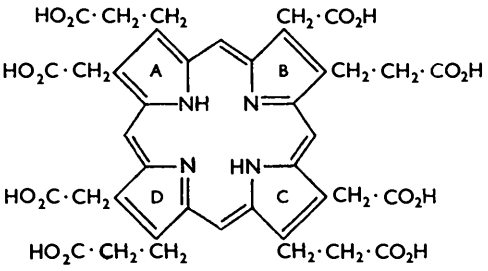
It has already been reported [1] that vigorous acid hydrolysis of vitamin B_{12} removes the nucleotide and aminopropanol residues, as well as amide groups, and yields mixtures of acids containing 4, 5, 6, and 7 carboxyl groups. These acids have now been separated by ion-exchange chromatography on Dowex 1 × 2 resin (chloride form), but none of them has so far been crystallised. Our knowledge of these acids is less than of the alkaline hydrolysis products, but it is clear that they are not identical with the latter and probably represent a separate series. No heptacarboxylic acid has ever been obtained except by acid hydrolysis; the hexacarboxylic acid obtained by alkaline hydrolysis is quite stable to further treatment with alkali.

In the initial phase of Dr. D. C. Hodgkin's *X*-ray analysis of the crystalline hexacarboxylic acid (as monochloride-monocyanide) obtained from alkaline hydrolysates as described above, the same central nucleus (II) containing four five-membered rings as occurs in vitamin B_{12} was recognised.[2] Further extension of the analysis determined the relative position of every atom (other than hydrogen) in the hexacarboxylic acid [3] and a projection of these positions is shown in Fig. 3. Much of the structure of the

hexacarboxylic acid follows at once from a consideration of Fig. 3 and the known chemical properties of the acid. It was known that the chlorine atom and the cyanide group were attached to the centrally situated cobalt atom, and analysis showed that the acid contained five nitrogen atoms in its molecule in addition to the one present in the cyanide group. The stability of the chromophoric system made it virtually certain that the other four atoms attached to the cobalt were nitrogen. The six carboxyl groups are clearly



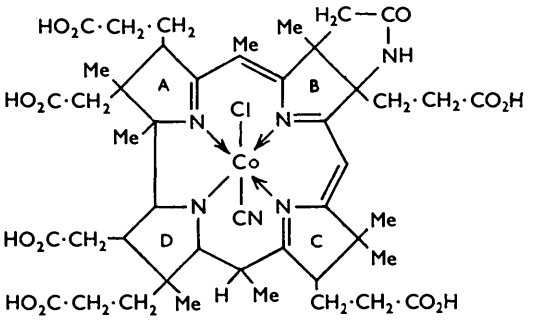
(II)



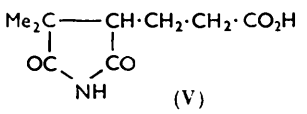
(III)

recognisable in Fig. 3 as occurring in four propionic acid and two acetic acid residues; all six residues form β-substituents on the five-membered rings, their arrangement being analogous to that known to occur in the natural porphyrin derivative (III) (uroporphyrin-III). Another projection of the electron-density pattern [3] shows that most of the inner portion of the molecule is flat but that the β-positions in the five-membered rings are all saturated; alternate β- and β′-positions are above and below the plane of the inner portion. All the propionic acid residues are situated on the same side of the molecule as the cyanide group, and the acetic acid residues and the chlorine atom project on the opposite side.

Ring B in the hexacarboxylic acid bears a β-methyl and a β′-propionic acid group as well as a ββ′-fused five-membered ring; the *X*-ray data suggest that this ring is either a lactone or a lactam and it is noteworthy that it does not occur in vitamin B_{12} itself which



(IV)



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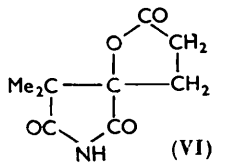
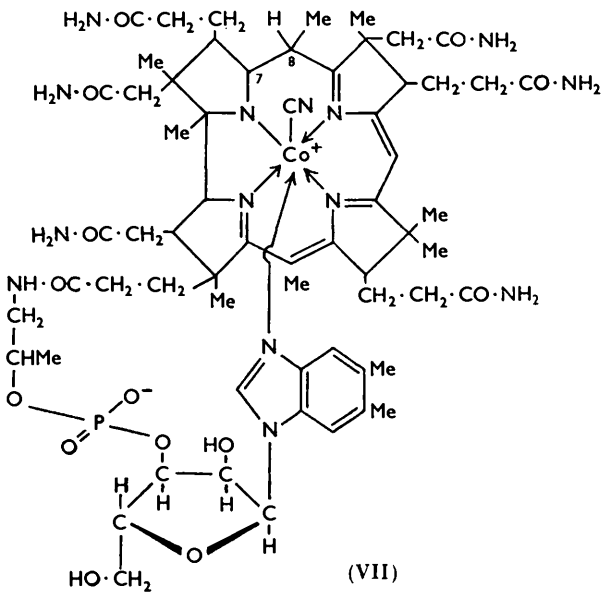


(VI)

appears to have methyl and acetamide groups as β-substituents and a propionamide group as a β′-substituent on ring B. The extra ring in the hexacarboxylic acid is therefore formed by a cyclisation involving either an acetamide or an acetic acid side-chain. The acid does not acquire an additional negative charge even at pH 11. This behaviour would be most remarkable for a lactone and, it being borne in mind that one of the nitrogen atoms in the acid is otherwise unaccounted for, the lactam formulation was adopted. Further support for this view comes from the presence of a band in the 1720 $cm.^{-1}$ region in the infrared spectrum of the hexacarboxylic acid. This frequency agrees with values quoted for the stretching frequency associated with the carbonyl group of a γ-lactam, but is very low for a γ-lactonic carbonyl group, which usually absorbs about 1770 $cm.^{-1}$. Further chemical

evidence for the γ-lactam structure derived from oxidation studies will be reported in a later communication. It should be noted that all the oxygen and nitrogen atoms required by the analytical values obtained for the hexacarboxylic acid monochloride-monocyanide (water of crystallisation being ignored) and the anhydrous dicyanide are accounted for by the foregoing considerations. In addition to the acetic and propionic acid side-chains there are eight single-atom (excluding hydrogen) substituents. It is reasonable to conclude



(VII)

that these are all methyl groups as the vitamin is re-formed on oxidation of its *leuco*-compound (apart from the displacement of the cyanide by a hydroxyl group [5]) and therefore contains no unsaturated side-chains. All these features were included in structure (IV) which was advanced for the hexacarboxylic acid in our preliminary communication.[6] Independent support for the structure of ring C in (IV) had also been provided by the isolation of the succinimides (V) and (VI) from the chromic acid oxidation products of a vitamin B_{12} acid hydrolysate,[7] and later the amide corresponding to (V) was isolated after a similar oxidation of the vitamin itself; [8] the imides (V) and (VI) are also found among the oxidation products of the pure hexacarboxylic acid.

The elucidation of the structure (IV) of the hexacarboxylic acid (we leave aside for the moment the question of number and location of double bonds) facilitated the interpretation of the electron-density pattern of vitamin B_{12} itself, and the combination of *X*-ray and chemical evidence allowed the complete structural formula (VII) to be advanced for the vitamin.[3,6] The electron-density patterns reveal that an exchange reaction occurs during the formation of the hexacarboxylic acid since the cyanide group in the acid occupies the same position relative to the rest of the molcule as does the benziminazole residue in the vitamin. The aminopropanol which is linked ester-wise to the phosphate and through an amide linkage to the central portion of the molecule [9] is found to be attached to the propionic acid side-chain of ring D. The vitamin is formulated as a diester of phosphoric acid, the remaining acid group of the phosphate being neutralised by a positive charge associated with the cobalt atom. This accords with the stability of the phosphate

[5] Kaczka, Wolf, and Folkers, *J. Amer. Chem. Soc.*, 1949, **71**, 1514.
[6] Bonnett, Cannon, Johnson, Sutherland, Todd, and Smith, *Nature*, 1955, **176**, 328.
[7] Kuehl, Shunk, and Folkers, *J. Amer. Chem. Soc.*, 1955, **77**, 251.
[8] Kuehl, Shunk, Moore, and Folkers, *ibid.*, p. 4418.
[9] Buchanan, Johnson, Mills, and Todd, *J.*, 1950, 426.

ester linkages, which excludes the possibility of a triester grouping,[10] and also with the properties of Factor B (vitamin B_{12} lacking only the nucleotide) which, in contrast to the vitamin, behaves electrophoretically as a monoacid base.[1]

The evidence so far presented does not permit any definite conclusion regarding the degree of unsaturation in either the hexacarboxylic acid or the vitamin. Structures (IV) and (VII) each contain five double bonds in conjugation although their location is different in the two cases but, in this sense only, they are tentative. That a conjugated system is present is certain from the absorption spectra and there is no reason to believe that it differs in extent in the two compounds. Although a system of six conjugated double bonds [*i.e.*, that in (VII) extended by a double bond between $C_{(7)}$ and $C_{(8)}$] has many attractions, not the least being that it would have two resonance forms making all the nitrogen atoms surrounding the cobalt equivalent, yet the five-double-bond system was suggested provisionally on the following grounds. From the first detailed *X*-ray patterns there appeared to be some departure from planarity in the neighbourhood of one nitrogen atom, and chlorination studies on vitamin B_{12} appeared to show that the vitamin reacted with 3 mols. of chloramine-T to give a product which contained only two atoms of chlorine and with a visible spectrum displaced towards the red.[11] The halogenation evidence seemed most readily explained by assuming that extension of the conjugated system had occurred by elimination of one mol. of hydrogen chloride from a halogenated derivative. This explanation would be feasible only on a structure containing five double bonds. There was, however, some doubt as to its validity since in the halogenation studies no pure compounds were isolated and the shift in absorption spectrum, although marked, might conceivably have some other explanation. Moreover, later *X*-ray evidence, although not decisive, tended to favour structures containing six double bonds. In view of these uncertainties it was clearly necessary to study the halogenation of the vitamin in more detail. This study is described in the following paper, together with an investigation of reactions leading to the formation of the lactam ring which occurs in the hexacarboxylic acid; it leads in fact to a preference for the structure with six double bonds.

Experimental

Vitamin B_{12}.—A sample was analysed (Found, in a sample dried to constant wt. at 80°/vac.: C, 55·4; H, 6·7; N, 14·2. Calc. for $C_{63}H_{90}O_{14}N_{14}PCo$: C, 55·8; H, 6·7; N, 14·5. Calc. for $C_{63}H_{88}O_{14}N_{14}PCo$: C, 55·8; H, 6·55; N, 14·5%).

Alkaline Hydrolysis of Vitamin B_{12}.—Crystalline vitamin B_{12} (air-dried; 2 g.) was dissolved in water (100 c.c.), and "AnalaR" sodium hydroxide (30 g.) was added. A quantity of a brown flocculent material separated and the mixture was heated under reflux (oil-bath temp., 145—150°) for 1 hr. The resulting brown solution became red on cooling, and it was then acidified with 17% hydrochloric acid (140 c.c.), diluted with water (400 c.c.), and shaken with phenol (4 × 100 c.c.) until the aqueous phase was colourless. The combined phenolic extracts were washed with water (2 × 200 c.c.), any coloured material in the resulting aqueous layer being re-extracted into phenol. The combined salt-free phenol solution was diluted with three volumes of ether and then shaken with 0·2N-aqueous ammonia until no more colour was removed from the organic layer. The cobalt-containing acids and the nucleotide were thus obtained in the aqueous layer as their ammonium salts. A small quantity of a reddish-purple compound remained in the ethereal solution. After washing and evaporation of the ether and removal of phenol from the residue, the product was obtained as an amorphous solid (68 mg. from several experiments using a total of 10·99 g. of vitamin B_{12}) which has not yet been induced to crystallise; it is apparently cobalt-free (no blue borax bead; no residue after ignition) and has not been identified. The compound is insoluble in light petroleum or water but soluble in aqueous alkali or chloroform to bright reddish-purple solutions.

The aqueous solution of the ammonium salts of the cobalt-containing acids from several experiments (10·99 g. of vitamin B_{12}) was washed with ether and evaporated under reduced

[10] Brown, Magrath, and Todd, *J.*, 1955, 4396.

[11] Ellis, Petrow, Beaven, and Holiday, *J. Pharm. Pharmacol.*, 1953, **6**, 60; Schmid, Ebnöther, and Karrer, *Helv. Chim. Acta*, 1953, **36**, 65.

pressure. A blue colour obtained in the distillate was probably due to traces of indophenol formed by the interaction of phenol and ammonia. The last traces of water were removed from the non-volatile residue by freeze-drying, leaving a dark red resin (11·325 g.). Examination of this product by electrophoresis on paper (0·1M-potassium cyanide on Whatman No. 4 paper with 4·6 v/cm. for 5 hr.; Part III, *loc. cit.*) revealed the presence of tetra-, penta-, and hexa-carboxylic acids lacking the nucleotide, as well as the free nucleotide.

The product was initially subjected to analysis by ion-exchange chromatography on a column (1 × 12 cm.) of Dowex 1 × 2 resin (through 200 mesh) in the chloride form. The hydrolysate (8 mg.) in water (0·5 c.c.) was treated with 2% aqueous hydrogen cyanide (2 c.c.), brought on to the column with 0·02% aqueous hydrogen cyanide (20 c.c.), and eluted with acetic acid (0·05N containing 0·02% of hydrogen cyanide). The flow was adjusted to 1·3 c.c./min. and fractions (10 c.c.) of the eluate were examined spectroscopically. Measurements of the optical density at 277 mμ showed that the nucleotide was eluted rapidly (fractions 2—6) together with a small quantity of coloured material. The further examination of the nucleotide will be described in a later paper. After 15 fractions had been collected the column was washed with 0·02% aqueous hydrogen cyanide (40 c.c.) and then eluted with 0·3M-sodium chloride containing 0·02% of hydrogen cyanide. Optical-density measurements were now taken at 364 mμ and three peaks were obtained in the fraction-number (fractions counted after introduction of sodium chloride solution)–optical-density graph, *viz.*: fraction 16 (tetracarboxylic acid as determined by electrophoresis); fraction 27 (pentacarboxylic acid); and fraction 50 (hexacarboxylic acid). Area measurements on the graph showed that the ratio of tetra- : penta- : hexa-carboxylic acid was 1 : 11 : 22. This technique was the basis of the large-scale separation of the cobalt-containing acids.

Separation of Cobalt-containing Acids by means of Ion-exchange Chromatography. Use of Dowex 1 × 2 Chloride and Aqueous Sodium Chloride for Elution.—The mixed hydrolysate (6·021 g. as ammonium salts from previous experiment) was dissolved in 0·8% aqueous hydrogen cyanide (100 c.c.) and added to a column (23 × 6·5 cm.) of Dowex 1 × 2 in the chloride form (50—100 mesh) and washed with 0·02% aqueous hydrogen cyanide (200 c.c.). The 5 : 6-dimethylbenziminazole nucleotide was eluted with 0·05N-acetic acid (1800 c.c.) containing 0·02% of hydrogen cyanide, and the column washed with 0·02% aqueous hydrogen cyanide (150 c.c.), followed by 0·3M-sodium chloride containing 0·02% of hydrogen cyanide, adjusted to flow at the rate of 17·4 c.c./min. The first runnings (1500 c.c.) were discarded and thereafter fractions (1 l. each) collected, the concentration of salt solution being slowly increased as shown in Table 1. A small portion of each fraction was extracted and concentrated by the phenol–ether method as outlined in the previous experiment and then subjected to electrophoresis on paper. Fractions 1—8 inclusive were purple (most intense in fraction 6), and fraction 9 was orange-brown.

Fraction 9, and to a smaller extent fraction 8, showed a yellowish spot in the pentabasic acid region after electrophoresis and an orange-purple spot in the hexabasic acid region. These are included in square brackets in Table 1.

TABLE 1.

Fraction	1	2	3	4	5	6	7	8	9
Normality of NaCl eluting soln.	0·3	0·3	0·3	0·4	0·4	0·6	0·6	1·0	1·0
No. of CO_2H in constituent acids (electrophoresis)	4, 5	5	5	5	5, 6	6	6	6[5, 6]	[5, 6]

A similar experiment with a mixed hydrolysate (5·304 g.) was carried out and the corresponding chromatography fractions were combined. Evaporation of the acetic acid solution yielded the 5 : 6-dimethylbenziminazole nucleotide (2·205 g. from 10·99 g. of vitamin B_{12}) as a dirty white amorphous solid [R_F 0·51 in a butan-1-ol–acetic acid–water (4 : 1 : 5); lit.,[9] 0·48]. The coloured fractions 1—9 were extracted by the phenol–ether method and combined as follows: fraction 1 (0·178 g.; free acid); fractions 2—5 (1·973 g.; ammonium salt); fractions 6—8 (5·492 g.; ammonium salt); and fraction 9 (0·159 g.; free acid). Each of these combined fractions was subjected to further purification.

Fractions 6—8 were combined and chromatographed on an ion-exchange column (23 × 6·5 cm.; 50—100 mesh), elution being with aqueous 0·3M-sodium chloride containing 0·02% of hydrogen cyanide. There were obtained (i) homogeneous (electrophoresis) hexacarboxylic acid (4·479 g.), (ii) mixed ammonium penta- and hexa-carboxylates (225 mg.) which were combined with fractions 2—5 (below), and (iii) tail fraction (245 mg.).

Fractions 2—5 (2·198 g. as ammonium salt) were combined and chromatographed as above and yielded (i) homogeneous (electrophoresis) pentacarboxylic acid (0·766 g.), (ii) a mixture (64 mg.) of tetra- and penta-carboxylic acids which was combined with fraction 1 (below), and (iii) mixed ammonium salts (1·023 g.) of the penta- and hexa-carboxylic acids. Fraction (iii) was again chromatographed on a column (2·5 × 11 cm.) of Dowex 1 × 2 (200-mesh; chloride form), and eluted with 0·3M-sodium chloride containing 0·02% of hydrogen cyanide at a rate of 2·2 c.c./min., 10 c.c. fractions being collected. This gave pure (electrophoresis) pentacarboxylic acid (672 mg.), hexacarboxylic acid (252 mg.), and a small amount of the mixture (57 mg.).

Fraction 1 (242 mg.) was subjected to further fractionation on a small ion-exchange column (2·5 × 11 cm.), and the various fractions were isolated as before. There were obtained pure (electrophoresis) tetracarboxylic acid (49 mg.), pure pentacarboxylic acid (147 mg.), and a mixture of tetra- and penta-carboxylic acids (31·5 mg.).

The overall yields of the pure acids were: tetracarboxylic 49 mg., pentacarboxylic 1·585 g., and hexacarboxylic 4·731 g.

Crystalline Dicyanide Derivative of the Pentacarboxylic Acid.—Electrophoretically homogeneous pentacarboxylic acid (500 mg.; previous experiment) was dissolved in the minimum amount of hot 50% aqueous acetone, and 4% aqueous hydrogen cyanide (5 c.c.) was added. The solution was cooled slowly and then kept at 0° for several days, then the dark red needles were separated and washed with a little water, both operations being conducted in subdued light. After a second crystallisation by the same method the crystalline *dicyanide* (240 mg.) was dried at 50°/0·05 mm. (Found, in a sample dried to constant wt.: C, 57·3; H, 6·3; N, 10·8. $C_{47}H_{59}O_{13}N_8Co$ requires C, 57·2; H, 6·0; N, 11·35. $C_{47}H_{61}O_{12}N_8Co$ requires C, 57·1; H, 6·2; N, 11·3%). Light absorption: (i) in H_2O, max. at 530, 500, 402, 353, 318—319, 223—224 mμ (log ε 3·86, 3·86, 3·59, 4·36, 3·95, 4·49 respectively); (ii) in 0·1M-KCN, max. at 579—580, 540, 413, 365, 348, 312, 302, 276, 215 mμ (log ε 3·94, 3·91, 3·30, 4·43, 4·14, 3·93, 3·91, 3·96, 4·50 respectively); (iii) in 0·1N-NaOH, max. at 576—577, 540—541, 412—413, 364, 312, and 276 mμ (log ε 3·85, 3·91, 3·34, 4·39, 3·92, 3·93, respectively). The infrared spectrum (Nujol mull) showed max. at 2119, 1709, 1695, 1580, 1504, 1399, 1304, 1258, 1185, 1160, 1143, 1120, 1058, 1047, 1021, 987, 973, and 946 cm.$^{-1}$. On the analytical ion-exchange column, the pentacarboxylic was obtained as a single band in tubes 20—25; on paper with butan-1-ol–acetic acid–2% hydrogen cyanide (4 : 1 : 5) it was obtained as a slightly elongated spot, R_F 0·62. On electrophoresis on Whatman No. 4 paper under standard conditions (0·1M-potassium cyanide solution for 5 hr. at 4·6 v/cm.) the pentacarboxylic acid migrated 10·1 cm. The acid did not melt below 300°; it was very soluble in aqueous acetone, phenol, and alkali, soluble in water, and insoluble in acetone or ether.

Crystalline Dicyanide Derivative of the Hexacarboxylic Acid.—Electrophoretically homogeneous hexacarboxylic acid was crystallised from aqueous acetone containing hydrogen cyanide as described for the pentacarboxylic acid, giving a *dicyanide* (Found, in a sample dried to constant wt.: C, 56·7; H, 6·0; N, 9·7. $C_{47}H_{58}O_{13}N_7Co$ requires C, 57·1; H, 5·9; N, 9·9. $C_{47}H_{60}O_{13}N_7Co$ requires C, 57·0; H, 6·1; N, 9·9%). Light absorption: (i) in H_2O, max. at 528, 497, 402, 380, 350—352, 318, 220—226 mμ (log ε 3·88, 3·91, 3·62, 3·62, 4·39, 3·99, 4·53 respectively); (ii) in 0·1M-KCN, max. at 576—577, 537, 412, 363, 348, 311, 301, 276, 212 mμ (log ε 3·97, 3·94, 3·32, 4·48, 4·16, 3·96, 3·92, 3·97, 4·62 respectively); (iii) in 0·1N-NaOH, max. at 532—539, 410—411, 360, 312—314, 275—276 mμ (log ε 3·91, 3·46, 4·34, 3·96, 3·99 respectively). The infrared spectrum (Nujol mull) showed max. at 2119, 1715, 1686, 1610, 1587, 1504, 1416, 1399, 1290, 1232, 1205, 1190, 1163, 1153, 1111, 1093, 1058, 1042, 1019, 1006, 974, and 810 cm.$^{-1}$. On the analytical ion-exchange column, the hexacarboxylic acid was obtained as a single band in tubes 44—50; on paper with the butan-1-ol–acetic acid–2% hydrogen cyanide it was obtained as a slightly elongated spot, R_F 0·76. Under standardised electrophoresis conditions (see under pentacarboxylic acid) it moved 11·7 cm. The solubilities of the hexacarboxylic acid in various solvents were similar to those of the pentacarboxylic acid.

Purification of Cobalt-containing Acids by means of Ion-exchange Chromatography. Use of Dowex 1 × 2 Acetate and Aqueous Ammonium Acetate for Elution.—A column of Dowex 1 × 2 acetate (18 × 2·5 cm.) was prepared and an aqueous solution (26 c.c.) of the ammonium salt of the hexabasic acid (490 mg.; obtained by electrophoresis on Whatman "seed-test" paper; Part III, *loc. cit.*) brought on to it. After being washed with water (100 c.c.) the column was developed with 0·5M-aqueous ammonium acetate until 3200 c.c. of eluate had been collected. A broad red band travelled slowly down the column and several minor bands were included in

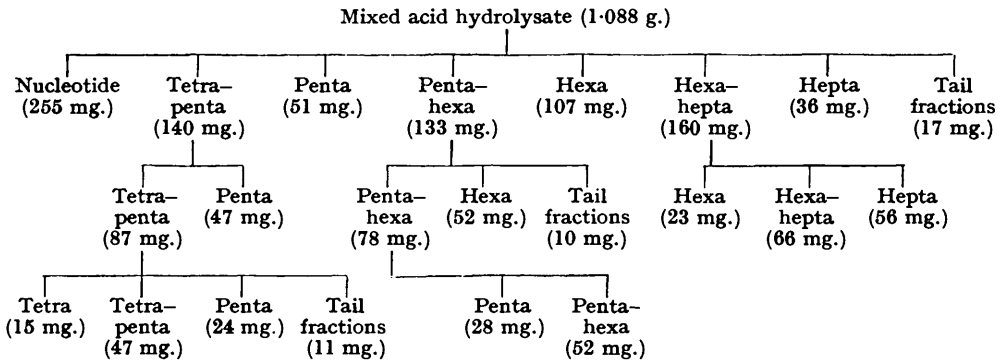
the eluate. Elution was continued with 0·6M-ammonium acetate, two major red fractions being obtained. The second and larger of these, obtained after 3200 c.c. of eluate had been collected, was acidified to pH 2 with concentrated hydrochloric acid and extracted with phenol. The phenolic extract was washed with N-hydrochloric acid to remove ammonium salts, and with water to remove chloride ions, then diluted with ether (3 vols.). The hexabasic acid was extracted with water, and the aqueous solution evaporated to dryness under reduced pressure. The red gum obtained was dissolved in water (7 c.c.), and acetone (100 c.c.) was added. Ether was added until a faint turbidity was produced and the solution was set aside for several weeks. The dark red prisms which separated were removed and sent to Dr. Hodgkin for *X*-ray crystallographic examination. The filtrate was again evaporated to dryness, the residue dissolved in aqueous acetone (1 : 4; 2 c.c.), and acetone (20 c.c.) added. After separation of a little amorphous material, ether was added to the solution until a turbidity was produced and, after scratching, fine red needles (130 mg.) of the *hexabasic acid* were deposited (Found, in material dried to constant wt. at 54°/0·05 mm.: C, 52·8; H, 6·3; N, ·7·85. $C_{46}H_{60}O_{13}N_6CoCl,2H_2O$ requires C, 53·2; H, 6·2; N, 8·1. $C_{46}H_{58}O_{13}N_6CoCl,2H_2O$ requires C, 53·5; H, 6·05; N, 8·1%). Light absorption: (i) in H_2O, max. at 528, 497, 402, 380—381, 350, and 317 mμ (log ε, 3·90, 3·92, 3·62, 3·59, 4·42, and 4·02 respectively) with an inflection at 264—270 mμ (log ε 4·34); (ii) in 0·1M-KCN, max. at 576, 537, 411, 363, 347, 311, 302, 287, and 277 mμ (log ε 3·97, 3·94, 3·29, 4·50, 4·18, 3·97, 3·93, 3·81, and 3·99 respectively). The infrared spectrum, determined on a mull in Nujol, showed max. at 2141, 2114, 1733, 1709, 1664, 1626, 1587, 1506, 1292, 1157, 1114, 1093, 1064, 1049, 1015, 976, and 813 $cm.^{-1}$.

A solution of the crystalline hexabasic acid (needles; 2·1 mg.) in 0·01N-ammonia was put on a column of Dowex 1 × 2 sulphate (9·5 × 1 cm.), washed with water, and eluted with 0·1M-sodium sulphate. Fractions (10 c.c.) of the eluate were collected and the intensity of light absorption at 351 mμ of each fraction was determined. Two main fractions were thus obtained, suggesting that some sulphate had been introduced into the molecule.

Acid Hydrolysis of Vitamin B_{12}.—Vitamin B_{12} (1·08 g.; air-dried) was dissolved in 2N-hydrochloric acid (50 c.c.) and heated in a boiling-water bath for 2 hr. The product was cooled and extracted by the phenol–ether method as described for the alkaline hydrolysate of vitamin B_{12} (above). From the aqueous solution there were obtained the combined ammonium salts (1·085 g.) of the mixed cobalt-containing acids and the nucleotide, and from the ethereal layer a small quantity of a crimson amorphous material (9·8 mg.).

The method of separation employed was similar to that described above for use with the alkaline hydrolysate. An examination of a small fraction of the product by the analytical Dowex 1 × 2 column showed that the nucleotide could be separated from the cobalt-containing acids and that these consisted of a mixture of tetra-, penta-, hexa-, and hepta-carboxylic acids in the approximate ratio, 1 : 6 : 9·5 : 4·5. These acids were eluted from the column in the same relative order as the acids obtained from the alkaline hydrolysis but the heptacarboxylic acid required 0·4M-sodium chloride for elution.

TABLE 2. *Ion-exchange separation of the acid hydrolysate from vitamin B_{12}.*



For the large-scale separation a column (10 × 2·5 cm.) of Dowex 1 × 2 (through 200 mesh) in the chloride form was used with a flow rate of 2·15—2·9 c.c./min. The experiment was conducted as before and 10 c.c. fractions were collected. Every tenth fraction was examined

by electrophoresis and the progress of the separation is illustrated in Table 2. The various tail fractions were not examined further; they contained material which could not be separated cleanly by electrophoresis. The combined yields of electrophoretically homogeneous material were: tetracarboxylic acid 15 mg.; pentacarboxylic acid 150 mg.; hexacarboxylic acid 182 mg.; heptacarboxylic acid 92 mg.; and nucleotide, 255 mg. containing a small amount of nucleoside.

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