227. *Chemistry of the Vitamin B_{12} Group. Part V.* The Structure of the Chromophoric Grouping.*

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Structures for vitamin B_{12} (I) and its hexacarboxylic acid degradation product (II) containing six rather than five conjugated double bonds are adopted and reactions bearing on this point are considered. With mild alkali in the presence of oxygen the acetamide side-chain of ring B of the vitamin undergoes cyclisation, to give dehydrovitamin B_{12}, a γ-lactam, whereas with an equivalent of a halogenating agent the same side-chain is converted into a γ-lactone; mechanisms for these cyclisations are considered. The reaction of vitamin B_{12} with more than one equivalent of halogenating agent has a marked effect on the chromophore; under analogous conditions, dehydrovitamin B_{12} gives a crystalline monohalogeno-derivative.

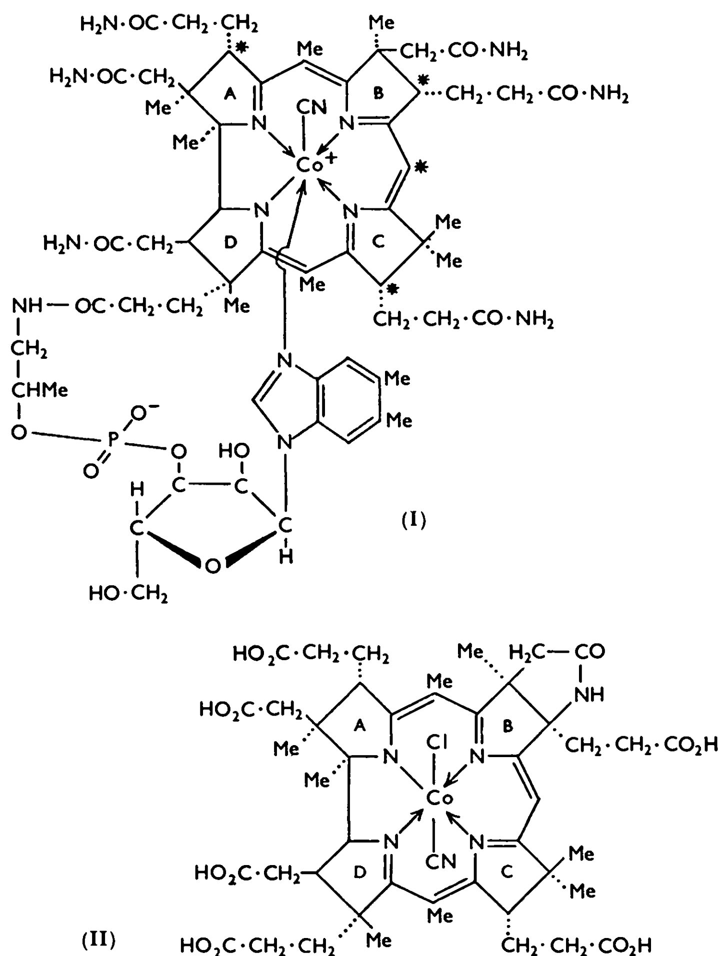
IN the preceding paper structural formulæ were presented for vitamin B_{12} and for the crystalline nucleotide-free hexacarboxylic acid obtained from it by treatment with 30% aqueous sodium hydroxide at 150°; both compounds were represented as containing five conjugated double bonds but it was emphasised that available evidence did not really permit of any clear decision between this degree of unsaturation and a system of six conjugated double bonds, the extended system having, indeed, certain attractions. It was hoped to reach a final decision by studying in detail the halogenation of the vitamin and some of its degradation products. This has now been achieved and we conclude that vitamin B_{12} and the hexacarboxylic acid degradation product each contain six conjugated double bonds. The same conclusion has been reached on *X*-ray crystallographic evidence.[1] They are therefore represented by structures (I) and (II) respectively, the conjugated system being shown arbitrarily in two alternative resonance forms. The chemical evidence upon which this conclusion rests comes primarily from halogenation experiments, but before dealing with these it is desirable to consider the formation of the lactam ring in the acid (II). Hydrolysis of all seven amide linkages in vitamin B_{12} to give a hepta-carboxylic acid occurs only under acidic conditions; under alkaline conditions no hepta-carboxylic acid is produced and the hexacarboxylic acid (II) is the end product. Clearly the formation of the lactam ring in this must have been the result of an oxidation at an early stage in the alkaline treatment of the vitamin and it is noteworthy that there are four positions (starred) in formula (I) which are " activated " by carbon–nitrogen double bonds.

* Part IV, preceding paper.

[1] Hodgkin, Kamper, Mackay, Pickworth, Trueblood, and White, *Nature*, 1956, **178**, 64.

In our preliminary communication [2] we suggested tentatively that cyclisation of the acetamide residue in ring B was preceded by hydroxylation at the β-position of the ring and involved dehydration or an equivalent reaction. This suggestion followed on our opinion that the neutral crystalline compound formed by aerial oxidation of vitamin B_{12} under mild alkaline conditions [3] was a monohydroxy-derivative of the vitamin. These views now require modification.

The oxidation product is very similar to vitamin B_{12} in its physical properties, *e.g.*, spectra, electrophoretic and chromatographic behaviour, but it is almost devoid of

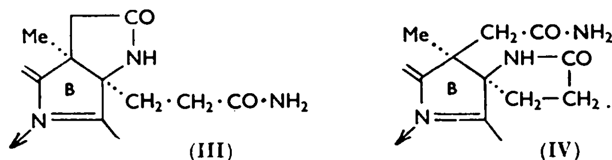


biological activity. Vigorous acidic hydrolysis of the oxidation product yields penta- and hexa-carboxylic acids but no hepta-carboxylic acid. Treatment of the oxidation product in the cold with alkali yields various acids formed by hydrolysis of amide groups; these are different from the corresponding acids obtained from the vitamin [4] and reconversion of the monocarboxylic acid fraction into the corresponding amide gives a biologically

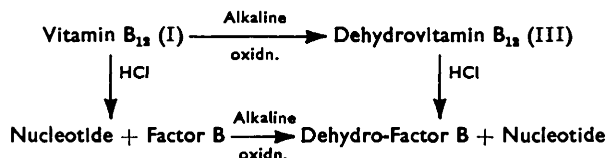
[2] Bonnett, Cannon, Johnson, Sutherland, Todd, and Smith, *ibid.*, 1955, **176**, 328; Hodgkin, Johnson, and Todd, *Chem. Soc. Special Publ. No.* 3, 1955, p. 109.
[3] Lester Smith, *Biochemical Soc. Symp.*, No. 13, 1955, p. 12.
[4] Armitage, Cannon, Johnson, Lester Smith, Parker, Stafford, and Todd, *J.*, 1953, 3849.

inactive oxidation product. Vigorous alkaline hydrolysis ultimately yields a hexacarboxylic acid which appears to be identical with the acid (II).

These results suggest that formation of the oxidation product in alkaline solution has involved direct cyclisation to yield the lactam (as in II) rather than hydroxylation at one of the starred positions in (I). Members of the chlorin series of chlorophyll derivatives are known to undergo hydroxylation at equivalent positions in the reduced ring,[5] but it is doubtful whether cyclisation of a hydroxyvitamin B_{12} would occur under the experimental conditions. Further examination has confirmed that a lactam ring is present in the oxid-



ation product which can be described as dehydrovitamin B_{12}. For convenience the dehydrovitamin is represented by the partial structure (III); only ring B is shown since the molecule is otherwise identical with vitamin B_{12} (I). The alternative structure (IV) is much less likely since it has been established by *X*-ray methods [6] that the hexacarboxylic acid (II), obtained by vigorous alkaline hydrolysis of the vitamin, contains the lactam grouping shown in (III) and the dehydrovitamin is almost certainly an intermediate in its production. The intensity of absorption at 1710 cm.$^{-1}$ in the infrared spectrum of the dehydrovitamin is increased relatively to that of vitamin B_{12}, and this is consistent with the formation of a γ-lactam as in (III) [cf. the hexacarboxylic acid (II) which shows a band at 1720 cm.$^{-1}$]. The dehydrovitamin is unaffected at 130° under reduced pressure. By short heating at 60° with concentrated hydrochloric acid the nucleotide is removed, giving a non-crystalline but homogeneous substance analogous to Factor B (obtained from vitamin B_{12} by similar treatment) but distinguishable from it both by chromatography and by microbiological assay. The same product is obtained by aerial alkaline oxidation of Factor B itself.



When the mixture of the three monocarboxylic acids obtained[4] by mild acidic hydrolysis of vitamin B_{12} is subjected to aerial alkaline oxidation and the products are examined electrophoretically at various pH's, no lactonic material can be detected. The known difference [7] in the rates of hydrolysis of γ-substituted as compared with β-substituted amides ($\gamma \gg \beta$) permits the assumption that the monocarboxylic acids obtained from the vitamin are isomers with one propionic acid residue on rings A, B, and C respectively. The above findings therefore argue against the presence of a free hydroxyl group in the β-position of one of these rings in the product of aerial alkaline oxidation and strongly support our thesis that partial structure (III) represents dehydrovitamin B_{12}.

The pronounced colour changes which are observed in presence of insufficient oxidising agent both in the vigorous alkaline hydrolysis of the vitamin (red ⟶ dark brown ⟶ red) and its mild alkaline oxidation (red ⟶ brown ⟶ red) strongly suggest that the cobalt

[5] Fischer *et al.*, *Annalen*, 1941, **547**, 234; 1943, **555**, 81.
[6] Hodgkin, Pickworth, Robertson, Trueblood, Prosen, and White, *Nature*, 1955, **176**, 325.
[7] Cason and Wolfhagen, *J. Org. Chem.*, 1949, **14**, 155; Cason, Castaldo, Clusker, Allinger, and Ash, *ibid.*, 1953, **18**, 1129.

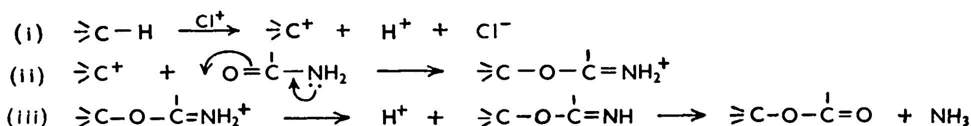
atom is involved in the initial phase of reaction in which oxidation occurs at the activated position on ring B. This is supported by the observation that the reaction both with vitamin B_{12} and Factor B is retarded by sodium mercaptoacetate and prevented by cyanide ions which stabilise the tervalent cobalt complex; this prevention of reaction recalls the inhibition of oxygen transfer on addition of cyanide to hæmoglobin.

The mechanism of the reaction is as yet unknown but would seem to be a one-electron oxidation involving formation of a radical at the starred position in ring B with simultaneous reduction of the cobalt from the tervalent to the bivalent state. The radical would then react with the acetamido-group to yield a lactam.

Knowledge of the nature and mode of formation of dehydrovitamin B_{12} is of value in understanding the reaction between vitamin B_{12} and halogenating agents. It has been reported [8] that *N*-chloroamides react with the vitamin and that three equivalents of the reagent are consumed before a product is obtained which is relatively stable in the presence of an excess of it. The product, although apparently micro-crystalline, was recognised as a mixture; analysis indicated that the halogen content corresponded to the introduction of two chlorine atoms into the molecule and, in the dicyanide form, the product appeared blue rather than red. Our early experiments showed that the normal Carius method for halogen analysis was unreliable in the B_{12} series and we developed a direct combustion technique in which the material was burned in a stream of oxygen and the halogen so produced estimated as halide ion after absorption in alkaline hydrogen peroxide.[9]

Subsequently, the reaction of the vitamin with halogens has been studied more closely. When treated with one equivalent of chloramine-T, vitamin B_{12} is converted into a red crystalline substance which is biologically inactive when assayed with *L. leichmannii*, *E. coli*, or *Ochromonas malhemensis*, and contains no halogen. The same product is obtained by the action of 1 equivalent of bromine water at pH 4 or by iodine in alkaline solution although in the latter case it is accompanied by dehydrovitamin B_{12} (III). [The lactam (III) is, indeed, the major product when the iodine concentration is low.]

This new substance is not isomorphous with the vitamin, and mixtures of the two can be separated by fractional crystallisation. It behaves as a neutral substance on electrophoresis at pH 6·5, but as a monobasic acid after treatment with aqueous sodium hydroxide for a few minutes. This change from a neutral to an acidic substance is reversible and strongly suggests that the halogenation product is a lactone. Both the neutral product and the acid into which it can be converted have been obtained crystalline and their absorption spectra confirm the postulated lactone–acid relationship. The infrared spectrum of the acidic form is very similar to that of vitamin B_{12} but that of the neutral form shows a new band at 1783 $cm.^{-1}$ which is consistent with the presence of a saturated γ-lactone group; the ultraviolet and visible spectra of the two forms are very similar to those of vitamin B_{12}, the principal bands being at 360 and 550 mμ for the acid and 359 and 552 mμ for the neutral form. The formation of this lactone evidently involves one of the amide groups in vitamin B_{12} since ammonia is also a product of the reaction and the lactone contains one nitrogen atom less than the vitamin. We interpret the course of



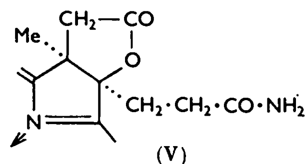
reaction as involving oxidation of one of the activated β-positions in (I) followed by reaction of the carbonium ion with the carbonyl-oxygen atom of the amide.[10] The sequence of

[8] Ellis, Petrow, Beaven, and Holiday, *J. Pharm. Pharmacol.*, 1953, **6**, 60; Schmid, Ebnöther, and Karrer, *Helv. Chim. Acta*, 1953, **36**, 65.
[9] Perold, *S. African Ind. Chemist*, 1953, **7**, 96.
[10] Winstein and Boschan, *J. Amer. Chem. Soc.*, 1950, **72**, 4669.

reactions is indicated on p. 1161; steps (ii) and (iii) are analogous to the reaction of acid halides with amides leading ultimately to anhydrides.[11]

All available evidence supports the view that the initial point of attack by the halogen is in ring B and that the lactone is a structural analogue of dehydrovitamin B_{12} (III), *i.e.*, that it has the partial structure (V).

The lactone can be recovered after treatment with hot dilute alkali under conditions used for the preparation of dehydrovitamin B_{12}, and when it is subjected to vigorous alkaline hydrolysis under the conditions which yield the hexacarboxylic acid (II) from vitamin B_{12} it yields a heptacarboxylic as well as a hexacarboxylic acid fraction. This is the only known instance of the formation of a heptacarboxylic acid by alkaline hydrolysis of a vitamin B_{12} derivative. These results not only accord with the lactone formulation but indicate with a high degree of probability that the formation of the lactone has blocked the position in ring B which is involved in lactam formation during the alkaline treatment discussed earlier. Furthermore, when dehydrovitamin B_{12} (III) is treated with one equivalent of halogenating agent it does not give a lactone; instead it is partly unchanged and partly converted into a new product which gives a blue cyanide derivative as does vitamin B_{12} after treatment with more than one equivalent of halogenating agent. Our preference for partial structure (V) is based on our choice of (III) rather than (IV) for dehydrovitamin B_{12}. Similar halogenation of two of the isolated isomeric monocarboxylic acids obtained [2] by mild hydrolysis of vitamin B_{12}, as well as of a mixture of all three isomers, yields in each instance lactonic acids, *i.e.*, the free acid groupings (one of which was almost certainly the propionic acid group on ring B) were not involved in the lactonisation. The lactone (V) was converted into derivatives of Factor B by warming it with concentrated hydrochloric acid for a short time. Although crystalline products were not isolated the nucleotide was removed and a neutral product (presumably the lactone derivative of Factor B) was produced together with mono- and di-basic acids formed by further hydrolysis of amide groups; all these products retained the lactone group, as shown by their electrophoretic behaviour before and after alkaline treatment.



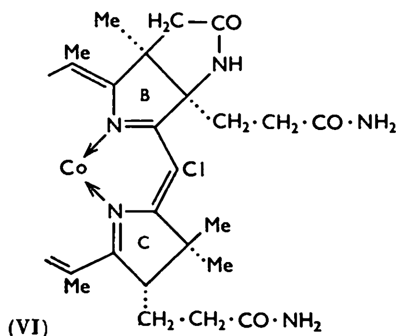
(V)

Whereas treatment with one equivalent of a halogenating agent has little effect on the ultraviolet and visible absorption of vitamin B_{12} (see above), addition of a second equivalent causes a marked bathochromic shift; during this reaction displacement of cyanide ion from the co-ordination shell may occur but this alone would result in a hypsochromic shift. In aqueous solution the bands at 361 and 550 mμ are displaced to 364 and 580 mμ, and in aqueous potassium cyanide (*i.e.*, as dicyanide) the bands at 368, 540, and 581 mμ are displaced to 370, 572, and 612 mμ respectively. Similar shifts in spectra are obtained when dehydrovitamin B_{12} (III) is treated with one equivalent of halogenating agent. Clearly it is only after the starred β-position in ring B is blocked (*e.g.*, by lactam or lactone formation) that attack by halogen seriously affects the chromophore. It was the pronounced bathochromic effect of halogenation that led us originally to favour a structure for vitamin B_{12} containing five conjugated double bonds (cf. Part IV); the magnitude of the shift suggested an increase in conjugation from five to six double bonds, which, if it were brought about by elimination of one mol. of hydrogen halide, would account for the apparent presence of only two chlorine atoms in the products of chlorination of the vitamin with three equivalents of halogen. The isolation of the lactone (V) as the halogen-free initial product in the halogenation reaction and our appreciation of the unreliable character of previous halogen analyses clearly undermined this argument and it became important to isolate the products of more extensive halogenation.

With dehydrovitamin B_{12} a bathochromic shift is apparent immediately upon introduction of the halogenating agent and, by using 1 mol. of chloramine-T, it is possible

[11] Titherley, *J.*, 1904, **85**, 1673.

to isolate a crystalline compound which contains one atom of chlorine and has lost the co-ordinated cyanide ion. The same monochloro-compound is obtained on using two and five mols. of chloramine-T. The action of excess of halogenating agent on vitamin B_{12} itself gives an amorphous halogen-containing lactonic product having spectral characteristics similar to those of the crystalline monochlorodehydrovitamin B_{12}, absorption bands being observed at 548 and 580 mμ in aqueous solution, whereas the vitamin has the corresponding bands at 520 and 550 mμ. From a consideration of electron-availability at the various positions in the chromophore [12] we presume that halogenation occurs at the free *meso*-position between rings B and C, to give the compound (VI), and the bathochromic shift is to be compared with that observed in passing from methyl vinyl ketone ($\lambda_{max.}$ 210 mμ) to methyl 2-chlorovinyl ketone ($\lambda_{max.}$ 228 mμ).[13]



The behaviour of the hexacarboxylic acid (II) towards chloramine-T is complicated by decomposition accompanied by a rapid fall in light absorption; in this case, no pure chlorinated compound has been isolated.

We conclude that the chromophoric anion in vitamin B_{12}, dehydrovitamin B_{12}, and the hexacarboxylic acid is degenerate, the 13 atom system containing six double bonds, and that the bathochromic shifts on halogenation are attributable to substitution rather than extension of the chromophore. Our failure to dehydrogenate the chromophoric system in the esterified hexacarboxylic acid by hydrogen-transfer to chloranil [14] is in accord with this interpretation.

When vitamin B_{12} is treated with silver oxide in ethanol–pyridine under the conditions that bring about hydroxylation of the chlorophyll molecule,[15] the main product is the same lactone that is formed from vitamin B_{12} by reaction with limited amounts of halogens; a smaller amount of the lactam is also formed. The lactone is converted into three uncharacterised products by prolonged heating with silver oxide in ethanol–pyridine under reflux.

EXPERIMENTAL

Dehydrovitamin B_{12}.—A solution of vitamin B_{12} (528 mg.; hydrated crystals) in aqueous 0·1N-sodium hydroxide (105 c.c.) was heated at 100° for 10 min. while a brisk current of air was drawn through the solution. After cooling to room temperature, the brown solution was acidified with dilute hydrochloric acid, and the red product extracted with small volumes of phenol until all the colour was removed from the aqueous layer. The combined phenolic extracts were washed with a little water to remove chloride ions, then diluted with ether, and the red product re-extracted into a small volume of water. 2% Aqueous hydrogen cyanide solution (2 c.c.) was added to the red aqueous extract which was then concentrated (to 20 c.c.) under reduced pressure on the water-bath. The product was then applied to a column (14 × 1 cm.) of Dowex 1 × 2 (acetate form) and eluted with water, the neutral product being obtained in the eluate and the acidic products adsorbed on the column. The aqueous eluate was concentrated as before (to 10 c.c.) and acetone added until a faint turbidity was produced. The solution gradually deposited long red needles (272 mg.) of *dehydrovitamin B_{12}* (Found, after drying at 50° *in vacuo* for 3 days: C, 55·3; H, 6·6; N, 14·2. $C_{63}H_{86}O_{14}N_{14}PCo$ requires C, 55·9; H, 6·4; N, 14·5%). Light absorption: (i) in H_2O, max. at 548, 517, 408, 359, 320, 304, 278 mμ (log ε 3·81, 3·80, 3·45, 4·35, 3·80, 3·87, and 4·09 respectively); (ii) in 0·1M-KCN, max. at 581, 541, 415, 366, 313, 303, 288, 278 mμ (log ε 3·87, 3·83, 3·21, 4·36, 3·86, 3·83, 3·92, and 4·02

[12] Cf. Longuet-Higgins, *J. Chem. Phys.*, 1950, **18**, 265, 275, 283.
[13] Bowden, Braude, and Jones, *J.*, 1946, 948.
[14] Braude, Jackman, and Linstead, *J.*, 1954, 3548.
[15] Fischer and Lautsch, *Annalen*, 1937, **528**, 247.

respectively), with inflections at 510 and 350 mμ (log ε 3·65 and 4·05). The infrared spectrum, determined in Nujol mull and as a potassium bromide plate, showed max. at 3350, 3155, 2920, 2140, 1670, 1580, 1507, 1387, 1370, 1215, 1146, and 875 cm.$^{-1}$. Dehydrovitamin B_{12} is indistinguishable chromatographically from vitamin B_{12} on paper when butan-1-ol–acetic acid–2% hydrogen cyanide (4 : 1 : 5) is used as solvent; but in butan-2-ol (1 l.) containing aqueous ammonia (*d* 0·88; 2 c.c.), 4% aqueous hydrogen cyanide (1 c.c.), and just sufficient water to saturate the system, it had R_F 1·15 relative to that of vitamin B_{12}. By the *E. coli* mutant plate assay a chromatographically pure sample of dehydrovitamin B_{12} showed 0·1—0·4% of the activity of vitamin B_{12}; it is impossible to be more precise because the response curves for the two substances have widely different slopes. The compound was inactive towards *Ochromonas malhemensis* and showed about 0·2% of the activity of vitamin B_{12} by the *Lactobacillus leichmannii* tube assay.

Prolonged Treatment of Vitamin B_{12} with Alkali.—Vitamin B_{12} (120 mg.; hydrated crystals) in 0·1N-aqueous sodium hydroxide (20 c.c.) was heated under reflux for 1 hr. The cooled solution was acidified and extracted with phenol as in the previous experiment. The final aqueous solution was applied to two sheets of Whatman seed-testing paper, as a streak *ca.* 20 cm. in length on each, and then submitted to electrophoresis in phosphate buffer (0·05N at pH 6·5, containing 0·01% of potassium cyanide) at 2·5 v/cm. for 16 hr. The papers showed a pattern of 5 red zones due to neutral products and mono- to tetra-basic acids, the mono- and di-basic acids being present in the largest amounts. Each of the red acidic zones was accompanied by a slightly faster-moving and partly overlapping purple zone due to the corresponding acid from which the nucleotide had been removed.[4] The zones were cut out separately as far as possible and the neutral and monobasic red fractions were submitted to electrophoresis in N-acetic acid at 4 v/cm. to remove remaining materials lacking the nucleotide.

The material which was neutral at pH 6·5 gave a main fraction (4·2 mg.), also neutral in acetic acid, and identical with the product obtained from brief treatment of vitamin B_{12} with alkali. The minor fraction was basic and gave an orange solution in acetic acid.

The red monobasic acids (8·1 mg.) were also purified by electrophoresis in acetic acid and then crystallised slowly from aqueous acetone. Chromatography on paper, solvent being butan-2-ol (1 l.) containing acetic acid (2 c.c.) and 4% aqueous hydrogen cyanide (10 c.c.), showed it to consist of a mixture of isomers, all different from those produced [4] by the action of cold dilute acids on vitamin B_{12} which were chromatographed on the same paper. The isomers from alkaline hydrolysis gave R_F values (relative to that of vitamin B_{12}) of 0·55 (weak), 0·95, and 1·3. The relative R_F values of those from acid hydrolysis were 1·2, 1·45, and 1·7 (weak).

Partial Synthesis of Dehydrovitamin B_{12}.—The monobasic acids (mixed isomers; 5 mg. of hydrated crystals) obtained in the foregoing experiment were dried at 120—130° under reduced pressure for 10 min. The dried material was dissolved in dry *NN*-dimethylformamide (0·15 c.c.) and triethylamine (0·015 c.c. of 20% v/v solution in dimethylformamide) was added, then cooled to −5°. Ethyl chloroformate (0·008 c.c. of 20% v/v solution in benzene) was added and the mixture kept at −5° for 15 min. It was then saturated with dry ammonia and kept for 3 hr. at room temperature. Acetone–ether (2 c.c.; 1 : 1) was added and the red precipitate separated and dissolved in a few drops of water. Examination of the solution by electrophoresis on Whatman 3MM paper showed that the main component was neutral. The *E. coli* mutant plate assay indicated 1·5—4% of the activity of the vitamin, confirming that the main product is dehydrovitamin B_{12} but containing a trace of the vitamin.

Treatment of Dehydrovitamin B_{12} with Concentrated Hydrochloric Acid.—(i) Dehydrovitamin B_{12} (5 mg.) was heated in concentrated hydrochloric acid at 65° for 4½ min. The acid was removed by evaporation under reduced pressure and the residue was chromatographed on Whatman 3MM paper in the acidic butan-2-ol system, alongside a similar hydrolysate of vitamin B_{12}, known to consist mainly of Factor B (vitamin B_{12} lacking the nucleotide) and corresponding mono-basic acids.[4] The two preparations gave patterns that were very similar in appearance, with different R_F values. The main fraction corresponding to dehydrovitamin B_{12} lacking the nucleotide had an R_F value (relative to that of vitamin B_{12}) of 1·85 compared with 1·7 for Factor B. The next strongest zones had relative R_F values of 1·5 and 1·3 respectively. The products were devoid of microbiological activity whereas Factor B is even more active than vitamin B_{12} in the *E. coli* plate assay.

(ii) Dehydrovitamin B_{12} (8·6 mg.) was heated with 6N-hydrochloric acid (1 c.c.) in a sealed

tube at 100° for 2 hr. The red solution was evaporated to dryness and the residue taken up in a few drops of N-ammonia. The solution was subjected to electrophoresis on paper at pH 10 and the product shown to be a mixture of penta- and hexa-carboxylic acids. No heptacarboxylic acid was present. A parallel hydrolysis of vitamin B_{12} under the same conditions gave a mixture of penta-, hexa-, and hepta-carboxylic acids (cf. ref. 4).

*Spectrophotometric Examination of the Action of Chloramine-*T *on Vitamin B_{12}, Dehydrovitamin B_{12}, and the Hexabasic Carboxylic Acid.*—The chloramine-T used was shown titrimetrically to be 99·6% $CH_3·C_6H_4·SO_2·NClNa,3H_2O$, and the procedure of Ellis *et al.*[8] was adopted. Dehydrovitamin B_{12} (10 mg.) was dissolved in 0·001N-acetic acid (100 c.c.) and to aliquot parts (20 c.c.) of this solution were added volumes (1, 2, 3, 4 c.c.) of a solution of chloramine-T (41 mg.) in water (100 c.c.), representing respectively 1, 2, 3, and 4 mols. of halogenating agent: each solution was made up to 25 c.c. with water, and, after 3 hr., examined spectrophotometrically between 500 and 600 mμ. The observations of Ellis *et al.*[8] on vitamin B_{12} were confirmed; however, in the case of dehydrovitamin B_{12} the first mol. of halogenating agent produded a bathochromic shift whereas with the hexacarboxylic acid accompanying destruction of the chromophore was rapid.

Action of Halogenating Agents on Vitamin B_{12}.—(i) (with Dr. G. W. MILLER) *Chloramine-*T (1 *mol.*). Vitamin B_{12} (100 mg.; air-dried crystals) was dissolved in hot water (15 c.c.; ammonia-free) and to the cooled solution were added chloramine-T (22 mg.) in water (10 c.c.) and then 0·1N-hydrochloric acid (1 c.c.). No obvious colour change occurred. After 2 hr. the solution was extracted with phenol (2 × 20 c.c.), and the aqueous layer set aside for subsequent determination of ammonia. The phenol extract was washed with a little water and diluted with ether, and the red product re-extracted into a small volume of water. This solution was freeze-dried and the residue (92·5 mg.) purified by preparative electrophoresis [16] in 0·1M-potassium cyanide solution on Whatman seed-testing paper (25 × 46 cm.) at 3 v/cm. for 10 hr. Two fractions were obtained, the minor corresponding to vitamin B_{12} and referred to as the neutral fraction (although in cyanide solution it travels as a singly charged anion) and the other, the major fraction, corresponding to a monocarboxylic acid of the B_{12} series (travelling as a doubly charged anion). This main band was excised, extracted into water (Waring blendor), and freed from inorganic and insoluble material by phenol–ether extraction. Re-extraction into water, followed by freeze-drying of the solution, yielded the product (71 mg.).

This was dissolved in 1 : 1 aqueous acetone (15 c.c.), a few drops of ether were added, and the solution was kept at 0° for several days. Large crystals of the *lactone* (23 mg.) were thus obtained (Found: C, 53·0; H, 6·9; N, 12·8%; C : N, 4·15. $C_{63}H_{85}O_{15}N_{13}PCo$ requires C, 55·9; H, 6·35; N, 13·4%; C : N, 4·17. $C_{63}H_{85}O_{15}N_{13}PCo,4H_2O$ requires C, 53·0; H, 6·5; N, 12·8%). Light absorption max.: (i) in H_2O, 552, 524—525, 407—409, 359—360, 320, 302—306, and 278—279 mμ (log ε 3·88, 3·87, 3·52, 4·41, 3·88, 3·92, and 4·14 respectively); (ii) in 0·1M-KCN, 584—586, 544—545, 418—420, 368—369, 314, 306—307, 288, and 278—279 mμ (log ε 3·94, 3·87, 3·32, 4·42, 3·93, 3·91, 4·01, and 4·08 respectively). The infrared spectrum showed main peaks at 2140, 1783, 1665, 1574, 1310, 1215, 1150, 1064, 1000, 905, 870, 850, and 815 cm.$^{-1}$.

On being subjected to electrophoresis at pH 6·5 in phosphate buffer this red product had zero mobility whereas it travelled as a purple doubly charged anion in 0·1M-potassium cyanide. Treatment of a small quantity (2 mg.) with 2N-hydrochloric acid (4 c.c.) at 100° for 2 hr. yielded, after electrophoresis, tetra-, penta-, hexa-, and hepta-basic acids: similar treatment with 30% sodium hydroxide solution yielded a mixture of hexa- and hepta-basic acids.

On chromatography on paper with butan-2-ol with a trace of acetic acid as solvent, the lactone had R_F 1·2 relative to vitamin B_{12}, but after treatment with alkali the R_F was 0·6, often with a faint streak of colour terminating in a weaker spot at R_F 1·2, suggesting partial reconversion into the ring form during the chromatography. The lactone showed no microbiological activity towards the *E. coli* mutant (in plate or tube assays), *Ochromonas malhemensis*, or *L. leichmannii*.

The aqueous solution previously set aside for the determination of the ammonia liberated during halogenation was extracted continuously with ether for 2 hr., to remove traces of phenol, separated, and left under reduced pressure for 1 hr. at room temperature. After filtration the solution was concentrated to 5 c.c. and a solution (0·5 c.c.) of chloroplatinic acid (10 g.) in water

[16] Cannon and Gilson, *Chem. and Ind.*, 1954, 120.

(10 c.c.) added. After evaporation, the residue was extracted with absolute ethanol (5 c.c.), transferred to a weighed crucible, and after further washing with absolute ethanol (10 c.c.) dried to constant weight at 130°. In this way were obtained 12·6 mg. of ammonium chloroplatinate, equivalent to a recovery of 0·77 mol. of ammonia. Controls with chloramine-T and vitamin B_{12} gave residues of 1·2 and 0·6 mg. respectively. With chloramine-T (22 mg.) and 0·0104M-ammonium chloride (10 c.c.), ammonium chloroplatinate (16·4 mg.) was obtained, equivalent to a recovery of 71% of the ammonia present.

(ii) *Chloramine*-T (3 *mols*.). Vitamin B_{12} (500 mg.) was dissolved in acetate buffer solution (1 l.) at pH 4 and a solution of chloramine-T (310 mg. : 3 mols.) in water (50 c.c.) added. After 3 hr. the solution was extracted as described above and the halogenated product isolated as an amorphous solid (460 mg.). On being subjected to electrophoresis at pH 7 the material remained at the origin whereas in 0·1M-potassium cyanide it behaved as a monobasic acid (*i.e.*, it moved as a doubly charged anion). Chromatography on paper with butan-1-ol–acetic acid–2% hydrogen cyanide (4 : 1 : 5) showed a single spot having R_F 0·40. The light absorption spectrum in 0·1M-potassium cyanide showed max. at 288, 370, 572, and 612 mμ (Found, in amorphous material: Cl, 4·35, 4·0. Calc. for monochlorovitamin B_{12}: Cl, 2·6%).

(iii) *Bromine* (1 *mol*.). To a solution of vitamin B_{12} (30 mg.; hydrated crystals) in water (200 c.c.) were added acetic acid (0·015 c.c.) and then 0·047N-bromine water (0·9 c.c.) dropwise during 1 min. After 1 hr. at room temperature the red product was extracted through phenol, and an aqueous solution subjected to electrophoresis at pH 6·5 and then in 0·1M-potassium cyanide. The neutral product was extracted and crystallised (17 mg.) from aqueous acetone. Analysis of the product showed only a trace of bromine.

(iv) *Iodine* (1 *mol*.) *and sodium hydroxide*. Vitamin B_{12} (13 mg.; hydrated crystals) was dissolved in N-aqueous sodium hydroxide (10 c.c.). One half of the solution was mixed with its own volume of water and the other with the same volume of 0·1N-iodine in aqueous potassium iodide. Both solutions were kept in the dark at room temperature for 30 min., then extracted with phenol as described above and subjected to electrophoresis at pH 6·5. The major neutral zones were eluted and assayed with the *E. coli* mutant; that (3·7 mg.) from treatment with alkali alone had 70% of the activity of vitamin B_{12} and that (2·5 mg.) from treatment with the alkaline solution of iodine had 7% of the activity of the vitamin.

The latter experiment was repeated on 8 times the scale and the product fractionated on 3 sheets of Whatman 3MM paper in the ammoniacal butan-2-ol system. A faint red zone, presumably of unchanged starting material, appeared at the same R_F as the vitamin B_{12} marker. The principal zone of dehydrovitamin B_{12} had an R_F of 1·1 relative to vitamin B_{12}, and a third zone of the halogenation product ran still faster. Several other faint zones were not investigated further. The main zone corresponded with dehydrovitamin B_{12} in chromatographic behaviour, in electrophoretic behaviour at all pH values, and in its slight microbiological activity towards three test organisms.

The fastest zone also corresponded in its properties with those of the products from either chloramine-T or bromine, including the lactonic behaviour.

In another experiment vitamin B_{12} (120 mg.) was treated with a 0·1N-solution of iodine in 0·1N-sodium hydroxide at room temperature for 2½ hr. The crude product was subjected to electrophoresis in potassium cyanide solution on Whatman seed-testing paper. The strongest zone travelled at the same rate as dicyanocobalamine and this was eluted and fractionated by chromatography on paper with, as solvent, butan-2-ol (1%) containing aqueous ammonia (*d* 0·88; 2 c.c., 4%), aqueous hydrogen cyanide (1 c.c.), and sufficient water to saturate the mixture. The zone was shown to consist mainly of the unchanged vitamin together with a little dehydrovitamin B_{12} (11 mg.). The weaker zone from the electrophoresis contained an additional acidic group, and was eluted and crystallised (32 mg.) from aqueous acetone. The electrophoretic and chromatographic properties of this substance were identical with those of the product obtained from vitamin B_{12} with either chloramine-T or bromine. The yield of product in this case greatly exceeded that of dehydrovitamin B_{12} in contrast to the previous experiment.

Acid Hydrolysis of the Lactone derived from Vitamin B_{12} by the Action of Bromine.—The product (5 mg.; hydrated crystals) from the action of bromine on vitamin B_{12} was dissolved in 11N-hydrochloric acid (0·2 c.c.) and kept at 65° for 5 min., then cooled, diluted, and extracted with cresol. The extract was washed with a small quantity of water (3 ×) to remove acid. The red product was precipitated by the addition of acetone and ether, and divided into two

parts. One portion was dissolved in 0·1N-sodium hydroxide and the other in 0·1N-hydrochloric acid and after 30 min. the solutions were brought to pH 6·5 and subjected to electrophoresis in 0·05N-phosphate buffer (pH 6·5) containing cyanide. The patterns of purple zones so obtained were as follows:

	Acid-treated	Alkali-treated
Neutral	Strong	—
Monobasic acid	Moderate	Strong
Dibasic acid	Faint	Moderate
Tribasic acid	—	Faint

Treatment of the Monocarboxylic Acids derived from Vitamin B_{12} with Bromine.—The monobasic acids (6 mg.; hydrated crystals; mixed isomers from treatment of vitamin B_{12} with cold dilute mineral acids) were dissolved in water (20 c.c.), and acetic acid (0·003 c.c.) and 0·082N-bromine water (0·18 c.c.) were added. After 45 min. at room temperature, the mixture was acidified, extracted with cresol, and subjected to electrophoresis at pH 6·5 as described in the previous experiment. The principal product showed the same mobility as the original monocarboxylic acid. This zone was eluted and portions made acid and alkaline (to 0·05N) with hydrochloric acid and sodium hydroxide solution, each kept for 15 min., then neutralised to pH 6·5 and subjected to electrophoresis. The acidified portion still behaved as a monobasic acid but the portion treated with alkali now displayed an extra acidic group.

The experiment was repeated, with similar results, with each of the two more abundant monocarboxylic acid isomers separated by chromatography on paper.[4]

Isolation of a Crystalline Chlorinated Derivative of Dehydrovitamin B_{12}.—Dehydrovitamin B_{12} (150 mg.: air-dried crystals) was dissolved in an acetate buffer solution (1 l.) at pH 4, and a solution of chloramine-T (63 mg.; 2 mols.) in water (50 c.c.) added. After 3 hr. at room temperature the solution was extracted through phenol and ether as for the vitamin (above), and the resultant aqueous solution was washed with ether, filtered, and evaporated to small bulk under reduced pressure at 50°. Acetone was added to opalescence (total vol., 5 c.c.) and the solution left at 0°, the *product* crystallising as delicate crimson needles (46 mg.). The infrared spectrum showed that the cyanide ion was no longer present, presumably having been replaced by an hydroxo-ligand (Found: C, 53·7; H, 6·0; N, 13·4; Cl, 2·8, 3·0. $C_{62}H_{86}O_{15}N_{13}ClPCo$ requires C, 54·0; H, 6·2; N, 13·2; Cl, 2·6%). Light absorption max.: (i) in H_2O, 580, 548, 417, 365, 288, 282 mμ (log ε 3·85, 3·82, 3·43, 4·44, 4·09, 4·07 respectively); (ii) in 0·1M-KCN, 609, 568, 424, 369, 316, 303, 289 mμ (log ε 3·93, 3·82, 3·87, 3·18, 4·45, 3·82, 4·06 respectively). The infrared spectrum, determined in a potassium bromide plate, showed maxima at 3200, 2930, 1670, 1550, 1480, 1415, 1387, 1335, 1220, 1145, 1048, and 1023 $cm.^{-1}$. On subjection to electrophoresis in 0·1M-potassium cyanide the product ran as a single spot alongside vitamin B_{12}: at pH 7 it remained at the origin.

Chromatography on paper with butan-1-ol–acetic acid–2% hydrogen cyanide (4 : 1 : 5) gave R_F 0·33. The same product was obtained by using two and using five mols. of chloramine-T. With chloramine-T (1 mol.), dehydrovitamin B_{12} (150 mg.) gave the crystalline chloro-derivative (35 mg.) (Found: Cl, 2·5%), identified electrophoretically and spectrophotometrically with the product obtained previously.

Attempted Dehydrogenation of the Chromophoric System in the Hexacarboxylic Acid.—The hexabasic carboxylic acid (100 mg.) from the alkaline hydrolysis of vitamin B_{12} (preceding paper) was heated in methanol (25 c.c.) containing 11N-hydrochloric acid (1 c.c.) under reflux for 20 hr. before being evaporated to dryness under reduced pressure. The red solid (106 mg.) so obtained was extracted from a Soxhlet thimble with anhydrous benzene for 1 hr. and the extract evaporated, to yield the hexamethyl ester (43 mg.) of the acid. Light absorption max.: (i) in C_6H_6, at 345, 368, 412, 432, 525, and 555 mμ; (ii) in benzene–methanol (2 : 1) in the presence of solid potassium cyanide, 315, 367, 417, 545, and 585 mμ. At pH 7 the ester behaved as an electrophoretically neutral entity containing a trace of a monobasic acid.

Solutions of the hexamethyl ester (10 mg.) in anhydrous benzene (100 c.c.) and of chloranil (recrystallised; 11·5 mg.) in the same solvent (100 c.c.) were prepared and to aliquot samples (10 c.c.) of the ester solution were added various amounts (2 c.c., 1 mol.; 4 c.c., 2 mols.) of the chloranil solution. Heating the mixed solutions in sealed tubes at 100° for 24 hr. caused no change in colour, and spectroscopic examination confirmed that no change had taken place. More vigorous conditions (150°; 48 hr.) had no effect save to broaden the absorption peaks slightly.

Reaction of Vitamin B_{12} with Silver Oxide.—Vitamin B_{12} (60 mg.), silver oxide (300 mg.), pyridine (3 c.c.), and ethanol (3 c.c.) were heated together under reflux for 7 min. The suspension was centrifuged and the supernatant liquid treated with ether (12 c.c.). The red precipitate was collected at the centrifuge, washed with ether, and dried. It was dissolved in water (0·5 c.c.) and streaked across four sheets of Whatman 3MM filter paper. After chromatography in the acidic butan-2-ol system (above), the main zone had R_F 1·25 (relative to that of vitamin B_{12}). There were also a weak slow zone, a fast purple zone (not further examined), and a moderately strong zone with the same R_F as vitamin B_{12}. The last zone was eluted, the solvent removed, and the residue crystallised from aqueous acetone and chromatographed again in the ammoniacal butan-2-ol system. There was then no colour at the level of the vitamin B_{12} marker spot, but all the colour was concentrated at the level of the dehydrovitamin B_{12} marker spot, with R_F 1·15 relative to that of vitamin B_{12}. This behaviour in the two solvent systems, and its neutrality on electrophoresis at pH 6·5, characterise this product as the lactam, dehydrovitamin B_{12}.

The major zone from the electrophoresis in the acidic solvent was also eluted and the solvent removed; the residue crystallised slowly from aqueous acetone. This product behaved in the same manner as the lactone (from vitamin B_{12} treated with one mol. of bromine) on chromatography and electrophoresis at pH 6·5, both in the lactone form and in the acid form (following brief treatment with aqueous alkali). The infrared spectrum also showed bands in the same positions, including the prominent band at 1785 cm.$^{-1}$ characteristic of γ-lactones.

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