785. *A Partial Synthesis of the Vitamin B_{12} Coenzyme and Some of its Analogues.*

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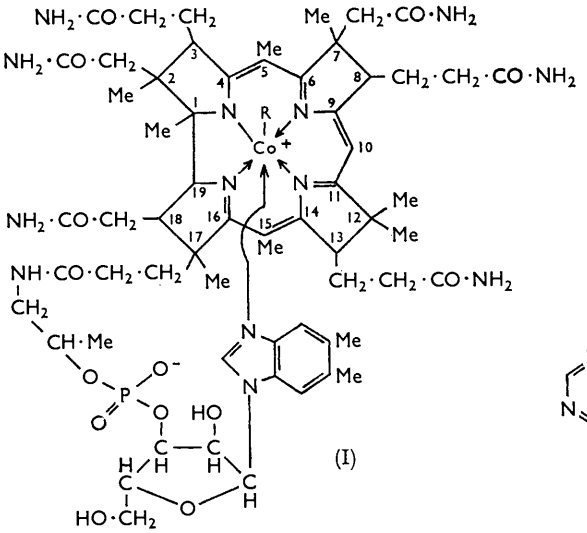
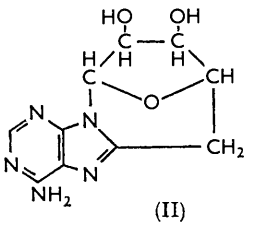
The vitamin B_{12} coenzyme has been partially synthesised from hydroxocobalamin (vitamin B_{12b}) by reduction followed by reaction with a 5′-tosyladenosine. Many analogues, including simple alkyl and acyl derivatives, have been obtained by similar methods. The reactive intermediate is postulated as a cobalt hydride on the basis of its addition to a variety of ethylenic and acetylenic compounds, yielding further analogues of the coenzyme. Reaction of the cobalt hydride with diazomethane gives the cobalt-methyl derivative. Certain reactions of the products are described.

The vitamin B_{12} coenzymes were first isolated by Barker and his colleagues,[1] and the chemical structure (I) of one of them, the 5,6-dimethylbenzimidazolylcobamide coenzyme was established by *X*-ray crystallographic and chemical analysis.[2] The other coenzymes of the group differ only in the nature of the base contained in the nucleotide fragment. The novel cobalt–carbon bond linking the metal to the 5′-deoxyadenosine group of the coenzyme (I; R = 5′-deoxyadenosyl) is easily broken by a variety of chemical reagents as well as by light, and many transformation products of the coenzyme have been characterised (see, *e.g.*, refs. 3 and 4). Apart from the possible addition of hydrogen at C_9–C_{10} in the corrin nucleus[2]* the main ambiguity of structure (I; R = 5′-deoxyadenosyl) concerns



(I)



(II)

the cobalt atom, which is probably tervalent on the basis of the observed chemical reactions, although there have been claims[5] that the coenzyme is paramagnetic, which would suggest the presence of bivalent cobalt.

* Until this ambiguity is resolved, the " cobalamin " nomenclature for the coenzymes and their analogues (I), *e.g.*, " methylcobalamin " for (I; R = Me), has been avoided:

[1] Barker, Weissbach, *et al.*, *Proc. Nat. Acad. Sci. U.S.A.*, 1958, **44**, 1093; 1959, **45**, 521; *J. Biol. Chem.*, 1960, **235**, 181, 480; Barker, " Vitamin B_{12} und Intrinsic Faktor," F. Enke, Stuttgart, 1962, p. 82.

[2] Lenhert and Hodgkin, *Nature*, 1961, **192**, 937; " Vitamin B_{12} und Intrinsic Faktor," F. Enke, Stuttgart, 1962, p. 105.

[3] Weissbach, Ladd, Volcani, Smyth, and Barker, *J. Biol. Chem.*, 1960, **235**, 1462.

[4] Johnson and Shaw, *J.*, 1962, 4608.

[5] Johnson and Shaw, *Proc. Chem. Soc.*, 1960, 420; Bernhauer, Gaiser, Müller, Müller, and Gunter, *Biochem. Z.*, 1961, **333**, 560; Nowicki and Pawelkiewicz, *Bull. Acad. polon. Sci.*, 1960, Cl. II, **8**, 433.

Even before the full structure of the coenzyme was established, attempts were made at a partial chemical synthesis from vitamin B_{12b}. Pawelkiewicz *et al.*,[6] who isolated the coenzyme form of cobinamide (factor B)[7] from *Propionibacterium shermanii*, reported that a compound with identical spectral and physicochemical properties was obtained by reduction of cobinamide with sodium dithionite in the presence of adenine. Bernhauer *et al.*,[8] obtained a substance, with an absorption spectrum similar to that of "vitamin B_{12r}"[9] (r = reduced; a reversible reduction product of vitamin B_{12b}), by catalytic reduction of hydroxocobalamin in the presence of adenine; the product obtained by Bernhauer *et al.*, in contrast to vitamin B_{12r}, was stable to oxygen in the absence of light. Barker and Brady,[10] and we also, have repeated these experiments and failed to obtain any product containing either adenine or an adenine nucleoside linked to the cobalt atom. On the basis of the known structure of the coenzyme, it seemed that a chemical partial synthesis might be achieved by the reaction of a suitable 5′-substituted adenosine with reduced hydroxocobalamin, and, following a suggestion from Dr. V. M. Clark, whom we thank for his interest, we then attempted to reduce the cobalamin, not just to the brown vitamin B_{12r}, believed to be a cobalt(II) derivative, but to the grey-green compound containing cobalt in a lower oxidation state. Preliminary alkylations using adenosine triphosphate failed, and it was apparent that a more reactive intermediate, such as 5′-tosyladenosine, was required. Introduction of the tosyl group unambiguously into the 5′-position of adenosine requires prior protection of the 2′- and the 3′-hydroxyl group; 2′,3′-*O*-isopropylidene-5′-tosyladenosine has been prepared[11] as an unstable resin, but unfortunately the conditions required for the removal of the isopropylidene protecting group also caused intramolecular cyclisation to the tosylate of cycloadenosine. It was therefore decided to remove this protecting group after the condensation with reduced hydroxocobalamin. Hydroxocobalamin was treated, under nitrogen, in aqueous-methanolic solution with sodium borohydride and then caused to react with 2′,3′-*O*-isopropylidene-5′-tosyladenosine. A crystalline product, isolated in moderate yield, had the properties expected of the isopropylidene derivative of the coenzyme (I; R = 2′,3′-*O*-isopropylidene-5′-deoxyadenosyl). A similar product (I; R = 2′,3′-di-*O*-acetyl-5′-deoxyadenosyl) was obtained from 2′,3′-diacetyl-5′-tosyladenosine. The protecting groups were removed by hydrolysis, and subsequent purification gave the crystalline vitamin B_{12} coenzyme. The absorption spectrum in neutral and acidic solution, and the paper-chromatographic and electrophoretic properties, were identical with those of the natural coenzyme. It was fully active in Abeles and Lee's[12] enzyme assay; degradation with cyanide yielded dicyanocobalamin, 2,3-dihydroxypent-4-enal, and adenine;[3,13] anaerobic photolysis yielded vitamin B_{12r} and an adenine nucleoside,[4,14] the so-called nucleoside A (II). These experiments have been reported in preliminary form,[15] and a similar partial synthesis has since been claimed in a German note.[16]

Analogous experiments with 2′,3′-*O*-isopropylidene-5′-tosyl-uridine and -inosine yielded crystalline products having properties similar to those of the coenzyme (I), but containing uracil and hypoxanthine residues, respectively, in place of adenine. Müller and Müller[17] recently described the synthesis of the latter analogue by deamination of

[6] Pawelkiewicz, Bartosinski, and Walerych, *Bull. Acad. polon. Sci.*, 1960, Cl. II, **8**, 123.
[7] Ford and Porter, *Biochem. J.*, 1952, **51**, v; Armitage, Cannon, Johnson, Parker, Smith, Stafford, and Todd, *J.*, 1953, 3849.
[8] Bernhauer, Gaiser, Müller, and Wagner, *Biochem. Z.*, 1960, **333**, 106.
[9] Diehl *et al.*, *Iowa State J. Sci.*, 1952, **26**, 555; *J. Amer. Chem. Soc.*, 1954, **76**, 4345.
[10] Barker and Brady, *Biochem. Biophys. Res. Comm.*, 1961, **4**, 373.
[11] Clark, Todd, and Zussman, *J.*, 1951, 2952.
[12] Abeles and Lee, *J. Biol. Chem.*, 1961, **236**, 2347.
[13] Johnson and Shaw, *Proc. Chem. Soc.*, 1961, 447.
[14] Bernhauer and Müller, *Biochem. Z.*, 1961, **334**, 199; **335**, 44; Hogenkamp, Barker, *et al.*, *Biochem. Biophys. Res. Comm.*, 1960, **2**, 143; *J. Biol. Chem.*, 1962, **237**, 1950; *Fed. Proc.*, 1962, **21**, 470.
[15] Smith, Mervyn, Johnson, and Shaw, *Nature*, 1962, **194**, 1175.
[16] Bernhauer, Muller, and Müller, *Biochem. Z.*, 1962, **336**, 102.
[17] Müller and Müller, *Biochem. Z.*, 1962, **335**, 340.

the natural coenzyme with dilute nitrous acid under mild conditions. We have repeated this experiment and obtained the deaminated product; as expected, it was identical with our inosine analogue. The adenosine, uridine, and inosine analogues can also be prepared without prior protection of the 2′,3′-secondary hydroxyl groups.

Treatment of the uridine and inosine analogues with potassium cyanide yielded uracil and hypoxanthine, respectively. Anaerobic photolysis of the isopropylidene derivative of the synthetic coenzyme gave the crystalline isopropylidene derivative of nucleoside A(II), and after mild acid hydrolysis the free nucleoside was obtained. Anaerobic photolysis of the inosine coenzyme analogue yielded a cyclic nucleoside of hypoxanthine which was identical with the product obtained by the action of nitrous acid on nucleoside A. Experiments designed to identify the gaseous products obtained from the action of cyanide and the anaerobic photolyses of the alkyl coenzyme analogues are not yet complete but acetic acid has been identified by chromatography as a photolysis product of the carboxymethyl analogue (I; R = $CH_2 \cdot CO_2H$).

Meanwhile biosynthetic methods have been discovered for the conversion of vitamin B_{12} or, better, vitamin B_{12b} into the corresponding cobamide coenzyme, and these methods are related closely to the chemical partial synthesis. Cell-free extracts of *Clostridium tetanomorphum* [18] and *Propionibacterium shermanii* [19] convert cyanocobalamin into its coenzyme form, and the use of tracer techniques established [20] that the additional 5′-deoxyadenosine originated from adenosine triphosphate. Adenine, adenosine, and adenosine monophosphate were all inactive in the synthesis, although adenosine diphosphate was utilised to a limited extent. Cofactors necessary for this conversion included flavin adenine dinucleotide, reduced glutathione, and reduced diphosphopyridine nucleotide, both oxidised forms being inactive. These experiments indicated that, for the biosynthesis of the vitamin B_{12} coenzyme, a phosphorylated adenosine, preferably ATP, was required, together with a reduced form of hydroxocobalamin. The latter requirement was cited [19] as additional evidence for the presence of bivalent cobalt in the coenzyme.

Two distinct reduction products of hydroxocobalamin have been reported. " Vitamin B_{12r}," [9] the red-brown reversible reduction product, is produced from hydroxocobalamin and chromous acetate at pH 5.[21] Chromous acetate in EDTA (ethylenediaminetetraacetic acid) buffer at pH 9·5, zinc dust and dilute acetic acid, and sodium borohydride all took the reduction, through " vitamin B_{12r}," to a grey-green product. Methyl iodide or dimethyl sulphate reacted rapidly with the latter in an atmosphere of nitrogen, the colour of the solution changing rapidly from grey-green to red, and examination of the resulting solution by paper chromatography showed complete conversion into the methyl coenzyme analogue (I; R = Me). In contrast, dimethyl sulphate failed to react with " vitamin B_{12r}," and subsequent oxidation gave hydroxocobalamin as the only product. In later preparations of the vitamin B_{12} coenzyme and its analogues, a recognition of the need for fully reduced hydroxocobalamin, combined with suitable solvent concentrations, has enabled virtually quantitative yields to be obtained.

A wide range of simple alkyl halides, as well as dimethyl sulphate and triethyl phosphate, have been allowed to react with reduced hydroxocobalamin, and crystalline products (structure I; R = alkyl) have been isolated containing the alkyl group linked directly to the cobalt atom. The reactions can be carried out either in presence of acetic acid or under alkaline conditions, and water or aqueous methanol are suitable solvents. ^{14}C-Labelled methyl iodide yielded a product (I; R = $^{14}CH_3$) with a molar specific radioactivity similar to that of the original methyl iodide. Reaction of reduced hydroxocobalamin with ethylene bromohydrin, chloroacetic acid, or acetyl chloride (or acetic anhydride) gave related products (I; R = $CH_2 \cdot CH_2 \cdot OH$, $CH_2 \cdot CO_2H$, or Ac, respectively).

[18] Weissbach, Redfield, and Peterkofsky, *J. Biol. Chem.*, 1961, **236**, PC.40.
[19] Brady and Barker, *Biochem. Biophys. Res. Comm.*, 1961, **4**, 464.
[20] Peterkofsky, Redfield, and Weissbach, *Biochem. Biophys. Res. Comm.*, 1961, **5**, 213.
[21] Beaven and Johnson, *Nature*, 1955, **176**, 1264.

It appears that electrophilic reagents of wide variety, steric conditions permitting, react with the reduction product of hydroxocobalamin to give compounds of the B_{12} coenzyme type containing cobalt–carbon bonds. Bromobenzene and t-butyl bromide did not react to an appreciable extent, probably for steric reasons. Although the cobalt–alkyl and cobalt–acyl derivatives described have been obtained in the crystalline state, elementary analyses have not been performed on most of them because of the difficulty we have experienced with the methyl coenzyme analogue. Microanalyses of this compound have been performed in three different laboratories but the results have not been consistent. This is attributed to the presence of cobalt and phosphorus in the compounds and to their high molecular weight (1400—1600) and hygroscopic properties, and we have had similar difficulty in earlier work with vitamin B_{12} and the coenzyme. However, one (out of four) set of satisfactory analyses was obtained on the synthetic coenzyme, and modifications of the normal microanalytical procedures which might be more applicable to this series are under investigation. Because of the identity of the partially synthetic and the natural coenzyme and the similar chemical behaviour (the oxidative photolytic fission to give hydroxocobalamin, the reaction with cyanide to give dicyanocobalamin, and the effect of acid) of the coenzymes and the alkyl analogues, there can be little doubt that the products are indeed alkyl–cobalt derivatives. For identification of individual compounds we have relied on spectra and chromatographic and electrophoretic behaviour. Almost all the cobalamins, coenzymes, and analogues change from red to yellow on acidification but the pH at which this occurs covers a wide range. For vitamin B_{12} (cyanocobalamin) complete conversion into the yellow form needs concentrated sulphuric acid, but the ethynyl analogue (I; R = C:CH; see below) changes colour in the presence of N-mineral acids, whereas the corresponding coenzyme (I) changes from red to yellow at pH ~3, as do many of our alkyl analogues. The acetyl analogue (I; R = Ac) changes colour pH ~4·5, and the AC coenzyme (I; R = 5′-deoxyadenosyl, but containing adenine in the nucleotide instead of 5,6-dimethylbenzimidazole) is permanently yellow. Barker and his colleagues [22] assume the red-yellow shift to be due to the breaking of the cobalt–nucleotide nitrogen bond, whereas Williams,[23] though agreeing with this, regards the change as more fundamental and involving protonation of the chromophore, possibly at position 10.

The alkyl–cobalt derivatives thus prepared were stable at room temperature in the absence of strong light and they are almost the first representatives of this class of compound to be made. The derivative $CH_3{\cdot}Co(CO)_4$ is reported [24] to be unstable above −35°, although the corresponding manganese [25] and rhenium [26] compounds are rather more stable. It is possible, however, that the success of the Fischer–Tropsch ("oxo") reaction [27] depends on the reactivity of the intermediate cobalt–alkyl derivatives. Several acylcobalt carbonyls have been described,[27] and a few arylcobalt derivatives are known,[28] but in these the presence of a bulky aryl substituent, *e.g.*, mesityl, was necessary for stability.

An understanding of the reaction mechanism involved in the alkyl halide reaction clearly requires a knowledge of the constitution of the two reduction products of hydroxocobalamin. Both "vitamin B_{12r}" and the grey-green reduction product have been stated to contain bivalent cobalt. It seemed possible, however, that the product of reduction by sodium borohydride, the reactive intermediate in the above synthesis, might be a cobalt hydride co-ordination compound, as are the intermediates of the "oxo"

[22] Ladd, Hogenkamp, and Barker, *J. Biol. Chem.*, 1961, **236**, 2114.
[23] Williams, "Advances in the Chemistry of Co-ordination Compounds," VIth Internat. Congr. Co-ordination Chemistry, Macmillan, New York, 1961, p. 65.
[24] Hieber, Vohler, and Braun, *Z. Naturforsch.*, 1958, **13**b, 192.
[25] Hieber *et al.*, *Z. Naturforsch.*, 1959, **14**b, 478; *Chem. Ber.*, 1961, **94**, 862.
[26] Hieber and Braun, *Z. Naturforsch.*, 1959, **14**b, 132.
[27] Heck and Breslow, *J. Amer. Chem. Soc.*, 1961, **83**, 4023; 1962, **84**, 2499; Sternberg and Wender, *Chem. Soc. Special Publ.*, No. 13, 1959, p. 35.
[28] Chatt and Shaw, *J.*, 1961, 285.

reaction.[27] The formation of alkyl- and acyl-cobalt derivatives would involve substitution of the alkyl or acyl groups for hydrogen, but to prove the validity of the cobalt hydride concept we have examined the addition of this intermediate to unsaturated compounds, which provides another synthetic route to the cobalt–alkyl coenzyme analogues. The addition proceeded readily at room temperature; acrylic acid, for example, reacted with the hydroxocobalamin reduction product to give an acidic coenzyme·analogue (I; R = $CH_2{\cdot}CH_2{\cdot}CO_2H$) which, on the basis of its chromatographic properties, was identical with the product obtained from β-bromopropionic acid. Likewise, reaction with ethylene oxide gave the 2-hydroxyethyl analogue, identical with the product obtained by reaction with ethylene bromohydrin. Acetylenes, which are more prone to nucleophilic addition than olefins, reacted readily with the cobalt hydride co-ordination complex; acetylene itself gave a good yield of the vinylcobalt compound (I; R = $CH{:}CH_2$) which was also obtained, though in poorer yield, by reaction of the cobalt hydride with vinyl bromide. Unlike acetylene, ethylene appeared not to react with the hydride. Bromoacetylene gave the ethynylcobalt compound (I; R = C:CH) (infrared band at 1996 $cm.^{-1}$, corresponding to the ethynyl group attached to cobalt) as the major product formed by substitution, but there was also a product which appeared by its chromatographic behaviour to be identical with that formed by reaction of the cobalt hydride with 1,2-dibromoethylene. The bromoacetylene thus reacted mainly by substitution, but to a smaller extent by addition. Reaction of the hydride with cyanogen bromide gave cyanocobalamin, vitamin B_{12}. A survey of the reaction of the cobalt hydride with a variety of unsaturated compounds suggests that, for addition to occur, the following conditions must be satisfied. (i) For ethylenic compounds the double bond must be activated by the presence of an adjacent electron-withdrawing group and the double bond must be terminal; the phenyl group (*e.g.*, in styrene) is not a sufficiently powerful activating group; if the unsaturated compound is not capable of reacting rapidly with the cobalt hydride by addition, it may be reduced and the cobalt hydride itself oxidised to the unreactive "B_{12r}" state. (ii) For steric reasons only small groups (*e.g.*, H, Me) can be present as substituents on the carbon atom attached to the cobalt; phenylpropiolic acid failed to yield an adduct, although propiolic acid reacted easily. (iii) With acetylenic compounds there is no need for further activation of the unsaturated linkage provided the addition is sterically feasible; reaction of the hydride with diazomethane gave the methyl coenzyme analogue, which suggests that the cobalt hydride has an acidic character. In a recent paper,[29] adducts from hydridopentacarbonylmanganese, $H(CO)_5Mn$, and various fluorinated olefins and acetylenes have been described.

With regard to the valency formulation of the cobalt in the active reduced form of hydroxocobalamin, the recent work of King and Winfield[30] is relevant. They obtained the complex ion $[Co(CN)_5H]^{3-}$ by reduction of $[Co^{II}(CN)_5]^{3-}$ with molecular hydrogen or sodium borohydride, but, because of the convention that the ligand should be regarded as contributing both electrons to the ligand–metal bond irrespective of the location of the electrons before addition of the ligand, their product was regarded as a complex of tervalent rather than of univalent cobalt. If this view is accepted, then the hydride reduction product of hydroxocobalamin should also be regarded as a complex of tervalent cobalt, and the formation of the alkyl derivatives would proceed with no change in valency of the cobalt. Experiments to test the hypothesis that the reduction also involves hydrogenation of the corrin chromophore are in progress.

The absorption spectra of the new coenzyme analogues all lack the prominent absorption peak at 350—370 mμ of the cobalamins, as does the natural vitamin B_{12} coenzyme. The spectra of the analogues containing modified nucleosides (Fig. 1) are identical above 300 mμ with that of the natural coenzyme; below 300 mμ the absorption maximum is dependent upon the nature of the base contained in the additional nucleoside. The

[29] Treichel, Pitcher, and Stone, *Inorg. Chem.*, 1962, **1**, 511.
[30] King and Winfield, *J. Amer. Chem. Soc.*, 1961, **83**, 3366.

analogues containing adenine and uracil show an absorption maximum at 260—264 mμ, whereas the analogue containing inosine has its maximum at 250 mμ owing to the absorption of the hypoxanthine base. The spectra of the coenzyme analogues containing simple alkyl substituents on cobalt lack this pronounced maximum (Fig. 2), although they contain a maximum in this region of much lower intensity; above 300 mμ they are similar to that of the natural product. The spectra of the analogues containing unsaturated alkyl groups are intermediate between those of the coenzyme and the normal cobalamins and show a relatively intense peak in the 350—370 mμ region.

The mode of participation of the vitamin B_{12} coenzyme in its biological reactions, or the active site in the molecule at which these reactions take place, is as yet unknown.

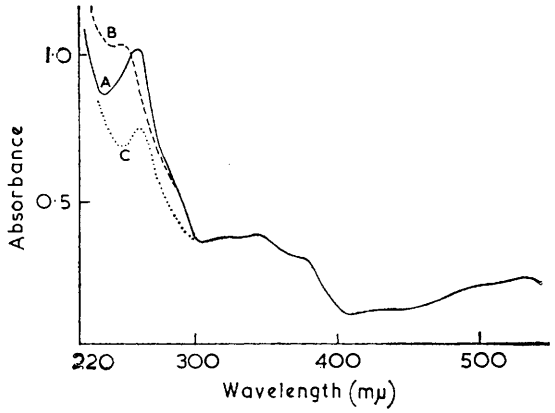


FIG. 1. Absorption spectra of vitamin B_{12} coenzyme and its analogues containing modified nucleosides.

Nucleoside bound by cobalt–carbon linkage: (A) adenosine; (B) inosine; (C) uridine.

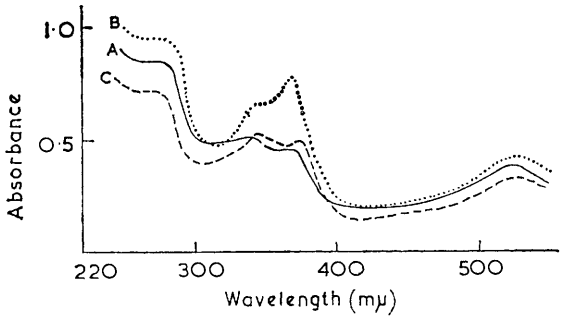


FIG. 2. Absorption spectra of vitamin B_{12} coenzyme analogues containing alkyl groups.

Alkyl group bound by cobalt–carbon linkage: (A) methyl; (B) ethynyl; (C) vinyl.

Most of the new coenzyme analogues described above have been tested in the Abeles system;[12] none showed coenzyme-like activity but on the contrary all behaved as competitive inhibitors of the coenzyme.[15] Thus, in this system at least, the only functional apoenzyme–coenzyme complexes are those containing adenosine linked to the cobalt. However, it has been demonstrated recently[31] that the methyl coenzyme analogue is fully active in the enzymic synthesis of methionine by extracts of *Escherichia coli*, a reaction which had previously been known to depend on the presence of a cobalamin derivative. Moreover, by the use of the ^{14}C-labelled methyl coenzyme analogue, it was established by isolation of labelled methionine that this was the methyl group which was transferred to the homocysteine. The methyl coenzyme analogue, however, has not yet been shown to occur naturally.

EXPERIMENTAL

Ultraviolet and visible absorption spectra were measured on aqueous solutions in Perkin-Elmer 137 and Unicam S.P. 500 spectrophotometers. Paper chromatography was carried out on Whatman's No. 1 paper by the ascending technique with the following solvent systems:

[31] Guest, Friedman, Woods, and Smith, *Nature*, 1962, **195**, 340.

solvent I; water–saturated butan-2-ol, solvent II: butan-1-ol–propan-2-ol–water, 10 : 7 : 10, solvent III: butan-1-ol–acetic acid–water, 4 : 1 : 5. $R_{B_{12}}$ values quoted for the coenzyme analogues are relative to cyanocobalamin. For other solvent systems the quoted R_F values are absolute. Electrophoresis was carried out on Whatman's No. 1 paper in a horizontal-type apparatus.

The technique of " purification by extraction through phenol " is used for freeing solutions of cyanocobalamin or the coenzyme and their analogues from any unwanted inorganic salts. The cobalamin solution (*e.g.*, 50 ml.) is extracted with small portions of 1 : 1 phenol–chloroform (10 ml.) until all the red colour is transferred to the phenolic phase. These extracts are combined and washed with an equal volume of water. The red components are then re-extracted into water by the addition of 1 : 1 chloroform–butan-1-ol (50 ml.), and the resulting aqueous solution is freed from traces of phenol by washing with ether (2 × 50 ml.).

DEAE cellulose = 2-diethylaminoethyl-cellulose; CM cellulose = methoxycarbonyl-cellulose.

Syntheses of the Coenzyme Analogues (I) *by Substitution Reactions.—Compound* (I; R = 2′,3′-*O*-isopropylidene-5′-deoxyadenosyl). 2′,3′-*O*-Isopropylideneadenosine, colourless needles (from acetone), m. p. 222—223°, was prepared from adenosine by Hampton's method; [32] it was treated with toluene-*p*-sulphonyl chloride by the method of Clark *et al.*[11] The product was obtained as a resin, which was not purified further but used directly.

Nitrogen was bubbled through a solution of hydroxocobalamin (250 mg.) in water (20 ml.) to ensure complete removal of oxygen, and then a solution of sodium borohydride (50 mg.) in ethanol (20 ml.) was added. The colour of the solution changed almost immediately to red-brown and then more slowly to grey-green. After 15 min. the vessel was protected from light and 2′,3′-*O*-isopropylidene-5′-tosyladenosine (220 mg.) in ethanol (10 ml.) was added. During the next hour, the colour of the solution reverted slowly to red. The ethanol was removed by distillation under reduced pressure and the residual aqueous solution purified by extraction through phenol. The solution was concentrated to small volume (5 ml.) and introduced on a column (10 × 1 cm.) of DEAE cellulose from which it was eluted rapidly with water. The eluate was then brought on a column (10 × 1 cm.) of CM cellulose. Elution with water removed the required product, leaving unchanged hydroxocobalamin adsorbed at the top of the column, from which it could be removed by elution with 0·02N-hydrochloric acid. The aqueous eluate was concentrated under reduced pressure and finally lyophilised, to yield an amorphous red solid (200 mg.). The 2′,3′-*O*-isopropylidene-5′-deoxyadenosyl-coenzyme analogue crystallised slowly from aqueous acetone as dark red rods, principal $\lambda_{max.}$ 263, 342, and 525 mμ ($10^{-4}\varepsilon$ 3·26, 1·17, and 0·73, respectively), identical with those of the natural vitamin B_{12} coenzyme derivative. In later experiments, almost quantitative yields (as indicated by paper chromatography) were obtained by using more concentrated solutions, *e.g.*, hydroxocobalamin (250 mg.) in 1 : 1 aqueous methanol (5 ml.). Under these conditions the molar ratio of 2′,3′-*O*-isopropylidene-5′-tosyladenosine : hydroxocobalamin was reduced to 1·3 : 1 (the ratio used in the initial experiment was 3 : 1).

Compound (I; R = 5′-deoxyadenosyl) (*the vitamin* B_{12} *coenzyme*, DBC). (a) Small samples of the above isopropylidene coenzyme derivative (1 mg.), dissolved in 0·1N-hydrochloric acid (0·1 ml.), were heated at 90° for 5, 10, 15, 20, 25, and 30 min. severally and the products were examined by paper chromatography (solvent I). After 15 min. the isopropylidene coenzyme analogue ($R_{B_{12}}$ 1·65) was completely hydrolysed to the vitamin B_{12} coenzyme ($R_{B_{12}}$ 0·73) with minimal formation of hydroxocobalamin ($R_{B_{12}}$ 0·37). Hydrolysis was incomplete in less than 15 min., and after this time appreciable quantities of hydroxocobalamin were produced.

2′,3′-*O*-Isopropylidene-5′-deoxyadenosylcobalamin (100 mg.) was dissolved in 0·1N-hydrochloric acid (5 ml.) and heated at 90° for 15 min. After cooling, the solution was neutralised with aqueous 0·1N-ammonia and desalted by extraction through phenol, and the product was separated from hydroxocobalamin by chromatography on CM cellulose (as above). The coenzyme solution was concentrated and crystallisation induced by the addition of acetone. During several days at 0°, the solution gradually deposited red crystals of the *vitamin* B_{12} *coenzyme* which were separated (45 mg.) (Found, on an undried sample: C, 50·3, 49·7; H, 7·45, 7·21; N, 13·0; P, 1·25; loss on drying at 100° *in vacuo*, 8·65. $C_{72}H_{100}CoN_{18}O_{17}P,8H_2O$ requires C, 50·15; H, 6·8; N, 14·6; P, 1·8; H_2O, 8·35%. Found, on a sample dried at 100° *in vacuo* to constant wt.: C, 52·65; H, 6·75; N, 15·5; P, 1·1. $C_{72}H_{100}CoN_{18}O_{17}P$ requires C, 54·75; H, 6·4; N, 15·95; P, 1·95%), principal $\lambda_{max.}$ in neutral solution 263, 342, and 525 mμ

[32] Hampton, *J. Amer. Chem. Soc.*, 1961, **83**, 3640.

($10^{-4}\varepsilon$ 2·82, 1·11, and 0·66, respectively) and, at pH 2, 265, 304, and 470 mμ ($10^{-4}\varepsilon$ 2·98, 1·74, and 0·69, respectively), all identical with those of the natural vitamin B_{12} coenzyme. The product was further characterised by treatment with cyanide, which yielded adenine and dicyanocobalamin, and by photochemical deactivation, which yielded nucleoside A and hydroxocobalamin, as described in an earlier paper.[4]

(*b*) Hydroxocobalamin (250 mg.) was reduced with sodium borohydride (125 mg.) in water (5 ml.) under anaerobic conditions and a solution of acetic acid (0·25 ml.) in water (2 ml.) was added. To this solution 2′,3′-di-*O*-acetyl-5′-tosyladenosine (from 267 mg. of adenosine) in 50% methanol (2 ml.) was added. The solution became dark red almost immediately and after 3 hr. the diacetyl-coenzyme was processed by conventional methods. Unchanged hydroxocobalamin was removed on a CM cellulose ion-exchange column after the solution had first passed through a column of DEAE cellulose. The eluate was freeze-dried, to yield the diacetyl compound as a red solid. The product was dissolved in methanol (20 c.c.), and anhydrous morpholine (10 mg.) was added. After the solution had been kept in the dark at room temperature for 2 hr., the product was isolated by precipitation with ether and purified as before. Its chromatographic behaviour was identical with that of the product of the previous experiment.

Compound (I; R = 5′-deoxyuridinyl). (*a*) 2′,3′-*O*-Isopropylidene-5′-tosyluridine [33] (215 mg.) was treated with reduced hydroxocobalamin (250 mg.), and the product was isolated and purified as outlined above. Crystallisation from aqueous acetone gave the 2′,3′-*O*-isopropylidene-5′-deoxyuridinyl-coenzyme analogue as red needles (240 mg.), principal $\lambda_{max.}$ 264, 342, and 525 mμ ($10^{-4}\varepsilon$ 2·44, 1·12, and 0·72, respectively).

Hydrolysis of the above isopropylidene derivative (100 mg.) with 0·1N-hydrochloric acid (5 ml.) at 90° for 15 min. and subsequent purification as detailed for the vitamin B_{12} coenzyme yielded the 5′-deoxyuridinyl-coenzyme analogue (65 mg.) as red needles from aqueous acetone. The absorption spectrum showed principal $\lambda_{max.}$ 264, 342, and 525 mμ.

(*b*) 2′,3′-*O*-Isopropylidene-5′-tosyluridine (250 mg.), dissolved in ethanol (5 ml.) and 0·1N-hydrochloric acid (5 ml.), was heated at 100° for 30 min. After cooling, the solution was neutralised with dilute aqueous ammonia and evaporated to dryness. The residue was taken up in ethanol (5 ml.) and added to reduced hydroxocobalamin (250 mg.) in 1 : 1 aqueous methanol (5 ml.). The 5′-deoxyuridinyl derivative was isolated by the usual procedure and crystallised from aqueous acetone as red needles (180 mg.). The physical properties of the product were identical with those of the foregoing preparation.

Compound (I; R = 5′-deoxyinosyl). (*a*) 2′,3′-*O*-Isopropylidene-5′-tosylinosine [34] was treated with reduced hydroxocobalamin (150 mg.) in 1 : 1 aqueous methanol (5 ml.), and the product isolated by the usual procedure. The aqueous solution of the 2′,3′-*O*-isopropylidene-5′-deoxyinosyl derivative was adjusted to N/10 with respect to hydrochloric acid and heated at 90° for 15 min. The 5′-deoxyinosyl-coenzyme analogue was isolated and crystallised from aqueous acetone as red needles (76 mg.), principal $\lambda_{max.}$ 250, 342, and 525 mμ ($10^{-4}\varepsilon$ 3·10, 1·10, and 0·72, respectively).

(*b*) The vitamin B_{12} coenzyme (20 mg.) was deaminated with sodium nitrite (200 mg.) in aqueous 5% acetic acid (5 ml.) as described by Müller and Müller,[17] and the products were separated on a CM cellulose column (10 × 1 cm.). Elution with water rapidly removed nitritocobalamin and, more slowly, two coenzyme fractions, the first of which contained the required deaminated product. The solution was lyophilised, to yield the 5′-deoxyinosyl derivative (5 mg.) as a red solid, which had properties identical with those of the synthetic material described above.

Compound (I; R = methyl) (the methyl-coenzyme analogue). (i) Hydroxocobalamin (50 mg.) in 1 : 1 aqueous methanol (25 ml.) was reduced with sodium borohydride under nitrogen. A 1% v/v solution (1 ml.) of methyl iodide in methanol was added; the colour of the mixture immediately changed from grey-green to red. The solvent was removed under reduced pressure and the aqueous residue desalted by extraction through phenol. Chromatographic examination of the product showed no unchanged hydroxocobalamin. The solution was concentrated and acetone added until a slight turbidity appeared. The methyl-coenzyme analogue crystallised rapidly as red needles (45 mg.), principal $\lambda_{max.}$ 268, 343, and 525 ($10^{-4}\varepsilon$ 2·27, 1·20, and 0·76, respectively).

(ii) Repetition of the experiment with methyl iodide (24 μc/μmole) gave the methyl-coenzyme

[33] Levene and Tipson, *J. Biol. Chem.*, 1934, **106**, 113.
[34] Levene and Tipson, *J. Biol. Chem.*, 1935, **111**, 313.

analogue, the purity of which (based on ε_{520} 10,300) was 70·5%. With an independent sodium [^{14}C] formate standard and a coincidence scintillation counter the observed activity was 14·9 μc/μmole which, after allowance for the purity of the sample, gave a true activity of 21·1 μc/μmole.

(iii) Experiments with equivalent amounts of dimethyl sulphate or methyl toluene-*p*-sulphonate in place of methyl iodide gave the same product.

(iv) Hydroxocobalamin (20 mg.) and chromous acetate (10 mg.) were dissolved in ethylenediaminetetra-acetic acid buffer (2 ml.) at pH 9·5 in an atmosphere of nitrogen. After 10 min. dimethyl sulphate (0·2 ml.), suspended in water (2 ml.), was added, the colour of the solution changing instantly from blue-green to red. After 15 min. the solution was decanted from the excess of dimethyl sulphate and extracted by the phenol–chloroform method to remove salts. The product (17 mg.) was recovered in water (~1 ml.) and crystallised by cautious addition of acetone. The absorption spectrum was identical with those of the products of the previous experiments.

The experiment was repeated with 0·05N-acetic acid instead of EDTA buffer. Under these conditions, the solution became brown and had the characteristic spectrum of vitamin B_{12r}. Adding dimethyl sulphate caused no colour change. After an hour the mixture was shaken with oxygen, and hydroxocobalamin was then isolated as the only product.

(v) Hydroxocobalamin (100 mg.) was reduced with an excess of zinc dust in aqueous 5% acetic acid until the colour of the solution was grey-green. The solution was filtered in an atmosphere of nitrogen into a vessel containing dimethyl sulphate (0·2 ml.), the colour of the solution immediately changing to red. After 15 min. the product was processed as in the previous examples to yield the methyl-coenzyme analogue (75 mg.).

(vi) (With Dr. F. WAGNER.) Hydroxocobalamin (40·5 mg.) was dissolved in water (50 ml.) in an atmosphere of pure nitrogen. Sodium borohydride (20 mg.) was added with exclusion of light and after 30 min. an ethereal solution (20 ml.) of diazomethane (from 1 g. of methylnitrosourea) was added. The solution was adjusted to pH 4 with acetic acid, and the product extracted through phenol. The re-extracted aqueous solution was concentrated to 5 c.c. under reduced pressure at 40° and the concentrate transferred to a CM cellulose column (1 × 10 cm.) which was then washed with water. The eluted methyl-coenzyme analogue (28 mg.) was crystallised from aqueous acetone and washed with dry acetone.

Compound (I; R = carboxymethyl). Cyanocobalamin (150 mg.) in water (10 c.c.) was treated with zinc dust (1 g.) and glacial acetic acid (1 ml.) while the flask was warmed gently under reduced pressure to remove hydrogen cyanide. When the reduction was complete, the solid material was separated in a closed vessel in an atmosphere of nitrogen, and the filtrate was added to monochloroacetic acid (250 mg.). The dark filtrate rapidly became red and after 30 min. the solution was percolated through charcoal (1 g.; Sutcliffe Speakman No. 5) and kieselguhr (1 g.); nearly all the red colour was adsorbed. After washing of the adsorbent with water, it was eluted with 70% acetone, and further acetone was added to the eluate to promote crystallisation of the crude product. This was dissolved in water (3 ml.) and brought on a small column (2″ × ½″) of DEAE cellulose. The acidic product was absorbed strongly, while a small quantity of red impurity was removed, and then was eluted with 0·01N-hydrochloric acid as a red solution. The red solution became yellow on acidification, and red again on exposure of the acid solution to light. The product (95 mg.) was purified in the usual manner and had principal $\lambda_{max.}$ 266, 342, and 525 mμ ($10^{-4}\varepsilon$ 2·04, 1·24, and 0·73, respectively). Substitution of β-bromopropionic acid in the above procedure yielded the carboxyethyl analogue.

Formation of Other Alkyl- and Acyl-coenzyme Analogues.—By similar methods the following halides and esters were caused to react with reduced hydroxocobalamin and converted into analogues of the natural coenzyme: ethyl iodide, triethyl phosphate, ethyl toluene-*p*-sulphonate (to give I; R = Et), n-butyl iodide (to give I; R = Bu^n), bromohydrin or ethylene oxide (to give I; R = $CH_2{\cdot}CH_2{\cdot}OH$), and acetyl chloride or acetic anhydride (to give I; R = Ac). Their electrophoretic and chromatographic properties are shown in the Table. The light absorption spectra of all the alkyl-coenzyme analogues were virtually identical with that of the methyl derivative. The spectrum of the acetyl compound showed a small peak at 350 mμ, probably due to the presence of hydroxocobalamin formed by photolytic decomposition. Several attempts at purification failed to eliminate this peak. Exposure of the acetyl compound to light confirmed that it was exceedingly photosensitive. Its absorption maximum

Paper-chromatographic and electrophoretic properties of the vitamin B_{12} coenzyme analogues.

	R_{B12} Solvent I	R_{B12} Solvent II	E		R_{B12} Solvent I	R_{B12} Solvent II	E
Hydroxocobalamin	0·37	0·45	52	*Coenzyme:* *			
Cyanocobalamin	1·0	1·0	0	Methyl	1·6	1·25	41
				Ethyl	1·65	1·3	41
Coenzyme: *				Butyl	2·4	1·5	41
2′,3′-Isopropylideneadenosyl	1·65	1·4	—	Hydroxyethyl ...	0·9	0·95	45
Adenosyl	0·73	0·75	58	Carboxymethyl	1·15	1·0	12
2′,3′-Isopropylideneuridinyl	1·3	1·25	—	Carboxyethyl ...	0·5	1·0	14
Uridinyl.....	0·62	0·75	28	Vinyl.....	1·9	1·5	30
2′,3′-Isopropylideneinosyl ...	1·22	1·23	—	Bromovinyl	1·9	1·45	32
Inosyl	0·52	0·7	25	Ethynyl	1·5	1·4	33
2′-Deoxyadenosyl	0·76	0·82	57	Acetyl	1·4	1·3	50

* The coenzyme analogue is denoted by the group co-ordinated to the cobalt. E = distance in mm. migrated during 4 hr. in 0·5M-acetic acid at a potential gradient of 10v/cm. Cyanocobalamin, which has $E = 0$, was used as a marker to correct for electro-osmosis.

in the visible region was at 510 mμ and accordingly in solution it was more orange in colour than the alkyl analogues which have their main absorption peak at 525 mμ in this region.

Bromobenzene and t-butyl iodide did not react with reduced hydroxocobalamin.

Formation of Alkyl-coenzyme Analogues by Addition Reactions.—The vinyl-coenzyme analogue (I; R = $CH{:}CH_2$). Hydroxocobalamin (400 mg.) was reduced with sodium borohydride (200 mg.) in water (8 ml.) under nitrogen. After a few minutes, when the colour had changed from red to very dark green, acetone (2 ml.) was added, and the flask evacuated and refilled with acetylene. The solution then became red again within a few min. and after 3 hr. it was extracted through phenol to remove salts. The product was further purified by chromatography on columns of DEAE and CM cellulose in series, and the red effluent was concentrated under reduced pressure and crystallised by addition of acetone. The vinyl compound was thus obtained as red crystals (320 mg.), principal $\lambda_{max.}$ 270, 340, 373, and 525 mμ ($10^{-4}\varepsilon$ 1·6, 1·16, 0·98, and 0·71, respectively).

The same compound was prepared by using vinyl bromide in place of acetylene. By the standard procedure, hydroxocobalamin (400 mg.) reduced with sodium borohydride (200 mg.) and treated with vinyl bromide (1 ml.), gave the crystalline vinyl-coenzyme analogue (190 mg.).

The ethynyl- and bromovinyl-coenzyme analogues (I; R = $C{:}CH$ and $CH{:}CHBr$). Hydroxocobalamin (500 mg.) was reduced with chromous acetate (0·5 g.) in EDTA buffer (pH 9·5) with the addition of N-sodium hydroxide (1 ml.). When the solution had become blackish-green, the flask was evacuated and an excess of bromoacetylene was admitted. After 30 min. the mixture was processed as above and paper chromatography of the crude product (in solvent I) showed a major band, R_{B12} 1·5, and a minor band, R_{B12} 1·9, which corresponded to the bromovinyl analogue. This compound was also obtained on reaction of reduced hydroxocobalamin with 1,2-dibromoethylene. The ethynyl coenzyme analogue had principal $\lambda_{max.}$ 265, 342, 368, and 525 mμ ($10^{-4}\varepsilon$ 1·5, 1·03, 1·25, and 0·7, respectively). The absorption spectrum of the bromovinyl analogue was identical with that of the vinyl analogue.

By similar methods, both propiolic and acrylic acid have been treated with reduced hydroxocobalamin, to yield coenzyme analogues (II; R = $CH{:}CH{\cdot}CO_2H$ and R = $CH_2{\cdot}CH_2{\cdot}CO_2H$, respectively). The latter analogue proved identical with that obtained previously by the reaction of β-bromopropionic acid with reduced hydroxocobalamin.

Reactions of the Coenzyme Analogues.—Reaction of the 5′-deoxyuridinyl-coenzyme analogue with potassium cyanide. The deoxyuridinyl-coenzyme analogue (3 mg.) was dissolved in 0·1M-potassium cyanide (0·5 ml.) and left in the dark for 24 hr. After neutralisation with dilute hydrochloric acid, the solution was examined by chromatography. The only visible component corresponded to cyanocobalamin (R_{B12} in water–saturated butan-2-ol, 1·0) but examination under ultraviolet light revealed another spot (R_F in solvent III, 0·46). This was eluted from the paper with water, and its solution showed $\lambda_{max.}$ 260 mμ at pH 7, and 285 mμ at pH 10. These spectral and chromatographic properties were identical with those of an authentic sample of uracil. Spraying a separate chromatogram of the product with the silver nitrate reagent revealed a carbohydrate (R_F in solvent III, 0·45).

Reaction of the 5′-deoxyinosyl-coenzyme analogue with potassium cyanide. This compound

was treated as in the foregoing experiment, and the products were shown to be cyanocobalamin, hypoxanthine (R_F in solvent III, 0·28, $\lambda_{max.}$ 250 mμ at pH 7 and 261 mμ at pH 10), and 2,3-dihydroxypent-4-enal (R_F in solvent III, 0·45).

2′,3′-O-Isopropylidene-nucleoside A. 2′,3′-*O*-Isopropylidene-5′-deoxyadenosyl-coenzyme analogue (300 mg.; above), dissolved in water (250 ml.), was photolysed for 8 hr. under nitrogen by exposure to sunlight. The solution was reduced to small volume by evaporation under reduced pressure, and the remainder lyophylised. The solid residue was extracted with methylene dichloride (2 × 50 ml.), the insoluble hydroxocobalamin separated, and the solution evaporated to dryness. The residue crystallised from methanol as colourless needles (42 mg.) of 2′,3′-*O*-isopropylidene-nucleoside A. A sample was further purified by sublimation at 200°/0·05 mm., being then obtained as prisms, m. p. 233—235°, R_F in solvent III, 0·75 (Found: C, 53·9; H, 5·1; N, 23·7. $C_{13}H_{15}N_5O_3$ requires C, 53·9; H, 5·2; N, 24·2%), $\lambda_{max.}$ 262 mμ at both pH 7 and 10 (10^{-4} ε 1·49).

The above nucleoside (22 mg.) was heated in 0·1N-hydrochloric acid (1 ml.) at 90° for 20 min. The solution was neutralised with 0·1N-aqueous ammonia and, after cooling, deposited colourless crystals (8 mg.) which were separated. Evaporation of the mother-liquors yielded a further quantity (6 mg.), R_F in solvent III, 0·36 (Found: C, 43·7; H, 4·9; N, 25·5. $C_{10}H_{11}N_5O_3,1\frac{1}{2}H_2O$ requires C, 43·6; H, 5·05; N, 25·4%), $\lambda_{max.}$ 262 mμ at both pH 7 and 10. These properties are identical with those of nucleoside A isolated after photolysis of the natural coenzyme.[14] The infrared spectra were also identical.

Photolysis of 5′-deoxyinosyl-coenzyme analogue. The inosyl-coenzyme analogue (75 mg.) was dissolved in water (50 ml.) and exposed to sunlight in an atmosphere of nitrogen. The colour of the solution gradually changed from red to brown, indicating the formation of vitamin B_{12r}. After 4 hr., the solution was evaporated to dryness and the residue taken up in methanol (10 ml.). A little insoluble material was separated and the solution concentrated and left at 0° for a few hours. A small amount (*ca.* 5 mg.) of colourless crystals was formed and was separated R_F in solvent III, 0·25; $\lambda_{max.}$ 253 mμ at pH 7 and 10. The compound decomposed slowly above 250° without melting.

Action of nitrous acid on the nucleoside A. To a solution of nucleoside A (25 mg.) in water (2 ml.) were added barium nitrite (50 mg.) and acetic acid (2 ml.), with external cooling. The solution was left in the dark for 2 days; a solid material separated which crystallised from water as colourless needles (7 mg.), R_F in solvent III, 0·25; $\lambda_{max.}$ 254 mμ at pH 7 and 10. The compound decomposed above 250° without melting. It was identical with the product isolated by photolysis of the inosyl-coenzyme analogue and the infrared spectra of the two products were identical.

Reaction of the Alkyl-coenzyme Analogues with Cyanide.—Small samples (1 mg.) of the methyl-, ethyl-, butyl-, and carboxymethyl-coenzyme analogues were dissolved separately in 0·1M-potassium cyanide (0·1 ml.) and left for 24 hr. The solutions were then neutralised with dilute hydrochloric acid, and examination by paper chromatography revealed cyanocobalamin (R_{B12} in solvent I, 1·0) as the only visible product.

Photolysis. Samples (1 mg.) of the above coenzyme analogues were dissolved separately in 0·1N-hydrochloric acid (10 ml.) and exposed to sunlight. The yellow solutions became pink in a few minutes and the light absorption spectra were then characteristic of hydroxocobalamin (principal $\lambda_{max.}$ 275, 351, and 525 mμ). These analogues were much less stable to light than analogues containing nucleosides.

The carboxymethyl-coenzyme analogue (25 mg.) was dissolved in water (25 ml.) and exposed to sunlight under nitrogen. After 2 hr. the solution was adjusted to pH 10 with dilute aqueous ammonia and evaporated to dryness. The residue was dissolved in a minimum amount of water and examined by chromatography in the following solvent systems: (i) ethanol–water–aqueous ammonia (*d* 0·88) (80 : 16 : 4), (ii) butan-1-ol–ethanol–water–aqueous ammonia (*d* 0·88) (40 : 40 : 16 : 4). Acetic acid, glycollic acid, succinic acid, and oxalic acid were used as standards. The chromatograms were sprayed with B.D.H. Universal Indicator, which revealed the acidic compounds as pink spots, when the following R_F values were obtained: photolysis product (i) 0·67, (ii) 0·41; acetic acid (i) 0·68, (ii) 0·43; glycollic acid (i) 0·57, (ii) 0·33; succinic acid (i) 0·37, (ii) 0·15; oxalic acid (i) streak at origin, (ii) 0·08.

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