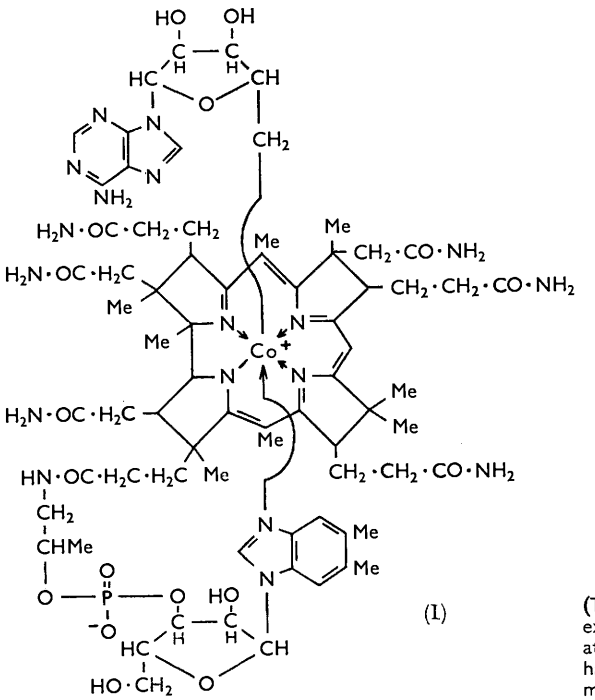
888. *Some Reactions of the Vitamin B_{12} Coenzyme.*

By A. W. Johnson and N. Shaw.

Several reactions of the vitamin B_{12} coenzyme are described and interpreted in the light of the structure determined recently by *X*-ray crystallography. The novel cobalt–carbon linkage is easily broken by the action of cyanide, dilute mineral acids, or light, and in the latter case it is suggested that a nucleoside free radical is obtained which undergoes intramolecular cyclisation.

The family of vitamin B_{12} coenzymes was first recognised by Barker and his associates [1,2] in their biochemical studies of the enzymic conversion of glutamate into β-methylaspartate by *Clostridium tetanomorphum*. The adeninylcobamide coenzyme (*i.e.*, containing an



(1)

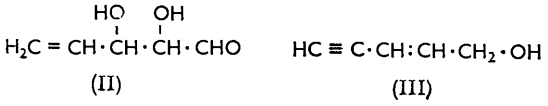
(The precise nature and extent of the conjugation in the chromophore have still to be determined with certainty.)

adenine nucleotide grouping and, therefore, related structurally to ψ-vitamin B_{12} [3]) was isolated originally [2] but the benzimidazole and 5,6-dimethylbenzimidazole analogues,[4] produced by growing *C. tetanomorphum* in the presence of the appropriate bases, were the first to be crystallised. Five other non-crystalline members of the family have been prepared by using *Propionibacterium arabinosum* in the presence of certain purines or benzimidazoles.[5] The coenzymes occur in several micro-organisms,[6,7] and their presence

[1] (a) Barker, Weissbach, and Smyth, *Proc. Nat. Acad. Sci. U.S.A.*, 1958, **44**, 1093; (b) Weissbach, Toohey, and Barker, *ibid.*, 1959, **45**, 521.
[2] Barker, Smyth, Weissbach, Munch-Petersen, Toohey, Ladd, Volcani, and Wilson, *J. Biol. Chem.*, 1960, **235**, 181.
[3] Dion, Calkins, and Pfiffner, *J. Amer. Chem. Soc.*, 1952, **74**, 1108; *Fed. Proc.*, 1952, **10**, 269.
[4] Barker, Smyth, Weissbach, Toohey, Ladd, and Volcani, *J. Biol. Chem.*, 1960, **235**, 480.
[5] Toohey, Perlman, and Barker, *J. Biol. Chem.*, 1961, **236**, 2119.
[6] Volcani, Toohey, and Barker, *Arch. Biochem. Biophys.*, 1960, **92**, 381.
[7] Abeles and Lee, *J. Biol. Chem.*, 1961, **236**, 2347.

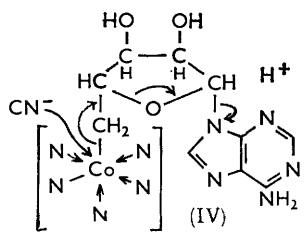
has been demonstrated by the glutamate isomerase coenzyme assay [8] or by direct isolation. Apart from their ability to bring about the rearrangement of glutamate to β-methylaspartate, the coenzymes appear also to be essential for the interconversion of *C*-methylmalonate and succinate [9] and of propane-1,2-diol and propionaldehyde [7] as well as a number of related reactions. Preliminary physical and chemical studies suggested that in the coenzymes the cyanide group of vitamin B_{12} was replaced by an adenine nucleoside,[2,4,10] and that the cobalt might be in a lower valency state.[11–13] The recent determination [14] of the complete structure (I) of the 5,6-dimethylbenzimidazolylcobamide coenzyme by *X*-ray analysis now permits most of the observed physical and chemical properties of the coenzyme to be interpreted. The existence of an essentially covalent bond between the cobalt and the 5′-carbon atom of the additional 5′-deoxyadenosine grouping gives the molecule the character of an organometallic compound, the first to be discovered in naturally occurring compounds.

It is presumed that the adeninyl and benzimidazolyl coenzymes have similar structures apart from the nature of the nucleotide base. All our work has been on the 5,6-dimethylbenzimidazolyl coenzyme, whereas Barker and his co-workers have studied the adeninyl as well as the benzimidazolyl coenzyme. It appears that in all of the compounds of this group the cobalt–carbon bond is readily broken, and examples of fission products formed by the action of acid, cyanide, and light have been described. Free adenine is released from the coenzymes by treatment with either hot dilute acid [4,10,15] or with cyanide [1b,5,10,11] and, apart from its spectra, the base has now been identified as the crystalline picrate.[11] In the case of the adeninyl coenzyme, treatment with 0·07N-hydrochloric acid at 85° for 10 minutes removes the adenine from the nucleotide preferentially,[10] whereas, by contrast, the corresponding nucleotide of the benzimidazolylcobamide coenzymes is stable under these conditions. Slightly more vigorous acid-hydrolysis (0·1N-hydrochloric acid at 100° for 90 min.) converts the adeninyl coenzymes into a mixture of hydroxocobalamin, free adenine, and a sugar. Treatment of the coenzymes with cyanide in the absence of light gives cyanocobalamin (initially in its dicyano-form) together with adenine and the sugar cyanhydrin. This sugar has been identified [16] as D-*erythro*-2,3-dihydroxypent-4-enal (II) by degradation and its structure now confirmed by a



(II)

(III)



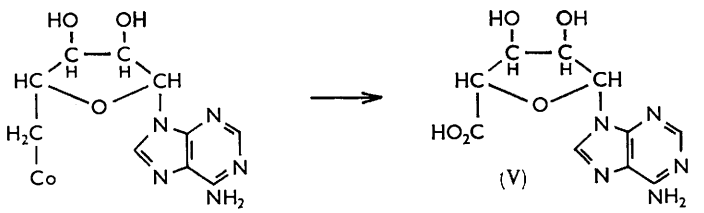
(IV)

synthesis of *erythro*-pent-4-en-1,2,3-triol, the racemic form of the product of the sodium borohydride reduction of the sugar. Pent-2-en-4-yn-1-ol (III), obtained by condensation of epichlorohydrin and acetylene,[17] was converted by performic acid into a monoformate

[8] Barker, Smyth, Wilson, and Weissbach, *J. Biol. Chem.*, 1959, **234**, 320.
[9] Eggerer, Overath, Lynen, and Stadtman, *J. Amer. Chem. Soc.*, 1960, **82**, 2643; *Biochem. Z.*, 1960, **333**, 1; Stjernholm and Wood, *Proc. Nat. Acad. Sci. U.S.A.*, 1961, **47**, 303; Hegre, Miller, and Lane, *Biochim. Biophys. Acta*, 1962, **56**, 538.
[10] Weissbach, Ladd, Volcani, Smyth, and Barker, *J. Biol. Chem.*, 1960, **235**, 1462.
[11] Johnson and Shaw, *Proc. Chem. Soc.*, 1960, 420.
[12] Bernhauer, Gaiser, Muller, Muller, and Gunter, *Biochem. Z.*, 1961, **333**, 560.
[13] Nowicki and Pawelkiewicz, *Bull. Acad. polon. Sci., Ser. Sci. chim. et biol.*, 1960, Cl. II, **8**, 433.
[14] Lenhert and Hodgkin, *Nature*, 1961, **192**, 937.
[15] Ladd, Hogenkamp, and Barker, *Biochem. Biophys. Res. Comm.*, 1960, **2**, 143.
[16] Hogenkamp and Barker, *J. Biol. Chem.*, 1961, **236**, 3097.
[17] Haynes, Heilbron, Jones, and Sondheimer, *J.*, 1947, 1583.

of DL-*erythro*-pent-4-yne-1,2,3-triol,[18] which was hydrogenated to give a monoformate ester of the DL-pentenal (II), and then hydrolysed to form the free ethylenic triol. The tris-phenylurethane of the triol[19] was identical, apart from optical properties, with that obtained from the reduction product of the sugar isolated from the coenzyme. The course of these hydrolyses of the coenzyme can be represented as in scheme (IV) which illustrates the production of the artefact sugar (II). This reaction is formally analogous to the alkaline hydrolysis of the sulphonium nucleosides related to active methionine and recently studied by Baddiley, Hughes, and their co-workers.[20]

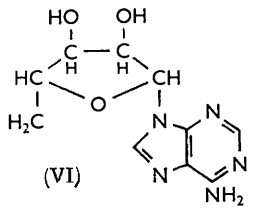
Fission products of the coenzyme, in which the sugar is still bound to the adenine, have been obtained by photolysis. The recognition of the instability of the coenzymes to light was of prime importance in their isolation, and this property undoubtedly contributed to the delay in the recognition of these compounds. Solutions of the coenzymes are decomposed in a few minutes by irradiation with direct sunlight but they can be handled easily in the laboratory when shielded from strong light. Photolysis of the 5,6-dimethylbenzimidazolylcobamide coenzyme at room temperature in the presence of air (oxygen) yields hydroxocobalamin, a mixture of two colourless nucleosides and a small quantity of free adenine, which can be separated on a Dowex-50 column.[11,15] The faster-running of the nucleosides and that present in the larger amount will be referred to as nucleoside A and the slower-running product as nucleoside B. The latter had a much lower R_F value than adenosine in paper chromatographic systems and its absorption spectrum showed that it was a 9-substituted adenine. Acid hydrolysis gave free adenine and a slow-running sugar which was positive to the periodate–Schiff's reagent. The electrophoretic behaviour of nucleoside B revealed the presence of a free acid-grouping, and by direct comparison [11] the nucleoside was shown to be adenosine-5′-carboxylic acid (V), recently prepared [21] by catalytic oxidation of adenosine. Hydrolysis of the acid (V) yielded adenine and D-riburonic acid, the latter having properties identical with those of the sugar obtained by hydrolysis of nucleoside B. From aerobic photolysis of the coenzymes, Hogenkamp and Barker [22] have recently obtained the corresponding aldehyde, which can be converted into the acid (V) by further oxidation.



When the 5,6-dimethylbenzimidazolyl coenzyme was photolysed under strictly anærobic conditions [23,24] there was produced vitamin B_{12r} [25,26] (an unstable reversible reduction product of vitamin B_{12}) and nucleoside A only. Nucleoside A has been obtained as a crystalline compound which moves slightly more slowly than adenosine on paper chromatograms. Nucleoside A consumes periodate in solution, and, although a chemical change occurs, the sugar is not liberated from the base by acid hydrolysis. Photolysis of the coenzyme presumably causes homolytic fission of the cobalt–carbon bond (see IX) with the

[18] Raphael, *J.*, 1949, S 45.
[19] Johnson and Shaw, *Proc. Chem. Soc.*, 1961, 447.
[20] Frank, Wieczorkowski, Hughes, and Baddiley, *Proc. Chem. Soc.*, 1961, 449.
[21] Reese, Schofield, Shapiro, and Sir Alexander Todd, *Proc. Chem. Soc.*, 1960, 290.
[22] Hogenkamp, Ladd, and Barker, *J. Biol. Chem.*, 1962, **237**, 1950.
[23] Brady and Barker, *Biochem. Biophys. Res. Comm.*, 1961, **4**, 373.
[24] Bernhauer and Muller, (a) *Biochem. Z.*, 1961, **334**, 199; (b) *ibid.*, **335**, 44.
[25] Diehl and Murie, *Iowa State J. Sci.*, 1952, **26**, 555; Jaselskis and Diehl, *J. Amer. Chem. Soc.*, 1954, **76**, 4345.
[26] Beaven and Johnson, *Nature*, 1955, **176**, 1264.

production of a nucleoside free radical (VI) and vitamin B_{12r}. Hogenkamp and Barker [27] suggest that this nucleoside free radical (VI) then cyclises with the 8-position of the adenine to yield (VII), nucleoside A, and this conclusion accords with our observations.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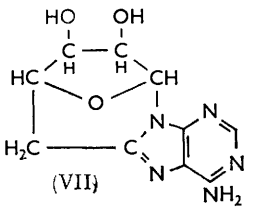
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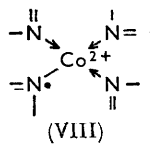
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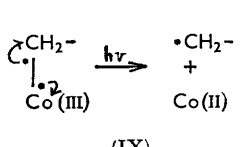
(IX)

Vitamin B_{12} is diamagnetic and it has been generally agreed that it contains tervalent cobalt; [28] on the other hand, vitamin B_{12r} [25-27] contains bivalent cobalt.[25] Several groups of workers [29] have pointed out the general resemblance of the visible absorption spectrum of the coenzymes to that of vitamin B_{12r} rather than B_{12}. In order to establish the valency of the cobalt in the coenzyme, magnetic susceptibility measurements have been made by several groups but, unfortunately, with conflicting results. Nowicki and Pawelkiewicz [13] found that the coenzyme form of cobamide (Factor B) was paramagnetic (1·3 B.M.) in solution and Bernhauer *et al.*[12] reported that the coenzyme itself was paramagnetic (1·63 B.M.) in solution, but diamagnetic in the crystalline state. On the other hand, we have found [11] that solid B_{12} coenzyme is paramagnetic (1·8 B.M.), and these results have been interpreted as indicating the presence of bivalent cobalt. Although Bernhauer [12] at first suggested that the compound might be dimeric in the solid, which is improbable on steric grounds, in a later paper,[24b] he favoured a " quasi-tervalent " state of the cobalt, the molecule being represented as a resonance structure of which (VIII) is one form.

This partial structure (VIII) emphasises the difficulty of formulating the coenzyme, in which the covalently-bound adenine nucleoside replaces the covalently-bound cyanide group of vitamin B_{12} with no marked change in the observed electrophoretic properties. The reactions of the coenzyme can be explained (*e.g.*, IX) more readily on the basis of the metal's being in the tervalent state, but clearly this aspect of the structure needs further investigation.

Experimental

M. p.s (Kofler block) are uncorrected. Ultraviolet and visible spectra were measured on aqueous solutions. Paper chromatograms were carried out by the ascending method, butanol–acetic acid–water (4 : 1 : 5) being used as solvent. Purines were detected on paper chromatograms by their ultraviolet absorption and sugars by their reaction with ammoniacal silver nitrate. " Coenzyme " in this section refers to the 5,6-dimethylbenzimidazolyl coenzyme.

Reaction of the Coenzyme with Potassium Cyanide.—(i) A solution of the coenzyme (3×10^{-5}M) in aqueous potassium cyanide ($3{\cdot}5 \times 10^{-2}$M) was kept at room temperature and the reaction followed by measuring the intensity of absorption at 367 mμ (dicyanocobalamin) with respect to time. The reaction required 160 min. for completion, whereas a similar reaction with hydroxocobalamin required only 8 min.

(ii) The coenzyme (100 mg.) was dissolved in aqueous potassium cyanide (0·02M; 10 c.c.), and the solution kept for 24 hr., the colour having then changed from red to purple. After careful neutralisation with dilute hydrochloric acid, hydrogen cyanide was removed in a stream of nitrogen. After concentration of the solution (to 3 c.c.) under reduced pressure, acetone was added gradually until a slight turbidity was produced. After the solution had been kept

[27] Hogenkamp and Barker, private communication.

[28] Diehl, Van der Haar, and Sealock, *J. Amer. Chem. Soc.*, 1950, **72**, 5312; Grun and Menasse, *Experientia*, 1950, **6**, 263; Wollmann, Cunningham, and Calvin, *Science*, 1951, **113**, 55; Boehm, Faessler, and Rittmayer, *Z. Naturforsch.*, 1954, **9**b, 509.

[29] George, Irvine, and Glauser, *Ann. New York Acad. Sci.*, 1960, **88**, 393; Bernhauer, Gaiser, Muller, and Wagner, *Biochem. Z.*, 1960, **333**, 106.

at 0° overnight, the red crystals (69 mg.) were recrystallised from aqueous acetone. The ultraviolet and visible spectra, paper chromatographic, and electrophoretic properties of the product were identical with those of authentic cyanocobalamin.

The mother liquors from the above experiment were chromatographed on Whatman 3 MM paper and the band corresponding to adenine was eluted with water (50 c.c.). The eluate was evaporated to small volume, and saturated aqueous picric acid solution (2 c.c.) was added. After several hours at 0° the yellow precipitate was separated, dried, and sublimed at 240°/0·03 mm.; it was then obtained as rosettes of yellow needles (5 mg.), m. p. 288—290° alone and on admixture with an authentic specimen of adenine picrate.

Also on the chromatogram was a compound (R_F 0·45) which gave a positive silver nitrate reaction and was produced concurrently with adenine. Occasionally a small amount of a related substance (R_F 0·74) was observed. The former was positive to the benzidine–periodate reagent and corresponded to the cyanohydrin of D-*erythro*-2,3-dihydroxypent-4-enal. An authetic sample (R_F 0·45) was obtained for comparison by the action of potassium cyanide on the aldehyde (R_F 0·74) obtained by mild acid hydrolysis of the coenzyme.

Reaction of the Coenzyme with Dilute Hydrochloric Acid.—The coenzyme (150 mg.), in 0·1N-hydrochloric acid (50 c.c.), was heated at 100° for 90 min. with exclusion of light. The solution was brought on to a column (10 × 1 cm.) of Dowex 50 and then eluted with water. The effluent was evaporated to dryness in a vacuum desiccator (KOH), and the pale-brown gum dissolved in water (1 c.c.). Paper chromatography of the solution showed a single sugar, R_F 0·74. The other products from the hydrolysis were removed from the column with 0·1N-ammonia solution and were shown by chromatography to be hydroxocobalamin and adenine. The sugar solution (0·1 c.c.) was treated with aqueous potassium cyanide (0·1M; 0·1 c.c.), and the mixture kept at room temperature for 3 hr. Chromatography of the product showed that the sugar had been completely converted into its cyanohydrin, R_F 0·45.

D-erythro-*Pent-4-ene-1,2,3-triol.*—(i) The sugar (prepared as above from 150 mg. of coenzyme) was dissolved in water (2 c.c.), and sodium borohydride (5 mg.) added. After 1 hr. at room temperature, the solution was added to a column (5 × 0·5 cm.) of Dowex 50 to remove sodium ions, the eluate evaporated to dryness, methanol (10 c.c.) added to the residue, and the solution again evaporated to dryness. This procedure was repeated three times and the residue was shown by paper chromatography to have an R_F value (0·63) identical to that of synthetic DL-*erythro*-pent-4-ene-1,2,3-triol. The triol was mixed with phenyl isocyanate (0·1 c.c.), and the solution heated at 100° for 5 min. After cooling, the residue was extracted with benzene (5 c.c.); evaporation of the extract gave a small quantity of crystalline material. After crystallisation from benzene, this had m. p. 205—207°, not depressed after admixture with an authentic specimen of the trisphenylurethane (see below).

(ii) Pent-2-en-4-yn-1-ol was obtained [17] as an oil, b. p. 68—70°/15 mm. [naphthylurethane, m. p. 108—110° (lit.[17] 110°), needles from light petroleum (Found: N, 5·6. Calc. for $C_{16}H_{13}NO_2$: N, 5·6%)], and was converted [18] into DL-*erythro*-pent-4-yne-1,2,3-triol monoformate, b. p. 128—130°/0·5 mm. A solution of the formate (4 g.) in methanol (50 c.c.) was shaken with hydrogen at room temperature in the presence of palladium–calcium carbonate (5%; 200 mg.) until 1 mole of hydrogen had been absorbed. The catalyst and the methanol were removed, and the residue was heated under reflux with 20% aqueous potassium hydroxide (10 c.c.) for 30 min. The solution was neutralised with dilute acetic acid, water removed, and the residue distilled. DL-erythro-*Pent-4-ene-1,2,3-triol* was obtained, b. p. 126—128°/0·5 mm. (1·84 g.; 56%). The *trisphenylurethane* had m. p. 207—208° (Found: C, 66·2; H, 5·25; N, 8·8. $C_{26}H_{25}N_3O_6$ requires C, 65·75; H, 5·4; N, 8·75%).

Light Inactivation of the Coenzyme.—(i) An aqueous solution (6×10^{-5}M) of the coenzyme in a 1-cm. silica cell was exposed to the light from a 100-w bulb at a distance of 30 cm. The ultraviolet spectrum was determined initially and at 15-min. intervals until no further change was observed. After 45 min., the spectrum was that of hydroxocobalamin, and the absorption curves showed well-defined isosbestic points at 243, 337, 367, and 475 mμ, indicative of a single photochemical reaction. A stronger solution (6×10^{-4}M) of the coenzyme required exposure for 4 hr. to sunlight for complete conversion at room temperature.

(ii) An aqueous solution of the coenzyme (166 mg. in 150 c.c.) was photochemically deactivated (sunlight) and the product concentrated and crystallisation induced by the addition of acetone. After several days at 0°, the solution gave a small yield (37 mg.) of dark-red crystals, which were separated and crystallised from aqueous acetone. The product

showed identical spectra (max. at 274, 352, and 525 mμ in the visible and the ultraviolet region) to those of authentic hydroxocobalamin, and the paper-chromatographic and electrophoretic behaviour of the two compounds were also identical.

(iii) A solution of the coenzyme (200 mg.) in water (200 c.c.) was deactivated by exposure to sunlight and then brought on to a column of Dowex 50 (30 × 1 cm.; previously equilibrated to pH 3 with 2N-sodium dihydrogen phosphate buffer), and washed with more water (200 c.c.). Elution was effected with 0·1N-ammonia solution, fractions (25 c.c.) were collected, and the absorption at 260 mμ of each fraction was measured. Ultraviolet-absorbing material was contained in fractions 8—11, and continued elution removed the hydroxocobalamin from the column. Fractions 8—11 were combined and concentrated, and the remainder freeze-dried to yield a faintly pink amorphous solid (43 mg.) which paper-chromatographic examination showed to be a mixture of three compounds: (i) R_F 0·49, $\lambda_{max.}$ 260 mμ at pH 7 and 267 at pH 10, was identified as adenine and was present only in traces; (ii) R_F 0·36, $\lambda_{max.}$ 260 mμ at pH 7 and 10, nucleoside A; and (iii) R_F 0·26, $\lambda_{max.}$ 260 mμ at pH 7 and 10, nucleoside B. The nucleosides were separated by chromatography on Whatman 3MM paper, the bands being eluted with water, and the eluates evaporated and freeze-dried. Both nucleosides were crystallised by keeping saturated aqueous solutions at 0° for several days, and the crystalline products were shown to be homogeneous by chromatography.

Paper Chromatographic and Electrophoretic Properties of Nucleosides A and B.—(*a*) Chromatography was carried out by the ascending technique on Whatman's No. 1 paper. Solvent I: n-butanol–acetic acid–water, 4 : 1 : 5. Solvent II: n-butanol–ethanol–water, 10 : 3 : 7. Solvent III: n-butanol–water, 21 : 4.

	R_F Values		
	Solvent I	Solvent II	Solvent III
Nucleoside A	0·36	0·43	0·26
,, B	0·26 *	0·23	0·05
Adenine	0·49	0·55	0·49
Adenosine	0·39 *	0·45	0·31
Deoxyadenosine	0·49	0·55	0·47
Adenosine-5′-carboxylic acid	0·26 *	0·23	0·05
Adenosine-5′-aldehyde	0·51 *	0·55 †	—

* Positive to the periodate–Schiff's reagent. (Nucleoside A did not react to periodate reagents on paper.)

† Indefinite because of streaking.

(*b*) *Electrophoresis.* This was carried out in 0·05M-disodium hydrogen phosphate (pH 8·5) for 3 hr. at 10 v/cm.

Nucleoside A did not move nor did adenosine; nucleoside B moved 4 cm. towards the anode; adenosine-5′-carboxylic acid moved 4 cm. towards the anode; adenosine-5′-phosphate moved 6 cm. towards the anode.

Nucleoside B.—This compound, which reacted with the periodate–Schiff's reagent, had electrophoretic and paper chromatographic properties identical with those of adenosine 5′-carboxylic acid.[21] Samples of nucleoside B (1 mg.) and another of adenosine-5′-carboxylic acid (1 mg.) were each dissolved in 0·1N-hydrochloric acid (0·5 c.c.) and heated at 100° for 8 hr. A sample of the products was examined each 30 min. by paper chromatography and hydrolysis to adenine (R_F 0·49) shown to be complete after 6 hr. Treatment of the papers with ammoniacal silver nitrate showed the presence of a carbohydrate (R_F 0·2) with properties identical with those of D-riburonic acid.

Nucleoside B was unchanged by being heated with 0·1N-potassium cyanide at 100° for 2 hr.

Anærobic Photolysis of the Coenzyme.—The coenzyme (10 mg.) was dissolved in freshly distilled water (50 c.c.), and purified nitrogen bubbled through the solution to ensure complete removal of oxygen. The flask was then sealed and exposed to sunlight; the colour of the solution gradually changed from red to brown. After 1 hr. the reaction was complete and a sample of the product was transferred to a silica cell which was then evacuated. The ultraviolet and visible spectra showed max. at 262, 313, and 475 mμ, identical above 300 mμ with the spectra of vitamin B_{12r}. The remainder of the product was concentrated and examined by chromatography. Apart from hydroxocobalamin, the only other component showing ultraviolet absorption had R_F 0·36, corresponding to the nucleoside A obtained by aerobic photolysis.

Nucleoside A.—The coenzyme (150 mg.) was dissolved in oxygen-free water (250 c.c.) and,

in an atmosphere of pure nitrogen, was exposed to sunlight. After 6 hr. the colour of the solution had changed to brown and the product was concentrated (to *ca.* 10 c.c.) and then freeze-dried. The residue was dissolved in methanol (50 c.c.), and a little insoluble material separated. The filtrate was concentrated and cooled, and the pink solid (23 mg.) obtained was crystallised from water to yield colourless crystals (10 mg.) of nucleoside A.

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The University, Nottingham. [*Received, April 13th,* 1962.]