228. *Chemistry of the Vitamin B_{12} Group. Part VI.* The Isomeric 5 : 6-Dimethylbenziminazole Nucleotides produced by Hydrolysis of Vitamin B_{12}.*

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Hydrolysis of vitamin B_{12} under acid or alkaline conditions gives a mixture of the 2'- and the 3'-phosphate of 5 : 6-dimethyl-1-α-D-ribofuranosylbenziminazole. These have been separated by ion-exchange chromatography and their properties are recorded. The formation of these isomeric phosphates involves intermediate formation of a cyclic 2' : 3'-phosphate as in the case of the pyrimidine and purine ribonucleotides.

The isolation of 5 : 6-dimethyl-1-α-D-ribofuranosylbenziminazole phosphate from vitamin B_{12} was first reported by Buchanan, Johnson, Mills, and Todd [1] who obtained it as an amorphous barium salt. The crystalline free acid was later obtained from an acid hydrolysate of the vitamin by Kaczka, Heyl, Jones, and Folkers [2] and again by Armitage, Cannon, Johnson, Parker, Smith, Stafford, and Todd.[3] In the meantime Brown and Todd,[4] in a discussion of the course of hydrolysis of ribonucleic acids, had shown that on treatment with acid, the individual pyrimidine or purine 2'- or 3'-ribonucleotides (the *a* and *b* nucleotides) readily formed an equilibrium mixture of the 2'- and the 3'-isomers, and that hydrolysis of their monoesters similarly yielded an equilibrium mixture, the cyclic 2' : 3'-phosphates being intermediates in the reactions. There were no obvious

* Part V, preceding paper.

[1] Buchanan, Johnson, Mills, and Todd, *Chem. and Ind.*, 1950, 426; *J.*, 1950, 2845.
[2] Kaczka, Heyl, Jones, and Folkers, *J. Amer. Chem. Soc.*, 1952, **74**, 5549.
[3] Armitage, Cannon, Johnson, Parker, Lester Smith, Stafford, and Todd, *J.*, 1953, 3849.

reasons why the benziminazole derivatives should not behave in a similar manner and it was therefore assumed by us that the crude phosphate obtained from the acid hydrolysis of the vitamin was a mixture of the two isomers and that the position of the phosphate in the crystalline degradation product (presumably one of the two isomers) was not necessarily the same as in the parent vitamin. Paper-chromatographic evidence for the existence of two isomeric phosphates was indeed obtained and it was reported [5] that each could be converted into a cyclic phosphate hydrolysable to a mixture of the two isomers. In view of their structural analogy to the natural ribonucleotides it has become common practice to refer to these phosphates as 5 : 6-dimethylbenziminazole nucleotides or even more briefly as benziminazole nucleotides.

In a later paper [6] Kaczka and Folkers claimed that only one isomer of the benziminazole nucleotide could be detected in the products obtained from vitamin B_{12} after

FIG. 1.

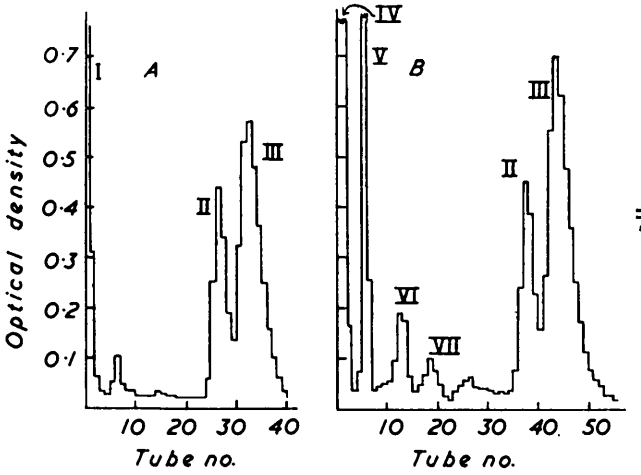

FIG. 2.

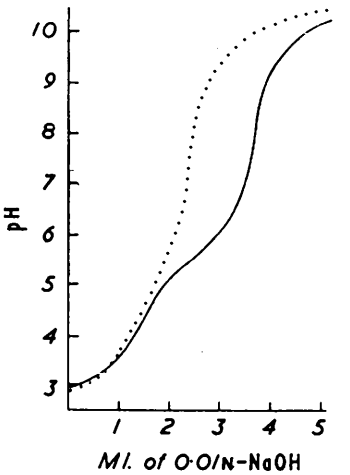

FIG. 1. *Ion-exchange separation of the 2′- and 3′-phosphate of* 5 : 6-*dimethyl*-1-α-D-*ribofuranosylbenziminazole on Dowex* 1 × 2 (*acetate*: 200—400 *mesh*: 7·5 *cm.* × 1·1 *sq. cm.*). Optical-density measurements were made at 277 mμ for the nucleotide fractions, and at 500 mμ for the coloured fractions. *A*, Separation of the isomeric nucleotides from an acid hydrolysate of vitamin B_{12}, the pigment acids being already removed (cf. Part IV); elution with water (40 c.c.), followed by 0·1N-acetic acid at a flow rate of 7 c.c./hr. *B*, Separation of the components of a previously unfractionated acid hydrolysate of vitamin B_{12}; elution with water (40 c.c.), 0·05N-acetic acid (200 c.c.) (both at 33 c.c./hr.), followed by 0·1N-acetic acid at 7 c.c./hr.

I, 5 : 6-Dimethyl-1-α-D-ribofuranosylbenziminazole; II and III, 2′- and 3′-phosphate of 5 : 6-dimethyl-1-α-D-ribofuranosylbenziminazole respectively; IV, unchanged vitamin B_{12} + Factor B; V, monobasic acid; VI and VII, dibasic acids.

FIG. 2. *Electrometric titration of the* 2′ : 3′-*cyclic phosphate of* 5 : 6-*dimethyl*-1-α-D-*ribofuranosylbenziminazole* (*broken line*). *After overnight treatment with* 4N-*hydrochloric acid, the secondary phosphoryl dissociation appears* (*full line*).

hydrolysis with 6N-hydrochloric acid at room temperature for 5—16 hr. However, treatment of the previously isolated crystalline phosphate with 80% acetic acid under reflux gave approximately equal quantities of the two isomers. They assumed therefore that the position of the phosphate group was the same in the product obtained from the hydrochloric acid hydrolyses, in the crystalline nucleotide, and in vitamin B_{12} itself. On the basis of the relative R_F values of the two isomers of the nucleotide obtained from the acetic acid isomerisation and by analogy with the properties of the *a* and *b* ribonucleotides,

[4] Brown and Todd, *J.*, 1952, 52.
[5] Todd and Johnson, *Rev. Pure Appl. Chem.*, 1952, **2**, 23.
[6] Kaczka and Folkers, *J. Amer. Chem. Soc.*, 1953, **75**, 6317.

it was suggested that the phosphate in the crystalline nucleotide and therefore in vitamin B_{12} itself was attached to the 3′-position in the sugar.

These results were clearly at variance with our own and we set out to isolate both isomers of the nucleotide in order to determine their properties and in particular their behaviour towards acid and alkali. By using a column of Dowex 1 × 2 acetate and eluting it with 0·1N-acetic acid we have shown that a mixture of the isomeric nucleotides can be resolved. Moreover, both isomers were obtained from the hydrolysis product of vitamin B_{12} with 6N-hydrochloric acid at room temperature for 18 hr. (Fig. 1). It is possible that Kaczka and Folkers's failure to detect both isomers by chromatography on paper was caused by the *a* isomer's being obscured by the coloured products of the reaction, a difficulty which does not arise with the Dowex 1 × 2 column.

Quantities of the individual nucleotides sufficient for analysis and chemical characterisation were obtained by separation of the nucleotide mixture formed [7] by alkaline hydrolysis of vitamin B_{12}. Dowex 1 × 2 was used in the chloride form in this case and the two isomers were purified by recrystallisation. As expected, each of the nucleotides was unaffected by saturated aqueous barium hydroxide at room temperature overnight, but each was isomerised to an equilibrium mixture by 6N-hydrochloric acid at room temperature overnight. The 2′ : 3′-cyclic nucleotide was obtained as an amorphous powder from either isomer by treatment with di*cyclo*hexylcarbodi-imide,[8] and treatment of it with either acid or alkali as above gave the equilibrium mixture of 2′- and 3′-nucleotides. The properties of the benziminazole 2′- and 3′-nucleotide thus parallel those of the corresponding nucleotides of the purine and pyrimidine series, apart from the variation in the ease of hydrolysis of the glycosylamine linkage, and the hydrolysis of vitamin B_{12} which yields them is strictly analogous to that of the esters of the 2′- and 3′-ribonucleotides. The position of the phosphate group in the benziminazole nucleotide of vitamin B_{12} was finally established as 3′ by *X*-ray crystallography.[9] As to which of the isolated benziminazole nucleotides is the 2′- and which the 3′-phosphate the evidence of Kaczka and Folkers [6] is, we believe, adequate to warrant the conclusion that the nucleotide isolated by them is the 3′-phosphate, and we have therefore described our materials accordingly as the 2′- and 3′-phosphate in the experimental portion of this paper.

Experimental

Chromatography of the Isomeric Nucleotides on Paper.—This was carried out with Whatman No. 1 paper in ascending chromatograms, and both layers of a mixture of 10% aqueous sodium carbonate and *iso*pentyl alcohol (1 : 1) as solvent.

Chromatograms were developed overnight, then sprayed with 20% acetic acid, and, while still moist, examined for fluorescent zones in ultraviolet light. 5 : 6-Dimethyl-1-α-D-ribofuranosylbenziminazole (the parent nucleoside) had R_F 0·27, the 2′-nucleotide R_F 0·68, and the 3′-nucleotide R_F 0·60 in this system. Aqueous sodium carbonate was found preferable to aqueous ammonium sulphate or disodium hydrogen phosphate in that better resolutions were obtained, and moreover qualitative tests for phosphate could be carried out on the paper. The above method was used throughout this work.

Separation of Nucleotides from an Acid Hydrolysate of Vitamin B_{12}.—Vitamin B_{12} (10 mg.) was treated with 6N-hydrochloric acid (2 c.c.) at room temperature for 18 hr. The product was evaporated to dryness under reduced pressure, the residue taken up in water (5 c.c.) and again taken to dryness, and the process repeated in order to remove the last traces of hydrochloric acid. The residue was adjusted to pH 9 by adding dilute aqueous ammonia, and the solution applied to a column (7·5 cm. × 1·1 sq. cm.) of Dowex 1 × 2 acetate (200—400 mesh). The column was first eluted with water (40 c.c.) which removed a mixture of unchanged vitamin

[7] Part IV, Bonnett, Cannon, Johnson, and Todd, *J.*, 1957, 1148.
[8] Dekker and Khorana, *J. Amer. Chem. Soc.*, 1954, **76**, 3522; Tener and Khorana, *ibid.*, 1955, **77**, 5349.
[9] Brink, Hodgkin, Lindsey, Pickworth, Robertson, and White, *Nature*, 1954, **174**, 1169.

B_{12} and factor B (electrophoresis) and then with 0·05N-acetic acid which removed a monocarboxylic acid and two separate dicarboxylic acids. The column was then practically colourless and 0·1N-acetic acid was applied at a flow-rate of 7 c.c./hr. in order to displace the nucleotides. Fractions (10 c.c.) were taken throughout the separation and the nucleotide-containing fractions detected by their absorption at 277 mμ. Two separate peaks were obtained in tubes 13 and 19 which corresponded respectively to the 2'- and the 3'-nucleotide in the proportion 7 : 17. The identification was confirmed by paper chromatography.

Separation of Nucleotides from an Alkaline Hydrolysis of Vitamin B_{12}.—Crystalline vitamin B_{12} (13·21 g. in all) was hydrolysed in portions with 30% sodium hydroxide solution at 150° for 1 hr. as described previously and the 5 : 6-dimethylbenziminazole nucleotides were separated by phenol-extraction and chromatography on a column of Dowex 1 × 2 (chloride [7]), being obtained as ammonium salts (2·74 g.) contaminated with a little red material. Paper-chromatographic examination of the product as described above showed that the two isomers were present together with a small quantity of the nucleoside. A solution of the product in water (80 c.c.; pH 9) was applied to a column of Dowex 1 × 2 chloride) 10 cm. × 4 sq. cm.; 200—400 mesh) and eluted with water which removed some coloured material together with the nucleoside (68 mg.). The eluting solvent was then changed to 0·05N-acetic acid and the first 200 c.c. of eluate were examined separately. This eluate contained the remainder of the pigmented material together with nucleotide and was purified by extraction into phenol, dilution with ether, and re-extraction into water. The resulting aqueous solution was freeze-dried and the product (0·379 g.) shown by paper chromatography to consist wholly of a mixture of the isomeric nucleotides. It was dissolved in the minimum of hot water, and acetone was added until the solution just remained clear at room temperature. When the solution was kept at 0°, the pure 2'-isomer (0·214 g.) crystallised, leaving a mixture of both isomers in the red mother-liquors. The *a* nucleotide, 5 : 6-*dimethyl*-1-α-D-*ribofuranosylbenziminazole* 2'-*phosphate* was separated; it recrystallised from aqueous acetone as small colourless needles, m. p. 226—228° (decomp.) (Found: C, 46·6; H, 5·6; N, 7·7; P, 8·5, 9·0. $C_{14}H_{19}O_7N_2P$ requires C, 46·9; H, 5·4; N, 7·8; P, 8·7%). Light absorption max.: in H_2O (*ca.* pH 2), 277 and 285 mμ (log ε 3·86 and 3·83 respectively). R_F in 10% aqueous sodium carbonate–*iso*pentyl alcohol, 0·68; in butan-1-ol–acetic acid–water (4 : 1 : 5), 0·49. The infrared spectrum, determined in Nujol and hexachlorobutadiene mulls, showed max. at 3320, 3100, 2980, 2920, 2860, 2720, 2000, 1605, 1548, 1455, 1395, 1324, 1306, 1270, 1238, 1212, 1175, 1145, 1106, 1055, 969, 957, 937, 877, 869, 854, 840, 810, 765, 707, and 699 cm.$^{-1}$.

The following litre of 0·05N-acetic acid eluate was collected and the nucleotide extracted into phenol and then again into water as above described. Evaporation of the resulting aqueous solution under reduced pressure caused the pure 3'-nucleotide (0·380 g.), 5 : 6-dimethyl-1-α-D-ribofuranosylbenziminazole 3'-phosphate, to crystallise. After cooling overnight it was separated and recrystallised from aqueous acetone as small colourless needles, m. p. 238—239° (decomp.) [lit., 240—241° (decomp.)] (Found: C, 46·8; H, 5·0; N, 7·9; P, 8·2, 8·8%). The mother-liquors from the original crystallisation were freeze-dried and yielded a mixture (1·287 g.) of the nucleotides. Light absorption of the 3'-nucleotide in water (*ca.* pH 2): max. at 277 and 285 mμ (log ε 3·90 and 3·88 respectively). R_F in 10% aqueous sodium carbonate–*iso*pentyl alcohol, 0·60; in butan-1-ol–acetic acid–water (4 : 1 : 5), 0·49. The infrared spectrum, determined in Nujol and hexachlorobutadiene mulls, showed max. at 3545, 3110, 2920, 1607, 1549, 1455, 1386, 1354, 1330, 1274, 1252, 1239, 1184, 1150, 1120, 1094, 1066, 1040, 1030, 974, 925, 885, 857, 848, 809, 793, 765, and 732 cm.$^{-1}$.

The red band remaining at the top of the ion-exchange column was eluted with M-aqueous sodium chloride, and the pigment extracted with phenol in the usual manner. Electrophoretic examination showed that it consisted mainly of a cobalt-containing tetracarboxylic acid.[7]

On a small scale it was possible to separate the isomeric nucleotides completely on a column (7·5 cm. × 1·1 sq. cm.) of Dowex 1 × 2 acetate (200—400 mesh) by using 0·1N-acetic acid as the eluant and a flow rate of 7 c.c./hr. The mixed nucleotides (5 mg.) were applied as aqueous solutions of their ammonium salts at pH 9 and independent sharp peaks were obtained (i) after 210 c.c. of eluate had been collected (2'-nucleotide, R_F in 10% aqueous sodium carbonate–*iso*pentyl alcohol, 0·60) and (ii) after 270 c.c. of eluate had been collected (3'-nucleotide, R_F as above, 0·68).

5 : 6-*Dimethyl*-1-α-D-*ribofuranosylbenziminazole* 2' : 3'-*Phosphate* (*cf. ref.* 8).—The mixed nucleotides (23 mg.) were dissolved in 10% aqueous pyridine (3·5 c.c.) and di*cyclo*hexylcarbodiimide (60 mg.) was added. The closed flask was shaken vigorously, then set aside for 2 days.

The mixture was diluted with water (5 c.c.), and the precipitated di*cyclo*hexylurea separated. The solution was extracted with ether (3 × 5 c.c.) and then lyophilised. The remaining slightly discoloured solid (19 mg.) had R_F 0·48 in the 10% aqueous sodium carbonate–*iso*pentyl alcohol system and was the 2′ : 3′-cyclic phosphate. On electrometric titration from pH 3 with 0·01N-aqueous sodium hydroxide (Fig. 2), unlike the 2′- and 3′-phosphate it did not show the secondary phosphoryl dissociation, but after treatment with cold 4N-hydrochloric acid it was reconverted into the mixed nucleotides.

Action of Acid and Alkali on the Individual Nucleotides.—(a) *Action of acid.* The nucleotide (2—3 mg.) was dissolved in 6N-hydrochloric acid and kept overnight at room temperature. Hydrochloric acid was removed under reduced pressure and the product examined by paper chromatography.

(b) *Action of alkali.* The nucleotide (2—3 mg.) was dissolved in saturated barium hydroxide solution and kept overnight at room temperature. Excess of solid ammonium carbonate was added to the product, the mixture filtered, and the filtrate examined by paper chromatography as before.

Under these conditions, the individual nucleotides were unaffected by alkali but isomerised to the equilibrium mixture by acid. The cyclic phosphate was converted into the mixed nucleotides by either acid or alkali.

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