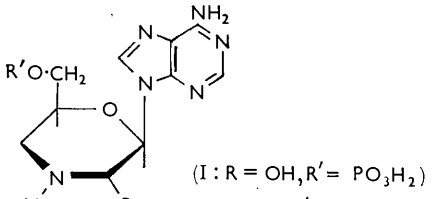
932. *Nucleotides. Part XLIX.*[1] *The Reduction of the Adduct of Periodate-oxidised Adenosine-5′ Phosphate and Methylamine*

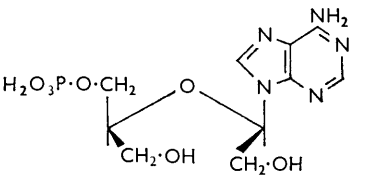
By D. M. Brown and A. P. Read

Sodium borohydride or catalytic hydrogenation converts the adduct of periodate-oxidised adenosine-5′ phosphate and methylamine into the *N*-methylmorpholine derivative (II).

In connection with a study of the mechanism of the elimination of phosphate, which occurs when a periodate-oxidised 5′-ribonucleotide is treated with an amine,[2,3] we have re-examined the substance produced by treating periodate-oxidised adenosine-5′ phosphate with methylamine and then with sodium borohydride.[4] Khym and Cohn[5] showed that periodate-oxidised adenosine-5′ phosphate and methylamine form an adduct which is stable at pH 9·5 but which rapidly eliminates phosphate under neutral or acid conditions. Sodium borohydride reduces this adduct to a stable substance which Khym[4] suggested was (I). Our results show that it is (II). When it was treated with 6N-hydrochloric acid at 100° for 30 minutes, adenine and a six-carbon amine were produced.[4] Hydrolysis of the glycosidic linkage in (I) should produce an extremely acid-labile fragment, which would not be recovered intact. Analysis by us supported structure (II).



(I: R = OH, R′ = PO_3H_2)
(II: R = H, R′ = PO_3H_2)
(III: R = R′ = H)



(IV)

We obtained the same product (II) by repeating Khym's preparation, and also by catalytic hydrogenation of a solution of periodate-oxidised adenosine-5′ phosphate and methylamine at pH 9·5. 5% Palladium–carbon caused no reduction, but with Raney nickel 2 moles of hydrogen were taken up per mole of adenine. Only 1 mole of hydrogen should be required to reduce an adduct of methylamine and the nucleotide dialdehyde to (I). Alkaline potassium ferricyanide, which should oxidise the carbinolamine (I), did not attack the compound.

The dephosphorylated compound (III) was prepared by treating (II) with *E. coli* alkaline phosphomonoesterase. Hydrogenation of a solution of periodate-oxidised adenosine and methylamine at pH 9·5 over Raney nickel also produced compound (III), with the consumption of 2 moles of hydrogen per mole of adenine.

The structure of (III) was proved by a nuclear magnetic resonance spectrum of its dihydrochloride, in deuterium oxide solution. The quadruplet splitting of the peak at τ 3·5—3·7, from the proton at C-2 of the morpholine ring, must be due to an adjacent methylene group, whilst the absence of other low-field multiplets also weighs against a structure having a hydroxyl group at C-3 or C-5. We conclude that the compound is 2-(9-adenyl)-6-hydroxymethyl-4-methylmorpholine (III), and that the compound prepared by Khym is its 6-*O*-phosphoryl derivative (II).

Paper electrophoresis in a series of buffers (see Experimental section) was used to compare the ionisation of (II) and (III) with that of the reduction product (IV) of adenosine-5′

[1] Part XLVIII, D. M. Brown and P. Schell, *J.*, 1965, 208.
[2] H. C. Neu and L. A. Heppel, *J. Biol. Chem.*, 1964, **239**, 2927, and refs. therein.
[3] D. M. Brown and A. P. Read, unpublished work.
[4] J. X. Khym, *Biochemistry*, 1963, **2**, 344.
[5] J. X. Khym and W. E. Cohn, *J. Biol. Chem.*, 1961, **236**, PC9; "Acides Ribonucléiques et Polyphosphates: Structure, Synthese, et Fonction," Colloques Internat. du C.N.R.S. (1961), Paris, 1962.

τ	Type of peak	No. of protons	Assignment
1·4, 1·5	Singlet	2	Adenine C-2 and C-8
3·50, 3·57, 3·62, 3·70	Quadruplet	1	$-O-\underset{\displaystyle CH_2}{\underset{\vert}{CH}}-N<$
5·4, 5·8	Complex	1	$-CH_2-\underset{\displaystyle CH_2}{\underset{\vert}{CH}}-O-$
5·95	Singlet	6	2 $-O-CH_2-CH<$
6·10—6·20	Doublet with shoulder		
6·30, 6·46, 6·65	Partially resolved singlets		4 $>N-CH_2-CH<$
6·85	Singlet	3	$>\overset{+}{N}-CH_3$

phosphate dialdehyde.[4] Compounds (II) and (III) each show an ionisation having a pK_a of *ca.* 5, which is absent from (IV). The pK_a's of (III), determined by titration, were 3·375 ± 0·004 and 5·38 ± 0·02, at 20° ± 0·5°. In adenosine, the pK_a of the amino-group is about 3·3. The second pK_a of (III) is presumably due to the morpholine nitrogen atom, but is considerably lower than in *N*-methylmorpholine (pK_a 7·41). Such a decrease, compared with *N*-methylmorpholine, is to be expected, because of the adenine ring and the extra oxygen function.[6]

Experimental

Paper electrophoresis was carried out in the apparatus described by Markham and Smith.[7] A G.H.S. 23 glass electrode was used in conjunction with an E.I.L. 33B/C33B meter (Electronic Instruments Ltd., Richmond, Surrey) to measure pH values. The n.m.r. spectrum was obtained on a Perkin-Elmer 60 Mc. machine; τ-values are relative to external tetramethylsilane. *E. coli* alkaline phosphomonoesterase was supplied by Worthington Biochemicals Corporation, New Jersey.

2-(9-*Adenyl*)-4-*methyl*-6-*phosphoryloxymethylmorpholine* (II).—(*a*) A sample sent by Mr. Khym was dried for 36 hr. *in vacuo* over phosphorus pentoxide at 50° [Found: C, 38·1; H, 5·95; N, 24·9. $C_{11}H_{17}N_6O_5P$ requires C, 38·4; H, 4·95; N, 24·5%. $C_{11}H_{17}N_6O_6P$ (I) requires C, 36·65; H, 4·75; N, 23·35%].

(*b*) Adenosine-5′ phosphate (500 mg.) and sodium metaperiodate (320 mg.) were shaken in water in the dark. After 15 min., barium chloride dihydrate (300 mg.), dissolved in a little water, was added, and the precipitate filtered off. Methylamine (40% aqueous solution) was added to the filtrate until a steady pH of 9·8 was reached. The solution was shaken with Raney nickel and hydrogen at 20°/758 mm. until uptake of gas ceased (4 hr.; 67 ml., 1·92 mol.). The catalyst was centrifuged off; paper electrophoresis in a series of buffers (see below) showed that the major product (*ca.* 90%) was *compound* (II). It was treated with a saturated solution of potassium ferricyanide at pH 9·0, for 22 hr. at 20°, and for 2 hr. at 85°. Paper electrophoresis at pH 5·1 and 10·3 of the solutions showed only spots running as the starting material.

2-(9-*Adenyl*)-6-*hydroxymethyl*-4-*methylmorpholine* (III).—(*a*) A solution of (II) (1 mg.) in pH 8·0 tris(hydroxymethyl)aminomethane buffer (1 ml.) was incubated for 12 hr. at 37° with *E. coli* alkaline phosphomonoesterase (1 ml. of a 1 : 1000 dilution of the preparation supplied). The *product* (III) ran as one spot on paper electrophoresis in a series of buffers, migrating as shown below. In each case the solution was adjusted to about the pH of the buffer to be used before it was spotted on to paper.

(*b*) Adenosine (500 mg.) was treated with sodium metaperiodate (400 mg.), barium chloride, methylamine, and Raney nickel, exactly as described above in the preparation of compound (II); 88 ml. of hydrogen were taken up in 4 hr., 1·94 moles per mole of adenine. The major product (*ca.* 90%) was electrophoretically identical with the enzymically dephosphorylated compound (II).

(*c*) Adenosine (3·0 g.) and sodium metaperiodate (2·45 g.) were dissolved in water and allowed to react at 20° in the dark. After 10 min. a solution of barium chloride (1·40 g. of the dihydrate) was added, and the precipitate centrifuged off. Methylamine (40% aqueous solution) was added dropwise to the supernatant liquid until a steady pH of 9·5 was reached. The solution was shaken with Raney nickel and hydrogen at 20° and 760 mm. until uptake of gas ceased

[6] J. Clark and D. D. Perrin, *Quart. Rev.*, 1964, **18**, 295.
[7] R. Markham and J. D. Smith, *Biochem. J.*, 1952, **52**, 552.

(48 hr.; 1·85 moles). The solution was filtered, evaporated to dryness under reduced pressure, and extracted with dry acetone. Paper electrophoresis showed that the material insoluble in acetone was a mixture, and it was discarded. Part of the acetone-soluble material (1·0 g., from a total of 1·73 g.) was dissolved in dilute hydrochloric acid (pH 1·5), evaporated to dryness *in vacuo*, and the solid extracted with dry methanol (3 × 10 ml.). The amorphous dihydrochloride (810 mg.) was electrophotetrically pure and had m. p. 218—220° (decomp.) (Found: C, 38·6; H, 5·35; Cl, 20·3; N, 24·35. $C_{11}H_{18}Cl_2N_6O_2$ requires C, 39·2; H, 5·3; Cl, 20·1; N, 25·0%).

1′-(9-*Adenyl*)-1-*phosphoryloxymethyldiethylene Glycol* (IV).—The compound (170 mg., 68%), prepared exactly as described by Khym [4] from adenosine-5′ phosphate (250 mg.) (Found: C, 34·8; H, 4·4; N, 20·0. Calc. for $C_{10}H_{16}N_5O_7P$: C, 34·4; H, 4·6; N, 20·0%), had m. p. 202° (lit.,[4] 201—203°).

Paper Electrophoresis of Products.—The distance in mm. through which the substance migrates in 1 hr. at 40v/cm., and the pole towards which it moves, are given.

pH	10·3	6·9	5·1	4·1	2·5
Compound (IV)	164+	168+	85+	70+	15—
Compound (II)	159+	159+	55+	13+	77—
Compound (III)	37—	29—	25—	105—	225—

Titration of Compound (III).—The analytically pure dihydrochloride of (III) (8·490 mg.) in water (3·13 g.) was titrated with 0·1008N-potassium hydroxide at 20° ± 0·5° under nitrogen, in the apparatus described by Albert and Serjeant.[8] Twenty additions, each of 0·025 ml. of alkali, were made, and the results calculated by Noyes's formula.[8]

We thank Mr. J. X. Khym for providing compound (I) and for personal communications, Professor N. Sheppard for advice, and the D.S.I.R. for a maintenance award to one of us (A. P. R.).

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[8] A. Albert and E. P. Serjeant, " The Ionisation Constants of Acids and Bases," Methuen, London, 1962.