122. *Nucleotides. Part I. Muscle Adenylic Acid and Adenosine Diphosphate.*

By J. Baddiley and A. R. Todd.

Muscle adenylic acid (adenosine-5′ phosphate) can be prepared in good yield by treatment of 2′ : 3′-*iso*propylidene adenosine with dibenzyl chlorophosphonate followed by removal of the benzyl and *iso*propylidene residues. 2′ : 3′-iso*Propylidene adenosine*-5′ *dibenzyl phosphate*, an intermediate in this preparation, can be converted by careful hydrolysis into *adenosine*-5′ *benzyl phosphate*. From this a synthesis of *adenosine*-5′ *pyrophosphate*, identical with natural adenosine diphosphate, has been effected by reaction of its silver salt with dibenzyl chlorophosphonate and subsequent debenzylation of the product.

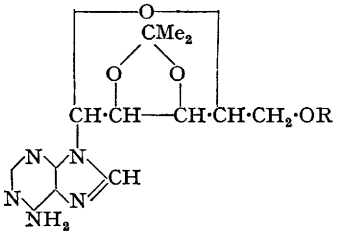
DURING recent years an extended series of investigations has been in progress in this laboratory having as its object the application of synthetic methods to the study of nucleotides. In a series of papers in this *Journal* we have reported results obtained in the synthesis of nucleosides and on the development of phosphorylation procedures suitable for application to sensitive molecules and offering the possibility of extension to synthesis of di- and tri-phosphoric esters. In a lecture recently delivered to the Society (Todd, *J.*, 1946, 647) a general review was given of the progress of these investigations and of their orientation. The present paper contains the initial results obtained in applying our methods to the preparation of nucleotides exercising coenzyme function. It is hoped that as a result of such work some of these nucleotides may be made more readily accessible and their structures rigidly established, and that by the study of synthetic analogues some light may be thrown on the problem of structural specificity in coenzymes. It is also clear that studies of this nature may assist in the clarification of some important aspects of polynucleotide structure. Given the structure of the nucleosides, the synthesis of nucleotides in general comprises three basic problems : (*a*) effective phosphorylation of nucleosides, (*b*) synthesis of esters of di- and tri-phosphoric acids, and (*c*) linkage of different molecules through phosphate or polyphosphate residues. In our initial studies muscle adenylic acid (III) and adenosine diphosphate (VI) were selected as compounds whose partial synthesis from natural adenosine involved a solution of problems (*a*) and (*b*).

Muscle adenylic acid (adenosine-5′ phosphate) was first isolated from rabbit muscle by Embden and Zimmermann (*Z. physiol. Chem.*, 1927, **167**, 137), and the location of the phosphate group was determined by degradation (Levene and Mori, *J. Biol. Chem.*, 1929, **81**, 215). Since adenosine has been shown to be 9-β-*d*-ribofuranosidoadenine (Levene and Tipson, *J. Biol. Chem.*, 1932, **94**, 809; Gulland and Holiday, *J.*, 1936, 765; Davoll, Lythgoe, and Todd, *J.*, 1946, 833; Lythgoe, Smith, and Todd, this vol., p. 355), the structure of muscle adenylic acid may be regarded as completely defined. This nucleotide is of considerable interest because of its role in muscle contraction and its effect on the heart and on blood pressure; preparations containing it have been employed clinically from time to time. It has been partially synthesised from adenosine by several workers, but in yields so poor that the nucleotide has remained accessible only by fermentative methods or by extraction from muscle. The synthetic methods employed have been direct phosphorylation of adenosine with phosphoryl chloride in pyridine (Jachimowicz, *Biochem. Z.*, 1937, **292**, 356; Gulland and Hobday, *J.*, 1940, 746), treatment of 2′ : 3′-*iso*propylidene adenosine with phosphoryl chloride followed by hydrolysis (Levene and Tipson, *J. Biol. Chem.*, 1937, **121**, 131), and treatment of 2′ : 3′-diacetyl adenosine with diphenyl chlorophosphonate followed by hydrolysis (Bredereck, Berger, and Ehrenberg, *Ber.*, 1940, **73**, 269); the last two methods being unambiguous as regards point of attachment of the phosphate residue confirm the allotted structure of muscle adenylic acid. Adenosine diphosphate was first obtained by Lohmann (*Biochem. Z.*, 1935, **282**, 109) by the action of a washed muscle preparation on adenosine triphosphate, and it has been isolated as a degradation product of cozymase (Vestin, Schlenk, and v. Euler, *Ber.*, 1937, **70**, 1369). Its formulation as adenosine-5′ pyrophosphate (VI), originally proposed by Lohmann on titration evidence, has recently been verified by Lythgoe and Todd (*Nature*, 1945, **155**, 695) by a periodate oxidation method, and by Gulland and Walsh (*J.*, 1945, 169) using a combination of titrimetric and enzymatic methods. So far no synthesis or partial synthesis has been described for this important nucleotide which functions in transphosphorylation systems by accepting phosphate and undergoing reversible transformation to adenosine triphosphate.

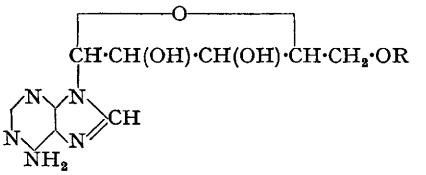
Earlier model experiments on phosphorylation (Atherton, Openshaw, and Todd, *J.*, 1945, 382) suggested that dibenzyl chlorophosphonate would be a suitable reagent to employ in the preparation of muscle adenylic acid from adenosine, and it seemed desirable to protect the hydroxyls at C_2' and C_3' in the nucleoside in order to avoid the formation of a mixture of products. Dibenzyl chlorophosphonate reacted at low temperature with 2′ : 3′-*iso*propylidene adenosine (I) in presence of pyridine giving 2′ : 3′-iso*propylidene adenosine*-5′ *dibenzyl phosphate* (II). Removal of benzyl groups by hydrogenolysis, followed by mild acid treatment to eliminate the acetone residue, gave adenosine-5′ phosphate, identical with muscle adenylic acid isolated from natural sources. Attention is drawn to the overall yield from adenosine (45%), which makes the nucleotide a comparatively accessible product.

The observation that organic pyrophosphates can be conveniently prepared by condensing a metallic salt of a diester of phosphoric acid with a halogeno-phosphonate, made in the course of other work by Mr. F. R. Atherton, suggested that 2′ : 3′-*iso*propylidene adenosine-5′ dibenzyl phosphate would be a suitable starting material for the preparation of adenosine diphosphate, since partial debenzylation would give a diester of phosphoric acid. One method of achieving

this object seemed to be controlled hydrogenolysis of (II), since this has been shown to be practicable with dibenzyl *iso*amyl phosphate (Atherton, Openshaw, and Todd, *loc. cit.*). It



(I.) R = H.
(II.) R = $PO(O{\cdot}CH_2Ph)_2$.



(III) R = PO_3H_2.
(IV.) R = $PO(OH){\cdot}O{\cdot}CH_2Ph$.
(V.) R = $PO(O{\cdot}CH_2Ph){\cdot}O{\cdot}PO(O{\cdot}CH_2Ph)_2$.
(VI.) R = $PO(OH){\cdot}O{\cdot}PO(OH)_2$.

was felt, however, that retention of the acetone residue during the projected synthesis would be undesirable, since its removal without fission of a pyrophosphate linkage might present difficulty. Accordingly, careful hydrolysis of (II) with dilute acid was carried out instead of hydrogenolysis. Under suitable conditions the acetone residue was removed together with one benzyl group giving *adenosine-5′ benzyl phosphate* (IV), which readily formed a silver salt. It was difficult to find a suitable solvent for the reaction of this salt with dibenzyl chlorophosphonate, but in warm glacial acetic acid reaction occurred smoothly with separation of silver chloride. The product, presumably adenosine-5′ tribenzyl pyrophosphate (V), was a brittle resin; catalytic hydrogenation in alcoholic solution gave *adenosine-5′ pyrophosphate* (VI), conveniently isolated as its *acridine* salt, which proved identical with the acridine salt of natural adenosine diphosphate.

Extension of this work with a view to the synthesis of adenosine triphosphate and other nucleotide coenzymes is in progress.

Experimental.

2′ : 3′-iso*Propylidene Adenosine*.—Levene and Tipson (*loc. cit.*) record for this substance m. p. 200—204° after softening at 190°, and $[\alpha]_D^{25°}$ −63·9°. Our product prepared by their method melted sharply at 220° without previous softening and had $[\alpha]_D^{18°}$ −65·0° (*c*, 1 in water) (Found: C, 50·9; H, 5·8; N, 23·0. Calc. for $C_{13}H_{17}O_4N_5$: C, 50·7; H, 5·5; N, 22·8%).

2′ : 3′-iso*Propylidene Adenosine-5′ Dibenzyl Phosphate*.—2′ : 3′-*iso*Propylidene adenosine (5 g. dried for 24 hours at 116°/10^{-3} mm.) was dissolved by warming in dry pyridine (60 c.c.) and the solution cooled in an acetone–solid carbon dioxide bath until the liquid at the sides of the flask began to solidify. Dibenzyl chlorophosphonate [prepared according to Atherton, Openshaw, and Todd (*loc. cit.*) by chlorinating 12 g. of dibenzyl phosphite in carbon tetrachloride solution and removing solvent under reduced pressure below 30°] was added rapidly with shaking and the mixture left for 3 hours in the cold bath at a temperature just above the freezing point of the mixture and then set aside at room temperature overnight. Water (20 c.c.) and sodium carbonate (*ca.* 5 g.) were added and the solution was evaporated under reduced pressure. The syrup so obtained was dissolved in chloroform (100 c.c.), and the solution washed thoroughly with water containing a small amount of sodium hydrogen carbonate, then with water, dried, and evaporated. After the residual syrup had been twice evaporated with alcohol to remove any traces of solvent, it was dissolved in a minimum amount of alcohol and treated with ether until slightly turbid. On standing at 0° for several hours, or more rapidly by seeding, the product separated in crystalline form. Recrystallised from alcohol–ether, 2′ : 3′-iso*propylidene adenosine-5′ dibenzyl phosphate* formed small, colourless needles (4·8 g.; 52%), m. p. 97—98° (Found in material dried at 50°: C, 57·0; H, 5·4; N, 12·4. $C_{27}H_{30}O_7N_5P$ requires C, 57·0; H, 5·3; N, 12·4%).

Adenosine-5′ Phosphate (*Muscle Adenylic Acid*).—2′ : 3′-*iso*Propylidene adenosine-5′ dibenzyl phosphate (0·7 g.) was dissolved in aqueous alcohol (50 c.c. of 50%) and shaken with hydrogen at atmospheric pressure using an Adams's palladium oxide catalyst; absorption of hydrogen was rapid, the theoretical amount for removal of two benzyl groups being taken up in 10 minutes. The catalyst was removed by filtration, the solution evaporated to dryness under reduced pressure, the residue dissolved in dilute sulphuric acid (100 c.c. of 0·1N), and the solution set aside for 2 days to hydrolyse the *iso*propylidene residue. The sulphuric acid was neutralised with the calculated amount of barium hydroxide, barium sulphate spun off, and the solution concentrated to small bulk. On standing overnight at 0° adenosine-5′ phosphate crystallised in fine needles (0·35 g.; 85%), m. p. 190°, undepressed in admixture with authentic muscle adenylic acid (m. p. 192°). Recrystallised from water it forms a dihydrate (Found in material dried at room temperature: C, 31·6; H, 5·0; N, 18·1; loss at 110°, 9·5. Calc. for $C_{10}H_{14}O_7N_5P,2H_2O$: C, 31·3; H, 4·9; N, 18·3; H_2O, 9·4%. Found in material dried at 110°: C, 34·3; H, 4·0; P, 8·7. Calc. for $C_{10}H_{14}O_7N_5P$: C, 34·6; H, 4·0; P, 8·9%). The acridine salt of the synthetic compound had m. p. 208°, undepressed by the acridine salt of natural muscle adenylic acid (m. p. 208°). The synthetic product had $[\alpha]_D^{20°}$ −45·5° (*c*, 0·72 in water) and the natural $[\alpha]_D^{20}$ −46·4° (*c*, 0·56 in water).

Adenosine-5′ Benzyl Phosphate.—2′ : 3′-*iso*Propylidene adenosine-5′ dibenzyl phosphate (5·4 g.) was dissolved in a mixture of dilute sulphuric acid (500 c.c. of N/50) and alcohol (150 c.c.) and the solu-

tion refluxed for 1 hour. Sulphate was removed by adding the calculated amount of barium hydroxide, the precipitated barium sulphate being removed by filtration. Concentrated to small bulk and set aside at room temperature, the solution deposited *adenosine-5′ benzyl phosphate*, which recrystallised from water as colourless needles (3·7 g.; 90%), m. p. 234° (decomp.) (Found in material dried at 110° C, 46·3; H, 4·8; N, 16·3. $C_{17}H_{20}O_7N_5P$ requires C, 46·5; H, 4·6; N, 16·1%). The *silver* salt was precipitated as a white powder on adding silver nitrate to a solution of substance in the requisite amount of aqueous sodium hydroxide. On catalytic hydrogenation, adenosine-5′ benzyl phosphate rapidly took up 1 mol. of hydrogen yielding muscle adenylic acid, identified by m. p. and mixed m. p. (208°) of its acridine salt.

Adenosine-5′ Pyrophosphate (*Adenosine Diphosphate*).—Dibenzyl chlorophosphonate (from 7 g. of dibenzyl phosphite) was added to a suspension of silver adenosine-5′ benzyl phosphate (4·2 g. dried for 24 hours at 116°/10^{-3} mm.) in anhydrous acetic acid (120 c.c.) at 50—60°. The mixture was shaken for 30 minutes, during which time the silver salt gradually disappeared and was replaced by silver chloride. The mixture was left overnight in the dark and then filtered, and the filtrate evaporated under reduced pressure. The residue was evaporated thrice with alcohol and triturated with ether; it then formed a brittle resin which could not be crystallised. This resinous product was dissolved in aqueous alcohol (100 c.c. of 50%) and hydrogenated at ordinary temperature and pressure using a palladium oxide catalyst. Hydrogenation was complete in 6 hours. Catalyst was removed by filtration and the filtrate concentrated to one-third volume under reduced pressure and decanted from a small amount of oil which separated. An equal volume of alcohol was then added, and aqueous barium hydroxide added to pH 8·5. The precipitated barium salt was collected by centrifugation, washed with aqueous alcohol (50%), and dried (yield, 5·1 g.).

The crude barium salt (5 g.) was suspended in a little water and dilute sulphuric acid (23·8 c.c. of N) added. Precipitated barium sulphate was spun off and washed with water, and the combined supernatant liquid and washings were treated with alcoholic acridine (1·1 g. in 50 c.c.). On standing, the *acridine* salt of adenosine-5′ pyrophosphate separated. Recrystallised from water it formed characteristic yellow prismatic needles (0·8 g.; 18%), m. p. 215° (decomp.), undepressed in admixture with an authentic sample of the acridine salt of natural adenosine diphosphate (m. p. 215° decomp.) prepared according to Wagner-Jauregg (*Z. physiol. Chem.*, 1936, **239**, 188) (Found in material dried at 110°: C, 44·9; H, 4·1; N, 13·5; P, 9·8. $C_{10}H_{15}O_{10}N_5P_2,C_{13}H_9N$ requires C, 45·5; H, 4·0; N, 13·9; P, 10·2%). Determination of acid-labile phosphorus (*i.e.*, phosphorus removed in 15 minutes at 100° with N-hydrochloric acid) and total phosphorus in a solution of adenosine diphosphate prepared from the synthetic acridine salt gave a ratio, acid-labile P/total P = 1·01/2 (calculated ratio for adenosine diphosphate = 1/2); these estimations were made using the colorimetric method of Allen (*Biochem. J.*, 1940, **34**, 858). For further confirmation of identity, the acridine salts of natural and synthetic adenosine diphosphate were examined by Dr. Clews and Mr. Nicol of the Department of Crystallography; *X*-ray powder photographs of the two materials were indistinguishable from one another.

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