124. *Nucleotides. Part II. A Synthesis of Adenosine Triphosphate.*

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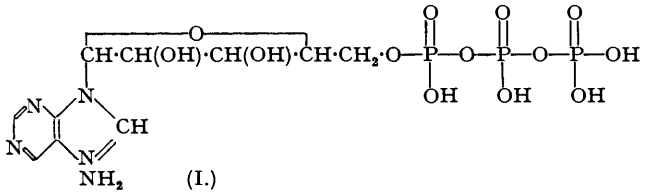
Adenosine-5′ triphosphate (I), identical with adenosine triphosphate isolated from natural sources, has been synthesised by bringing the silver salt of *adenosine-5′ dibenzyl pyrophosphate* (IV) into reaction with dibenzyl chlorophosphonate and submitting the product to hydrogenolysis. The synthetic material has been characterised as its *triacridinium* salt, m. p. 209°, an *acridinium* salt, m. p. 218°, having the composition (adenosine-5′ triphosphate)$_2$, (acridine)$_5$ and as the hexahydrate of its *dibarium* salt. Its biological activity corresponds to that of the natural product.

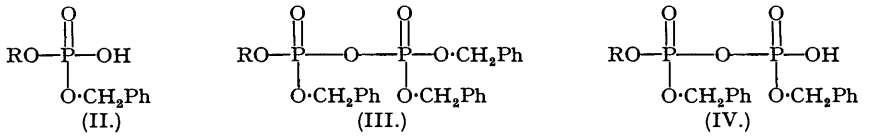
In Part I of this series (Baddiley and Todd, *J.*, 1947, 648) methods for the synthesis of muscle adenylic acid (adenosine-5′ phosphate) and adenosine diphosphate (adenosine-5′ pyrophosphate), identical with the naturally occurring coenzymes, were described, and it was stated that an extension of the investigations in the direction of adenosine triphosphate was in progress. The present memoir deals with these further studies, which have resulted in the total synthesis of this biologically important substance. Adenosine triphosphate, first isolated from muscle extracts in 1929 (Lohmann, *Naturwiss.*, 1929, **17**, 624; Fiske and Subbarow, *Science*, 1929, **70**, 381), plays a vital part in many biological processes, and according to current views its breakdown provides the energy used in muscular contraction; it has been employed from time to time in clinical medicine. By acid hydrolysis of adenosine triphosphate, 1 mol. of adenine and 2 mols. of phosphoric acid are liberated rapidly, the third phosphorus atom appearing as D-ribose-5 phosphate, and Lohmann's investigations (*Biochem. Z.*, 1931, **233**, 460; 1932, **254**, 381; 1935, **282**, 120) led him to formulate it as adenosine-5′ triphosphate (I). This structure was not universally accepted, it being maintained by certain workers (Satoh, *J. Biochem. Japan*, 1936, **21**, 19; Barrenscheen and Jachimowicz, *Biochem. Z.*, 1937, **292**, 350) that adenosine triphosphate showed properties incompatible with such a structure. Further titrimetric and enzymatic evidence in favour of structure (I) was provided by Gulland and Walsh (*J.*, 1945, 169), and finally its correctness was established by Lythgoe and Todd (*Nature*, 1945, **155**, 695) by the method of periodate oxidation. Quite apart from its great biological interest, adenosine triphosphate is unique among organic compounds in that it is the only known derivative of triphosphoric acid. As might be expected both from structure (I) and from its biological function, it is an extremely unstable compound, being very readily degraded by alkalis to muscle adenylic acid. It is normally isolated from muscle as its barium salt, but even in this form it undergoes slow decomposition; at room temperature it loses much of its biological activity by hydrolysis in a few weeks. This instability makes its preparation in a pure state from muscle a matter of difficulty, and complicates the problem of its synthesis by chemical methods.

Since adenosine (9-β-D-ribofuranosidoadenine) has already been synthesised (Davoll, Lythgoe, and Todd, *J.*, 1948, 967), the outstanding problem in an adenosine triphosphate synthesis is the attachment of a triphosphate residue to the 5′-position in that compound. In Part I (*loc. cit.*), adenosine-5′ pyrophosphate was synthesised by condensing silver adenosine-5′ benzyl phosphate (II) with dibenzyl chlorophosphonate in glacial acetic acid solution and directly hydrogenating the crude resinous adenosine-5′ tribenzyl pyrophosphate (III), adenosine-5′ pyrophosphate being finally isolated as its crystalline acridinium salt. The route envisaged for the synthesis of adenosine triphosphate involved partial debenzylation of adenosine-5′ tribenzyl pyrophosphate to adenosine-5′ dibenzyl pyrophosphate (IV), and condensation of the latter in the form of a metallic salt with dibenzyl chlorophosphonate followed by hydrogenolysis of the adenosine-5′ tetrabenzyl triphosphate (V) produced. There were certain obvious difficulties to be surmounted before such a scheme could be realised. Selective monodebenzylation of a pyrophosphate such as (III) was likely to be a more difficult matter than monodebenzylation of adenosine-5′ dibenzyl phosphate, and step (IV) ⟶ (V) was expected to give trouble, if only because fully esterified polyphosphoric acids had been observed in other studies in these laboratories to be even more unstable (*i.e.*, more potent phosphorylating agents) than the free acids themselves. As a preliminary, the methods used by Baddiley and Todd (*loc. cit.*) for the synthesis of adenosine diphosphate were re-examined with the object of improving yields so that such intermediates as (III) might be more readily available. It was found that the yield of 2′ : 3′-*iso*propylidene-adenosine-5′ dibenzyl phosphate previously recorded (Part I, *loc. cit.*) was too low; in many subsequent experiments the yield has been 70—75%. The use of glacial acetic acid as a medium for condensing the silver salt of adenosine-5′ benzyl phosphate (II) with dibenzyl chloro-

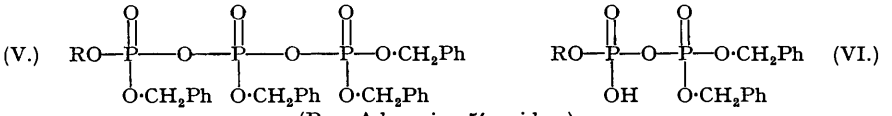
phosphonate gave consistently low yields. A variety of alternative solvents was tried in its place, and finally a mixture of phenol and methyl cyanide was selected for regular use; using it the yield of pure adenosine diphosphate, isolated as its acridinium salt, is normally 55%.



(I.)



(II.) (III.) (IV.)



(V.) (VI.)

(R = Adenosine-5′ residue.)

In the original synthesis of adenosine diphosphate (*loc. cit.*), the intermediate adenosine-5′ tribenzyl pyrophosphate (III) was not isolated in pure form. Upon attempting to purify the condensation product of adenosine-5′ benzyl phosphate (II) with dibenzyl chlorophosphonate in glacial acetic acid it was evident that it was far from pure and that it contained acidic material the proportion of which increased during purification experiments. The acidic material was isolated, and from its analysis and behaviour it was undoubtedly *adenosine-5′ dibenzyl pyrophosphate* (IV). The same tendency to debenzylation was shown by the purer specimens of (III) obtained when phenol–methyl cyanide was used as the condensation medium in their preparation. In view of this, and since for an adenosine triphosphate synthesis (IV) is the key intermediate, attempts to isolate (III) in a state of purity were not further pursued. Instead, the crude condensation product containing (III) was directly treated with *N*-methylmorpholine, according to the quaternisation procedure of Baddiley, Clark, Michalski, and Todd (forthcoming publication),* so that all the tribenzyl ester present was mono-debenzylated to (IV), which could be isolated as a crude silver salt. It is considered that the adenosine-5′ dibenzyl pyrophosphate so obtained is correctly formulated as (IV). The mono-debenzylation of mixed pyrophosphates has not yet been thoroughly investigated, and if the quaternisation were random, in the sense that any one of the benzyl groups might be removed, then one might expect to find that the debenzylated material, although mainly (IV), would contain a proportion of an isomer having structure (VI). On various grounds, however, we expected that the mono-debenzylation of (III) would not be random and that the main reaction would be removal of one of the adjacent terminal benzyl residues to yield (IV); that some of the isomeric (VI) may have been formed is, of course, possible, but the successful synthesis of adenosine triphosphate [which from titration results must have structure (I) and not the isomeric one derived from (VI)] and our failure to find any evidence for the presence of an isomeric triphosphate in the synthetic product from our adenosine-5′ dibenzyl pyrophosphate are strong evidence for the correctness of our view of its nature.

In initial attempts to prepare adenosine triphosphate by condensing the crude silver salt of (IV) with dibenzyl chlorophosphonate, followed by direct hydrogenation to avoid isolating the unstable tetrabenzyl ester (V), choice of solvent for the condensation gave some trouble owing, apparently, to the fact that the particular sample of phenol used in the first trials gave products which could not be hydrogenated. This difficulty has not since been encountered, but as a result the first evidence of successful adenosine triphosphate synthesis was obtained from experiments in which glacial acetic acid was used as a reaction medium. This procedure gave crude products which contained low and variable amounts (7—15%) of the desired product as assayed enzymatically by means of adenosine triphosphatase. Subsequently, using phenol–

* The term " quaternisation " is here used to indicate debenzylation by a process depending on the transfer of a benzyl residue from oxygen to nitrogen with formation of a quaternary salt.

methyl cyanide as reaction medium and carrying out the hydrogenation step in aqueous dioxan, we obtained crude products (isolated as barium salts) containing *ca.* 40% of adenosine triphosphate by enzymatic assay. As the chief impurities (also assayed enzymatically) were adenosine diphosphate and inorganic pyrophosphate, it is reasonable to assume that the initial condensation product contains a higher proportion of (V) than that corresponding to the adenosine triphosphate isolated and that considerable loss occurs by decomposition in the later stages; it seems possible that some modification of these later stages or more rapid working might lead to improved yields of final product.

From the crude barium salt obtained as above, pure *adenosine-5′ triphosphate* was prepared in the form of its *triacridinium* salt (Wagner-Jauregg, *Z. physiol. Chem.*, 1936, **239**, 188) and as a second *acridinium* salt in which the molar ratio adenosine triphosphate : acridine is 2 : 5. Both these salts were identical with the corresponding salts prepared from natural adenosine triphosphate, showed no depression in m. p. when mixed with them, and had the correct ratio of acid-labile phosphorus (*i.e.*, phosphorus liberated as phosphate in 15 minutes at 100° with N-hydrochloric acid) to acid stable phosphorus. For further characterisation the barium salt was prepared; this had the properties recorded by Lohmann (*Biochem. Z.*, 1931, **233**, 460) and was obtained as the known hexahydrate. All the synthetic materials were submitted to enzymatic assay, the acridinium or barium salts being converted in solution into sodium salt and assayed by the three enzyme systems, adenosine triphosphatase, adenosine triphosphatase + myokinase, and yeast inorganic pyrophosphatase (Bailey, *Proc. Biochem. Soc.*, 1948, **42**, lviii). The analytically pure synthetic salts showed by this method biological activity comparable with that of the pure salts of natural adenosine triphosphate.

Wagner-Jauregg (*loc. cit.*) recorded two acridinium salts of adenosine triphosphate. One of these, the triacridinium salt, we have also obtained, but not the other in which the molar ratio adenosine triphosphate to acridine was 1·2 : 2. It seems possible that Wagner-Jauregg's product may have been an impure specimen of the well-defined salt of composition (adenosine triphosphate)$_2$, (acridine)$_5$ which was obtained in our investigations from both natural and synthetic material. At any rate, we found no evidence for the formation of any other definite compound. As yet we have made no thorough study of the stability of salts of adenosine triphosphate, but it seems clear that all the metal salts so far known are hydrated. This fact probably explains their tendency to decompose on storage, and in order to achieve stability it would be desirable to use some base which would yield a crystalline anhydrous salt.*

Experimental.

Adenosine-5′ Pyrophosphate.—Replacement of the acetic acid as condensation medium in the method of Baddiley and Todd (*loc. cit.*) by a mixture of phenol (40 g.) and dry methyl cyanide (8 c.c.) and reduction of the amount of dibenzyl chlorophosphonate used by half, gave a 55% yield (calculated on silver adenosine-5′ benzyl phosphate) of the acridine salt of adenosine-5′ pyrophosphate, m. p. 215° (decomp.).

Adenosine-5′ Dibenzyl Pyrophosphate.—(*a*) Dibenzyl chlorophosphonate (from 4 g. of dibenzyl phosphite) was added to a suspension of silver adenosine-5′ benzyl phosphate (Baddiley and Todd, *loc. cit.*) (4 g., dried for 12 hours at 110°/1 mm.) in anhydrous acetic acid (100 c.c.) at 50—60°, and the mixture shaken for 30 minutes. After standing overnight at room temperature in the dark, the mixture was filtered and the filtrate evaporated under reduced pressure to give a residue which was evaporated thrice with alcohol and the gummy residue stirred with chloroform. Insoluble material (1·4 g., m. p. *ca.* 72°) was filtered off (Found in material dried for 12 hours at 50°/1 mm. : C, 43·9; H, 5·1; N, 13·9. Adenosine-5′ tribenzyl pyrophosphate, $C_{31}H_{33}O_{10}N_5P_2$, requires C, 53·4; H, 4·7; N, 10·0; adenosine-5′ dibenzyl pyrophosphate, $C_{24}H_{27}O_{10}N_5P_2$, requires C, 47·5; H, 4·5; N, 11·5%). The chloroform solution was washed with dilute sodium hydrogen carbonate solution till neutral and then with water, dried (Na_2SO_4), and freed from solvent under reduced pressure. The white semi-solid residue was dissolved in alcohol and filtered into ether. On standing at 0° overnight a small quantity of a fine white powder separated. This product was strongly acidic and had m. p. *ca.* 115° (Found in material dried for 12 hours at 50°/1 mm. : C, 45·8; H, 4·8; N, 11·3; P, 10·0. $C_{24}H_{27}O_{10}N_5P_2,H_2O$ requires C, 46·1; H, 4·6; N, 11·2; P, 9·9%). The silver salt was precipitated as a white powder on adding silver nitrate to a solution of the acid in a mixture of dimethylformamide and dilute aqueous sodium hydroxide.

(*b*) Dibenzyl chlorophosphonate (from 4 g. of dibenzyl phosphite) was added to a solution of silver adenosine-5′ benzyl phosphate (5 g., dried for 12 hours at 110°/1 mm.) in a mixture of absolute phenol (50 g.) and methyl cyanide (10 c.c.) at 50°, and the solution kept at this temperature for 15 minutes, during which time silver chloride separated. The mixture was then poured into dry ether (300 c.c.) and the precipitated solid filtered off, washed well with ether, and dried at room temperature/1 mm. for 3 hours. The crude adenosine-5′ tribenzyl pyrophosphate was dissolved in dry dimethylformamide

* *Added in proof.*—Since the above was written, we have kept a specimen of the air-dried triacridinium salt of synthetic adenosine triphosphate for eight months at room temperature in a cork-stoppered tube without any special precautions; enzymic assay has disclosed no appreciable diminution in its activity.

(40 c.c.), *N*-methylmorpholine (1·5 c.c.) added, and the slightly yellow solution heated on the water-bath in a stoppered round-bottomed flask for 15 minutes. Silver chloride was removed by filtration of the hot solution through " Hyflo supercel "; the filtrate, on being poured into dry ether (300 c.c.), deposited a gum which, after being washed with ether, was dissolved in aqueous dimethylformamide (100 c.c. of 10%), a little sodium hydroxide (3 c.c. of N) being added to bring the solution to neutrality. To the filtered solution, silver nitrate (5 g.) in water (20 c.c.) was added, and the crude silver salt (6·2 g.) filtered off after 2 hours, washed well with water then acetone, and dried for 12 hours at 60°/1 mm. (Found : Ag, 16·8. Calc. for $C_{34}H_{26}O_{10}N_5P_2Ag$: 15·1%). This crude salt was not further purified but was used directly for adenosine triphosphate synthesis.

Crude Barium Adenosine-5′ Triphosphate (ca. 40%).—To a solution of the above silver salt [6 g., prepared as in (*b*) above] in a mixture of phenol (50 g.) and methyl cyanide (10 c.c.) at 50°, dibenzyl chlorophosphonate (from 4 g. dibenzyl phosphite) was added, and the solution kept at 50° during 15 minutes and then poured into dry ether (350 c.c.). The precipitated solid was filtered off, washed well with ether, dried under reduced pressure at room temperature for 2 hours, and dissolved in aqueous dioxan (200 c.c. of 50%). Silver chloride was spun off, and the solution hydrogenated at room temperature and atmospheric pressure with a mixture of palladium oxide and palladised carbon catalysts. After absorption of hydrogen had ceased (365 c.c. in *ca.* 2 hours), catalyst was removed by filtration, and barium hydroxide added to the strongly acidic solution to bring it to pH 6; the precipitated barium salt was centrifuged off, washed twice with water, dissolved in cold hydrochloric acid (approx. 170 c.c. of N/10), centrifuged from insoluble matter, and reprecipitated with two volumes of 95% ethanol. The collected barium salt was again dissolved in cold hydrochloric acid (*ca.* 200 c.c. of N/20) and centrifuged, and 20% barium acetate solution was added to the supernatant liquid to bring it to pH 4·6. The barium salt was centrifuged off, washed with water, once with 50% ethanol, twice with 95% ethanol, and once with ether, and dried in a vacuum at room temperature over phosphoric oxide. Yield, 1·8—2·0 g. Enzyme tests by the method of Bailey (*loc. cit.*) indicated a composition (expressed as % of 7-minute phosphorus) : adenosine triphosphate 37 molar %; adenosine diphosphate 23 molar %; inorganic pyrophosphate 18 molar %.

Preparation of Acridinium Salts from Crude Barium Adenosine-5′ Triphosphate.—To the crude barium salt (1·125 g.) suspended in water (2 c.c.), dilute sulphuric acid (5·1 c.c. of N) was added. Barium sulphate was spun off and washed several times by centrifugation with small quantities of water. Acridine (0·705 g.), dissolved in a little warm ethanol, was added to the collected supernatant liquids, and the mixture of acridine salts warmed to solution (total vol. *ca.* 30 c.c.). Fractional crystallisation yielded a small amount of an egg-yellow *triacridinium adenosine-5′ triphosphate*, m. p. 209° (decomp.), from the middle fractions (Found in material dried overnight at room temperature/1 mm. over phosphoric oxide : N, 10·2; P, 9·0. $C_{10}H_{16}O_{13}N_5P_3,3C_{13}H_9N$ requires N, 10·7; P, 8·9; a monohydrate requires N, 10·5; P, 8·8%). Wagner-Jauregg (*loc. cit.*) reports a hexahydrate of the triacridinium salt obtained by drying over calcium chloride in a vacuum, but gives incorrect calculated figures for this compound. 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 triphosphate prepared from this acridinium salt gave ratios $P_{total}/P_{labile} = 1·505$, and $P_{labile}/P_{stable} = 1·98$; the calculated ratios for adenosine triphosphate are 1·5 and 2·0, respectively. Inorganic phosphate was absent. These and all other phosphorus determinations recorded in this paper were carried out by the colorimetric method of Allen (*Biochem. J.*, 1940, **34**, 858). The least soluble fraction yielded a lemon-yellow *acridinium* salt, m. p. 218° (decomp.), identical with that obtained from authentic natural barium adenosine triphosphate by Wagner-Jauregg's method (using 3·2 mols. of acridine) (Found : N, 11·5; P, 9·9; P_{total}/P_{labile}, 1·59. $2C_{10}H_{16}O_{13}N_5P_3,5C_{13}H_9N$ requires N, 11·0; P, 9·8%; P_{total}/P_{labile}, 1·5). This salt differs in solubility and m. p. from the substance $1·2C_{10}H_{16}O_{13}N_5P_3,2C_{13}H_9N,4H_2O$ reported by Wagner-Jauregg (*loc. cit.*). The action of adenosine triphosphatase on sodium salts from both the synthetic and the natural acridinium salts of m. p. 218° indicated high purity. Inorganic phosphate was absent from both.

Purification of Crude Barium Adenosine-5′ Triphosphate via *the Mercury Salt.*—To the crude barium salt (1·5 g., purity *ca.* 40%) in water, dilute sulphuric acid (7·5 c.c. of N) was added, barium sulphate spun off and washed well by centrifugation, and the supernatant liquids treated with acridine (0·96 g.) in ethanol to remove pyrophosphate as the very soluble acridine pyrophosphate. After standing overnight at 0°, the deposited solid (0·54 g., m. p. *ca.* 205°; enzyme assay 58% adenosine triphosphate, 16% adenosine diphosphate) was filtered off, washed with a little water, and suspended in water (100 c.c.). N/10-Sodium hydroxide was then added until the yellow colour had completely disappeared (pH 6), and the acridine removed by 3 successive washings with ether. The solution was then acidified to pH 1 with nitric acid, and mercuric nitrate solution (34·6 g. in 12·5 c.c. of water + 12·5 c.c. of concentrated nitric acid) added, drop by drop, the precipitate being allowed to settle each time, until no more was produced (0·5 c.c.). The mercuric salt was spun off, washed with water, and then suspended in water (100 c.c.) and cooled in ice, and hydrogen sulphide passed through for 45 minutes. Mercuric sulphide was spun off, re-suspended in water (25 c.c.), hydrogen sulphide passed for a further 15 minutes, and the mixture again centrifuged. To the combined supernatant liquids barium acetate solution was added to pH 5 (10 c.c. of 20%), and the precipitate of barium salt centrifuged off and dissolved in cold hydrochloric acid (*ca.* 40 c.c. of N/10). Insoluble material was spun off, and the barium salt reprecipitated by addition of barium acetate solution (1 drop) and sodium hydroxide (3 c.c. of N) to pH 4, centrifuged, redissolved in hydrochloric acid (10 c.c. of N/10), insoluble material again spun off, and barium acetate solution (0·6 c.c.) added to bring the solution to pH 4·2. The precipitated barium salt was collected and again redissolved in cold hydrochloric acid (N/10), and finally precipitated at pH 6 by addition of barium acetate and sodium hydroxide (N/10), washed twice with water, once with 50% ethanol, twice with 95% ethanol, and once with ether, and dried in a vacuum at room temperature over phosphoric oxide (0·18 g.; Enzyme assay, 80% adenosine triphosphate) (Found : N, 7·8; P, 10·5%; P_{total}/P_{labile}, 1·5. $C_{10}H_{12}O_{13}N_5P_3Ba_2,6H_2O$ requires N, 7·9; P, 10·5%; P_{total}/P_{labile}, 1·5). Inorganic phosphate was absent.

Triacridinium Adenosine-5′ Triphosphate.—To the above purified barium salt (0·082 g.) in water

(0·15 c.c.) dilute sulphuric acid (0·396 c.c. of N) was added, barium sulphate spun off and washed thoroughly, acridine (0·054 g., 3·2 mol.) in a little ethanol added to the collected supernant liquids, and the crude acridine salt recrystallised twice from water. The pure triacridinium salt formed egg-yellow needles, m. p. 208—209° (decomp.) (Found in material dried over phosphoric oxide at 0°/1 mm. for 12 hours: N, 10·4; P, 8·9; P_{total}/P_{labile}, 1·5; Inorganic P, nil. $C_{10}H_{16}O_{13}N_5P_3,3C_{13}H_9N,H_2O$ requires N, 10·5; P, 8·8%; P_{total}/P_{labile}, 1·5). The salt was hygroscopic. It is formulated as a monohydrate on the basis of its nitrogen and phosphorus content, which can be determined accurately. Carbon and hydrogen determinations are known to be unreliable in the adenosine triphosphate series, the difficulty of combustion leading to low results. In the present case the values obtained (C, 52·2; H, 4·9) correspond more closely with a tetrahydrate (C, 52·7; H, 4·6), but we prefer the monohydrate formulation in the circumstances. Confirmation of purity was also provided by enzymic assay with adenosine triphosphatase, which indicated that the material regenerated from the synthetic triacridinium salt contained at least 90% of adenosine triphosphate. A value of 100% is unlikely to be achieved from the nature of the enzyme test employed. The synthetic salt was identical in m. p. and had undepressed mixed m. p. with the triacridinium salt (Found: N, 10·4%) prepared from authentic natural barium adenosine triphosphate, or by treatment of the acridinium salt, m. p. 218°, of natural adenosine triphosphate with excess of acridine and recrystallising twice from water.

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