522. *Nucleotides. Part IV. A Novel Synthesis of Adenosine Triphosphate.*

By A. M. Michelson and A. R. Todd.

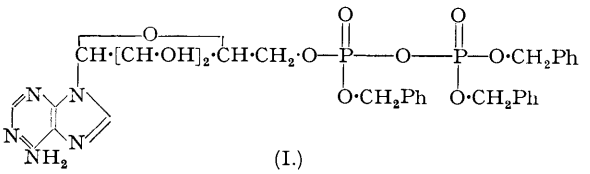
Condensation of dibenzyl chlorophosphonate with disilver adenosine-5′ phosphate, followed by removal of benzyl groups by hydrogenation, yielded adenosine-5′ triphosphate (IV), identical with the natural adenosine triphosphate obtained from muscle and not, as had been expected, the isomeric ester (VII). The reasons for formulating natural adenosine triphosphate as (IV) are discussed and its production by the synthesis described is attributed to the isomerisation of (VII) through the anhydro-derivative adenosine-5′ trimetaphosphate (VIII).

In Part II of this series (Baddiley, Michelson, and Todd, this vol., p. 582) a synthesis of the co-phosphorylase adenosine triphosphate (IV) was described. This synthesis depended on the selective monodebenzylation of adenosine tribenzyl pyrophosphate (I) (Baddiley and Todd, *J.*, 1947, 648) by reaction with 4-methylmorpholine, condensation of the resulting adenosine dibenzyl pyrophosphate (II) in the form of its silver salt with dibenzyl chlorophosphonate, and removal of the protecting benzyl groups from the initially formed adenosine tetrabenzyl triphosphate (III). The validity of formulating the adenosine dibenzyl pyrophosphate intermediate as essentially (II) and not (V) has already been discussed (Baddiley, Michelson, and Todd, *loc. cit.*) but the possibility remained that some of the isomeric ester (V) might have been formed; if so, the further steps in the synthetic route used would have produced a certain amount of an isomeric adenosine triphosphate (VII) by way of the intermediate tetrabenzyl ester (VI). Although we found no evidence for the presence in our final product of such an isomeric compound, it was possible that this was due to its presence in such small amount that it had escaped detection. In any case, the isomeric adenosine triphosphate (VII) was of sufficient interest to warrant an attempt at its synthesis by unambiguous methods. It should contain labile phosphate groupings just as does the natural compound (IV) and it would be of considerable interest to study its behaviour in biological systems where adenosine triphosphate normally functions. The present paper records our experience in endeavouring to synthesise (VII).

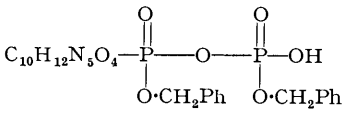
The disilver salt of muscle adenylic acid (adenosine-5′ phosphate) was condensed with an excess of dibenzyl chlorophosphonate. Silver chloride was precipitated and the amorphous product, which one would expect to have been (VI) or a mixture of this with partly debenzylated material (cf. the analogous case of adenosine tribenzyl pyrophosphate discussed by Baddiley, Michelson, and Todd, *loc. cit.*), was subjected to catalytic hydrogenation in the usual manner to remove the protecting benzyl groups. The final product was purified *via* its barium salt and isolated in a pure condition as its acridinium salt. This proved to be identical with the triacridinium salt of natural adenosine triphosphate (IV). Identity was established by direct comparison with a sample of the synthetic triacridinium salt of (IV); m. p., mixed m. p., *X*-ray powder photographs, infra-red absorption spectra, and biological activity of the regenerated free acid were all identical for the two materials. The yield of adenosine triphosphate (IV) obtained by this synthesis was, moreover, more than twice that obtained by the original procedure of Baddiley, Michelson, and Todd (*loc. cit.*).

The decisive evidence that natural adenosine triphosphate, and hence the synthetic product, has structure (IV), *i.e.*, that it is adenosine-5′ P^1-triphosphate, comes from the careful titrimetric studies of Lohmann (*Biochem. Z.*, 1932, **254**, 381) who showed that it exhibits three primary and one secondary phosphoryl dissociations. Although no triphosphate analogous to (VII) (adenosine-5′ P^2-triphosphate) is known, it can be assumed with reasonable certainty that such a compound, if it were obtained, would show two primary and two secondary dissociations. This view is supported by the titration data for pyrophosphoric acid ($K_1 = 0{\cdot}14$; $K_2 = 0{\cdot}011$; $K_3 = 0{\cdot}21 \times 10^{-6}$; $K_4 = 0{\cdot}406 \times 10^{-9}$ at 18°) to which (VII) containing no acidic hydroxyl on P^2 bears a structural analogy. Structure (IV) for adenosine triphosphate is also supported by the formation of a triacridinium salt—again indicative of three primary dissociations. Moreover, the crude barium salt precipitated from the hydrogenation solution in the attempted synthesis of (VII) contained, in addition to barium adenosine triphosphate, some 30% of barium pyrophosphate. Pyrophosphate could not arise from breakdown of (VII), although it could be readily derived from (IV); nor could it have been produced as a by-product in the condensation of disilver adenylate with dibenzyl chlorophosphonate, since both pyrophosphoric acid and its benzyl esters are soluble in ether, and the crude reaction

mixture was poured into a large volume of ether to precipitate (V). Our conclusion is therefore that adenosine triphosphate, prepared from muscle, by the synthesis of Baddiley, Michelson, and Todd (*loc. cit.*) or by the method described in this paper, has structure (IV).



(I.)



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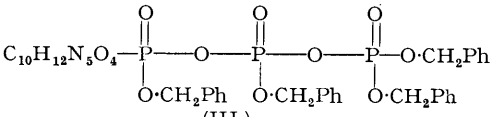

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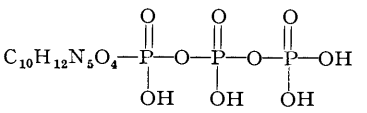
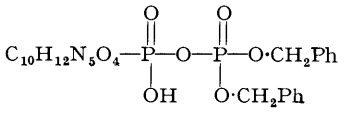
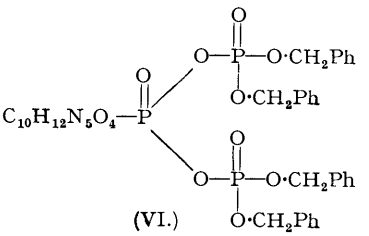


(IV.)

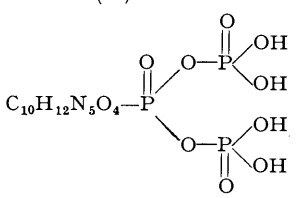


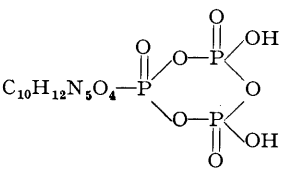
(V.)



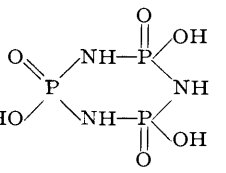
(VI.)

(VII.)

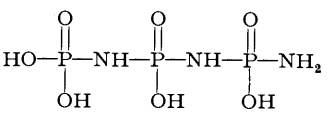




(VIII.)



(IX.)



(X.)

The production of (IV) by the synthesis here described must involve a rearrangement at some stage. In our view the most probable explanation is that the initially formed (VII) undergoes rearrangement *via* a cyclic anhydro-form (VIII). It is, of course, also possible that formation of the cyclic form occurs in a partly debenzylated material and that after removal of the remaining benzyl groups the product (VIII) passes on hydration or on salt formation into (IV). Cyclic structures have frequently been postulated for polyoxy-acids of phosphorus and in certain cases at least such structures seem to be well established. The hypothetical intermediate (VIII) is simply the adenosine-5′ mono-ester of trimetaphosphoric acid if we accept for that acid the structure corresponding to its trisodium salt (Treadwell and Leutwyler, *Helv. Chim. Acta*, 1938, **21**, 1450; Rudy and Schloesser, *Ber.*, 1940, **73**, 484). Whether the initial product formed from trimetaphosphates in acid solution is the linear triphosphoric acid does not seem to have been established, but it seems reasonable to suppose that it is; further slow hydrolysis yields orthophosphate and pyrophosphate (cf. Beans and Kiehl, *J. Amer. Chem. Soc.*, 1927, **49**, 1878.) A rather similar state of affairs is found in the metaphosphimino-acids. According to Stokes (*Z. anorg. Chem.*, 1899, **19**, 36) the trimeric acid has the cyclic structure (IX) and undergoes hydrolysis to the linear structure (X). Cyclic polymetaphosphates may, indeed, be intermediate products in some other recorded syntheses of polyphosphates—for example, in the co-carboxylase syntheses of Weiljard (*J. Amer. Chem. Soc.*, 1942, **64**, 2279) and of Karrer and Viscontini (*Helv. Chim. Acta*, 1946, **29**, 711), and in the aneurin triphosphate

synthesis reported by Velluz, Amiard, and Bartos (*Bull. Soc. chim.*, 1948, 871). In all these cases the phosphorylating agents used undoubtedly contained polymetaphosphoric acids.

Our findings in the present work prompt the question whether such a compound as (VIII) plays any rôle in biological systems commonly associated with adenosine triphosphate. Although evidence is lacking as yet, it would seem not unreasonable to suppose that it may; evidence on this might well be obtained by examining the behaviour of synthetic adenosine triphosphate containing a radioactive phosphorus atom at P^2 or P^3 in biological systems. Lipmann (*Adv. Enzymology*, 1941, **1**, 99) has already suggested that the increase in stability consequent upon ring-closure to such a structure would have a large effect on the intramolecular energy distribution and might well have a bearing on the problem of the utilisation of " energy-rich " phosphate bonds in muscular contraction. It is of some interest in this connexion that inorganic metaphosphates have been isolated from yeast (Wiame, *J. Biol. Chem.*, 1949, **178**, 919), *Neurospora* (Houlahan and Mitchell, *Arch. Biochem.*, 1948, **19**, 257), and other micro-organisms (Mann, *Biochem. J.*, 1944, **38**, 339, 345) and that Umschweif and Gibaylo (*Acta Biol. Exper.*, 1937, **11**, 6; *Compt. rend. Soc. biol.*, 1937, **125**, 275; see also *Z. physiol. Chem.*, 1937, **246**, 163) found that alkaline hydrolysis of adenosine triphosphate from both muscle and yeast liberated a compound behaving like metaphosphate, in addition to pyrophosphate.

A point of practical interest from our experiments is that the crude barium adenosine triphosphate isolated as initial product in the synthesis always contained large amounts of inorganic pyrophosphate. As the latter almost certainly arose through the breakdown of the triphosphate, it is possible that a careful study of methods of isolation and purification might lead to a marked increase in the yield of isolated adenosine triphosphate.

Experimental.

Preparation of Crude Barium Adenosine-5′ Triphosphate from the Reaction Product of Dibenzyl Chlorophosphonate and Disilver Adenosine-5′ Phosphate.—To a solution of disilver adenosine-5′ phosphate (2 g., dried for 12 hours at 80°/1 mm.) in phenol (90 g.) at 60°, dibenzyl chlorophosphonate (prepared from 5 g. of dibenzyl phosphite, according to Atherton, Openshaw, and Todd, *J.*, 1945, 382) and methyl cyanide (10 c.c.) were added. The mixture was maintained at 60° for 5 minutes with exclusion of moisture and then poured into dry ether (500 c.c.). The precipitated solid was filtered off, washed well with ether, dried under reduced pressure at room temperature, and dissolved in aqueous dioxan (200 c.c. of 50%). Silver chloride was removed by filtration through Hyflo supercel, 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, catalyst was removed by filtration, and N-sodium hydroxide and barium acetate solution were added to pH 6·5. The precipitated barium salt was centrifuged off, washed with water, dissolved in cold hydrochloric acid (N/10), and centrifuged from insoluble matter, and N-sodium hydroxide solution added to pH 5. The collected barium salt was again dissolved in cold hydrochloric acid (N/10) and the barium salt reprecipitated by adding two volumes of absolute ethanol. Water, barium acetate (20%), and N-sodium hydroxide solution were added to the collected barium salt (which contained a certain amount of acidic monobarium adenosine triphosphate) to pH 6·5, and the barium salt was centrifuged off, washed twice with water, once with 50% ethanol, twice with 95% ethanol, and once with ether, and dried under reduced pressure at room temperature over phosphoric oxide (yield, 1·5 g.). Enzymic assay (by Dr. K. Bailey, School of Biochemistry, Cambridge) indicated *ca.* 30% of inorganic pyrophosphate and 40—45% (slight variation in successive runs) of an adenosine triphosphate split by myosin adenosine-triphosphatase.

Preparation of Pure Salts of Adenosine-5′ Triphosphate from the Crude Barium Salt.—To the above crude barium salt (2·2 g.) suspended in water (5 c.c.), dilute sulphuric acid (11 c.c. of N.; check with rhodizonic acid) was added with vigorous shaking. Barium sulphate was spun off and washed several times by centrifugation with small quantities of water. Acridine (1·5 g.), dissolved in a little warm ethanol, was added to the collected supernatant liquids, and the mixture of acridinium salts warmed to dissolution (total volume, *ca.* 50 c.c.), filtered and set aside at 0° overnight. The deposited solid *A* [1·0 g.; m. p. 200—204° (decomp.)] was collected, and the mother liquors were taken to small volume under reduced pressure, whereupon crystalline acridinium pyrophosphate [0·28 g.; m. p. 258° (decomp.)] separated. This was removed by filtration, ethanol (40 c.c.) added to the filtrate, and the precipitated acridinium salt (0·673 g.), which contained considerable quantities of the acridinium salt of adenosine triphosphate, collected and recrystallised from water (in which it was now only moderately soluble), giving *B* (0·34 g.; darkens at 190—200°, blackens and shrinks at 205°). The solids *A* (recrystallised from water) and *B* were combined (*ca.* 1·1 g.) and suspended in water, and N-sodium hydroxide solution was added until the yellow colour of the solution had completely disappeared. Liberated acridine was removed by washing with ether, and nitric acid was added to the solution to give a final acid concentration of N/10. Mercuric nitrate solution (Lohmann reagent) was now added, drop by drop, until precipitation was complete. The mercuric salt was spun off, washed with water, and decomposed at 0° in the usual way. To the combined supernatant liquids, after removal of mercuric sulphide, barium acetate solution (20%) and N-sodium hydroxide were added to pH 6, the barium salt was spun off, dissolved in cold hydrochloric acid (N/10), and centrifuged from insoluble matter, and N-sodium hydroxide and barium acetate solution (1 drop) were added to pH 5. The barium salt was

again spun off, washed with water, redissolved in cold hydrochloric acid (N/10), reprecipitated by adding two volumes of absolute ethanol, and collected by centrifugation. The salt was stirred with a mixture of aqueous sodium hydroxide and barium acetate solution (pH 6·5), then spun off, washed twice with water, once with 50% ethanol, twice with 95% ethanol, and once with ether, and dried at room temperature (yield, 0·5 g.; enzymic assay, *ca.* 75% of adenosine triphosphate).

Triacridinium Salt.—The above barium salt (0·39 g.), suspended in water (1 c.c.), was treated in the usual way with sulphuric acid (1·88 c.c. of N.), acridine (0·26 g.) in a little warm ethanol added to the combined supernatant liquids, and the acridinium salt recrystallised from water (0·35 g.); it had m. p. 209° (decomp.), undepressed in admixture with the acridinium salt of natural adenosine triphosphate. *X*-Ray powder photographs of the two specimens were identical, as also were the infra-red absorption spectra (Found, in material dried for 24 hours over phosphoric oxide at room temperature/1 mm.: N, 10·7; P, 8·7%; P total/P labile = 1·54, 1·51. Calc. for $C_{10}H_{16}O_{13}N_5P_3,3C_{13}H_9N,H_2O$: N, 10·5; P, 8·8%; P total/P labile = 1·50). Enzymic assay, >80% of adenosine triphosphate.

Dibarium Salt.—The above acridinium salt (20 mg.) was dissolved in aqueous sodium hydroxide (N/10), and the acridine removed by extraction with ether. To the filtered solution barium acetate (20%) was added and the dibarium salt spun off, washed thrice with water, twice with ethanol, and once with ether, and dried (Found: N, 7·6%; P total/P labile = 1·50. Calc. for $C_{10}H_{12}O_{13}N_5P_3Ba_2,6H_2O$: N, 7·9%; P total/P labile = 1·50).

Grateful acknowledgment is made to the Department of Scientific and Industrial Research for a Maintenance Allowance (to A.M.M.) and to Roche Products Ltd. for gifts of material. Our thanks are also due to Dr. K. Bailey for enzymic tests, and to Dr. N. Sheppard and Mr. A. Vallance-Jones for infra-red data.

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