The Preparation of Adenosine 5'-Pyrophosphate by a Non-Enzymic Method

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1. A non-enzymic method for the preparation of adenosine 5'-diphosphate is described, in which the terminal phosphate of adenosine 5'-triphosphate is transferred to methanol in the presence of hydrochloric acid. The final purified product can be obtained in 60% yield. 2. Experiments with [^{14}C]methanol showed that no methylation of the adenosine diphosphate occurs during the reaction. 3. Confirmation that the pyrophosphate moiety of the adenosine diphosphate produced was in the 5'-position was obtained by: (a) periodate oxidation; (b) treatment with apyrase and examination of the resulting adenylic acid isomer by paper chromatography. 4. The method appears to be generally applicable to the preparation of nucleoside 5'-diphosphates from the corresponding nucleoside 5'-triphosphates.

Two established methods have been used for the preparation of adenosine 5'-pyrophosphate (ADP) from ATP. In both of these a partially purified adenosine triphosphatase from lobster tail (Lohmann, 1935) or rabbit muscle (Bailey, 1942) is used to hydrolyse ATP, and the ADP is then isolated from the incubation medium.

A recent observation that the terminal phosphate moiety of ATP can readily be transferred to methanol in the presence of hydrochloric acid catalyst (Eichberg & Dawson, 1964) suggested an alternative non-enzymic method for the preparation of ADP. The present paper describes such a method and provides evidence that no phosphoryl migration or methylation occurs during the reaction.

METHODS

Preparation of ADP. Both the sodium and barium salts of ATP could be used to prepare ADP. The disodium salt of ATP (0.6 g.) was dissolved in 5 ml. of water and the solution slowly added with stirring to 410 ml. of methanol containing 1.05 ml. of aq. 11N-HCl. The barium salt of ATP (0.6 g.) was dissolved in 5 ml. of 2.5N-HCl, and a solution of 0.24 g. of Na_2SO_4 (anhydrous) in 10 ml. of water was slowly added, followed by 500 ml. of methanol. Either mixture was heated at 50° for 2 hr. with occasional swirling. The initial cloudy precipitate cleared, leaving a more granular precipitate that did not redissolve. After being cooled, the methanolic solution was adjusted to approx. pH 7 with aq. NH_3 solution (sp.gr. 0.88) and concentrated to dryness

on a rotary evaporator. Water (50 ml.) was added and the solution adjusted to pH 7.0 with aq. NH_3 solution. The ADP was then precipitated as the barium salt and purified with Lohmann's reagent, as described by LePage (1950a). When the barium salt of ATP had been used as the starting material, care was taken to ensure that sufficient barium acetate was added to precipitate any excess of SO_4^{2-} ions as well as ADP. The BaSO_4 precipitate was removed after the barium salt of ADP had been dissolved in 0.2N- HNO_3 before precipitation by Lohmann's reagent. The dried barium salt of ADP was obtained in a yield of about 60%. It was converted into the sodium salt by double decomposition with the calculated amount of Na_2SO_4 (LePage, 1950b).

Ionophoresis and chromatography. High-voltage ionophoresis (80 min. at 50 v/cm. and pH 4) was carried out in the acetate buffer system described by Heald (1956). Ascending paper-chromatographic separations were performed in the following solvents: *A*, propan-1-ol-aq. NH_3 (sp.gr. 0.88)-water (11:7:2, by vol.) (Baddiley, Buchanan & Letters, 1958); *B*, saturated ammonium sulphate-0.1M-ammonium acetate-propan-2-ol (79:19:2, by vol.) (Markham & Smith, 1951); *C*, aq. 5% Na_2HPO_4 -isopentanol (two-phase system) (Carter, 1950); *D*, water-saturated phenol-ethanol-acetic acid (50:6:5, by vol.) (Dawson, Hemington & Davenport, 1962).

Nucleotides were located by inspection under ultraviolet light, or by photography with reflex paper if necessary (Markham & Smith, 1949). Phosphorus-containing compounds were detected by means of an acid molybdate spray followed by irradiation in ultraviolet light. Radioactive components were located by the Nuclear-Chicago Actiograph strip-scanner or by radioautography with X-ray film.

Dephosphorylation of ADP with apyrase. Purified apyrase

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was prepared from new-season potatoes by the method of Krishnan (1949) and used to hydrolyse the ADP prepared as described above. The barium salt of ADP (5mg.) was dissolved in 1 ml. of 0.02 *N*-acetic acid, and 1 ml. of 0.188 *M*-sodium acetate was added to buffer the system. Then 0.5 ml. of 0.5% CaCl_2 and 0.5 ml. of the purified enzyme solution were added, and the mixture was incubated at 30° long enough to liberate all the labile phosphate (10–20 min.). The reaction was stopped by adding 0.5 vol. of 20% (w/v) trichloroacetic acid solution. After removal of the precipitated protein by centrifuging, the supernatant was washed four times with an equal volume of diethyl ether to remove excess of trichloroacetic acid. The AMP was then separated by preparative strip electrophoresis, located by inspection in ultraviolet light and eluted (Dawson & Dittmer, 1961).

Periodate oxidation of ADP. To achieve satisfactory ionophoresis of the reaction mixture after periodate oxidation, it was found necessary to convert the prepared barium salt into the sodium salt. The solution of the disodium salt of ADP (2.4 mg. in 0.8 ml.) was adjusted to pH 4, and 0.2 ml of 0.03 *M*- KIO_4 was added. The reaction mixture was incubated for 30 min. at room temperature and then treated with 0.05 ml. of ethylene glycol to remove excess of periodate. The dialdehyde formed was then treated with monomethylamine (Khym & Cohn, 1961) by adding 1 ml. of 0.04 *M*-monomethylamine hydrochloride in 0.2 *M*-2-amino-2-methylpropane-1,3-diol (pH 9.6). After 1 hr. at room temperature the solution (pH 9) was acidified to pH 5 with HCl to decompose the addition product. The inorganic pyrophosphate formed was identified by direct paper ionophoresis of 0.4 ml. of the reaction mixture at pH 4 ($M_{\text{P}} 1.25$).

Estimation. Phosphorus was determined by the method of Fiske & Subbarow (1925). Spots on chromatograms were initially wet-digested with HClO_4 (Dawson *et al.* 1962).

Materials. [^{14}C]Methanol (0.31 mg., 0.1 mc) was obtained from The Radiochemical Centre, Amersham, Bucks., as a vapour in a sealed ampoule. It was dissolved in 0.5 ml. of unlabelled carrier methanol by a standard vacuum-line technique.

Phosphate esters were obtained from the following commercial firms: monomethyl phosphate and adenosine 5'-monophosphate (also the 2'- and 3'-isomers) (L. Light and Co. Ltd., Colnbrook, Bucks.); ATP (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany); ADP (Sigma Chemical Co., St Louis, Mo., U.S.A.).

RESULTS AND DISCUSSION

Table 1 shows the results of experiments in which ATP was incubated at various temperatures and times with aqueous and methanolic hydrochloric acid. More extensive decomposition at a given temperature always occurred in methanolic hydrochloric acid. The transphosphorylation from ATP to methanol was a much faster reaction than the subsequent decomposition of ADP to AMP. By choosing suitable conditions of time and temperature the ATP could be converted into ADP in excellent yield with only minimal further dephosphorylation.

ADP prepared in this way on a macro-scale can be separated by precipitation with barium acetate from monomethyl phosphate as well as any AMP produced, as these compounds have soluble barium salts. The further purification of ADP by precipitation of its mercuric salt with Lohmann's reagent removed any inorganic phosphate.

Purity of the prepared ADP. On examination of the prepared ADP by ionophoresis at pH 4, virtually all the phosphorus migrated as a single spot with a mobility identical with that of an ADP marker ($M_{\text{P}} 0.57$). Traces of ribose monophosphate and inorganic phosphate were present.

When the sodium salt of the prepared ADP was hydrolysed with *N*-hydrochloric acid (8 min. at 100°), 49% of the total phosphate was converted into inorganic phosphate. The ionophoretic pattern of the acid-hydrolysis products was exactly equivalent to that obtained with authentic ADP, the AMP released being nearly all further hydrolysed to the sugar phosphates (mainly ribose monophosphate).

Evidence for the lack of methylation of prepared ADP. The prepared sodium salt was hydrolysed in *N*-hydrochloric acid (8 min. at 100°). The hydrolysate was then examined by chromatography in phenol-acetic acid-ethanol-water followed by ionophoresis at pH 4, and no monomethyl phosphate was observed. The latter might have been expected

Table 1. *Products formed on the acid hydrolysis of ATP (disodium salt) under various conditions*

Hydrolysis was always carried out in the presence of 0.25% (v/v) of aq. conc. HCl.

Conditions of hydrolysis			Products (% of total phosphate)			
Temp.	Time (hr.)	Medium	ATP	ADP	AMP	Inorganic phosphate + monomethyl phosphate
—	No hydrolysis	—	97	14	—	1
Room	16	Aqueous	77	14	—	8
Room	16	Methanolic	11	50	5	34
37°	4	Methanolic	8	39	6	47
50	1	Methanolic	16	44	4	36
50	2	Aqueous	86	12	—	2
50	2	Methanolic	1	57	5	38
50	4	Methanolic	—	46	12	42

if some of the terminal phosphate of the ADP had become esterified while in contact with the methanol-hydrochloric acid mixture, as monomethyl phosphate is quite stable to acid hydrolysis (Eichberg & Dawson, 1964).

To confirm this point and in addition to test for methylation of the adenosine portion of the molecule, the reaction was conducted in the presence of [^{14}C]methanol and the reaction mixture directly separated by ionophoresis. No radioactivity associated with the ADP could be detected either by examining the strip with a flow-counter scanner or after radioautography for 10 days. A highly-labelled monomethyl phosphate spot (M_R 1.02) and also a minor phosphorus-free radioactive component with M_R 0.06 were present. The AMP spot also contained a trace of radioactivity.

Position of the pyrophosphate moiety in prepared ADP. It was necessary to exclude the possibility that migration of one or both phosphate moieties from the 5'-position of the ribose moiety had taken place. The occurrence of such migrations during the acid and alkaline hydrolysis of nucleotides (Baddiley *et al.* 1958; Lipkin, Markham & Cook, 1959) are well-documented phenomena.

Adenosine 5'-pyrophosphate is decomposed on reaction with periodate by oxidation of *cis*-diol groups at the 2'- and 3'-positions on the ribose ring (Lipkin *et al.* 1959). When ADP was oxidized with periodate, the resulting dialdehyde pyrophosphate migrated on ionophoresis to exactly the same position as unchanged ADP. However, when the dialdehyde formed was treated with monomethylamine and the resulting product decomposed by acid (Khym & Cohn, 1961), the pyrophosphate and orthophosphate formed were readily separable from ADP by ionophoresis. When this procedure was applied to samples of prepared and authentic ADP, examination of the resulting reaction mixture by ionophoresis showed that both had been completely decomposed and pyrophosphate and orthophosphate produced. This indicated that, in the prepared ADP, the 2'- and 3'-positions were not esterified and that all the pyrophosphate was at the 5'-position.

This result was confirmed by enzymic hydrolysis of the prepared ADP with purified apyrase. The AMP in the incubation mixture was isolated by preparative ionophoresis and examined by paper chromatography in solvent systems (*A*, *B* and *C*; see the Methods section) that separate adenosine 5', 3'- and 2'-monophosphate. Two incubation conditions were chosen: (1) when the ADP had almost completely disappeared; (2) soon after the reaction had gone to completion. The AMP isolated was esterified in the 5'-position, showing that the prepared ADP was all adenosine 5'-pyrophosphate.

ENVOY

The present method provides a simple means of preparing adenosine 5'-pyrophosphate from the barium or sodium salt of ATP without the tedium of making enzyme preparations. It is possible that an equivalent method could be devised based on the catalytic hydrolysis of ATP by metal ions (Tetas & Lowenstein, 1963); with Cd^{2+} a particularly favourable ADP/AMP ratio results. However the reaction is comparatively slow, and it would have to be ascertained that no phosphoryl migration occurs. Bielschowsky (1950) in fact described a method of preparing ADP in which a solution of the magnesium salt of ATP adjusted to pH 4.5 was heated at 100°. We have tried to repeat this method twice without success. Conversion into ADP was minimal and considerable AMP was also formed. It is to be expected that a limited amount of phosphoryl migration would occur even at this pH (Baddiley *et al.* 1958).

Experiments with CTP and GTP showed that an analogous transphosphorylation takes place in methanolic hydrochloric acid. CDP and GDP were the predominant products shown by ionophoresis to be formed on heating these nucleoside triphosphates in methanolic hydrochloric acid at 50° for 2 hr.; only minimal amounts of nucleoside monophosphates were present. It seems reasonable to conclude that this reaction could be used to prepare many nucleoside pyrophosphates from the corresponding triphosphates.

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