Preparation of ^{32}P -labelled Adenosine Triphosphate

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In experiments on the metabolism of adenosine triphosphate (ATP) it became desirable to use the ^{32}P -labelled compound. ATP labelled with ^{32}P has been isolated from the rabbit after injection of radioactive phosphorus by Dounce, Rothstein, Beyer, Meier & Freer (1948), but the specific activity of the product was too low to be of much further experimental use. Therefore a laboratory method of preparing this substance quickly in small quantities of high specific activity was elaborated. It is based on the observation that ATP readily exchanges the two terminal phosphate groups only when incubated with inorganic phosphate in respiring tissue suspensions (Krebs, Ruffo, Johnson, Eggleston & Hems, 1953). In the presence of $^{32}\text{PO}_4$ such suspensions convert added ATP in 0.0025 M solution, within about 20 min. incubation, into ATP equally labelled in the two terminal phosphate groups. The active material can be recovered as barium salt and purified by paper chromatography.

The three phosphorus atoms of adenosine triphosphate are distinguished as α , β and γ , thus

PROCEDURE

Substrate solution. This is made from 2.5 ml. of a 0.02 M solution of the sodium salt of ATP; 2.5 ml. of 0.02 M MgCl_2 ; 1.0 ml. of 0.1 M-potassium phosphate buffer, pH 7.4; 0.5 ml. of 0.5 M-sodium α -ketoglutarate; 'carrier free' $\text{KH}_2^{32}\text{PO}_4$ (about 5 mc), and water to a final volume of 11 ml.

Tissue suspension. A sheep heart collected immediately after the slaughter of the animal is transported in ice-cold 0.9% KCl to the laboratory where 50 g. are immediately blended in a Waring Blender for 2 min. with 200 ml.

0.9% KCl. Smaller quantities cannot be handled in the standard size Blender.

Incubation. A 10 ml. sample of a freshly prepared tissue suspension and 11 ml. of substrate solution are measured into a 250 ml. conical flask fitted with two glass tubes serving as gas inlet and outlet, and the mixture is shaken at 20° for 40 min. whilst a rapid stream of O_2 is blown through the flask. At the end of the incubation time 2.5 ml. of 30% (w/v) trichloroacetic acid is added and the mixture is chilled in ice. After standing for about 10 min. at 0° the precipitated protein is removed by centrifugation (10 min. at 2000 g) in a refrigerated centrifuge at -5° . The supernatant is poured into a 50 ml. conical flask and stored at 0° and the precipitated protein is extracted on the centrifuge with about 5 ml. of water. The combined supernatants are brought to pH 6.8 by the cautious addition of N-NaOH, the pH being followed with a glass electrode. Excess M-barium acetate (about 1 ml.) is added to the solution and the material is stored in a deep-freeze overnight. The purification of the Ba precipitate closely follows the procedure described by LePage (1949), the ATP being once precipitated as the Hg salt by the addition of Lohmann's reagent (100 g. $\text{Hg}(\text{NO}_3)_2$, $8\text{H}_2\text{O}$ + 25 ml. conc. HNO_3 (70% (w/w)) + 25 ml. water; the HNO_3 is added to the salt before the water) and once as the Ba salt. The latter is converted to the Na salt by solution in N-HCl and double decomposition with the calculated amount of Na_2SO_4 (0.24 ml. of 0.5 M). The resulting solution (approx. 5 ml.) is placed on a line 10 cm. long at the end of a chromatogram paper washed with ethylenediaminetetraacetic acid as described by Eggleston & Hems (1952). This is most effectively done with a Pasteur pipette drawn down to a capillary fine enough to prevent any escape of liquid unless the pipette is actually in contact with the filter paper. Cold air from a hair-dryer is blown over the surface of the filter paper to facilitate drying. The chromatogram is then developed for 1 hr. by upward paper chromatography using the ethyl ether:formic acid solvent described by Bartley (1953).

Table 1. *Distribution of phosphate and of radioactivity among ATP, ADP and orthophosphate in the solution of ^{32}P -labelled ATP*

(Duplicate 5 μl . samples of the solution were chromatographed and the paper areas corresponding to ATP, ADP and orthophosphate were wet-ashed and analysed for phosphorus by the method of Berenblum & Chain (1938), and for radioactivity.)

Substance	Activity in 5 μl . sample (counts/min.)	Phosphorus content (μg .)	Specific activity (counts/ μg . P/min.)	Calculated exchangeable phosphorus (μg .)	Calculated specific activity of exchangeable P (counts/ μg . P/min.)	Radioactivity in 1.5 ml. of ATP (counts/min.)
ATP	421 000	2.02	208 200	1.34	313 000	126 300 000
ADP	70 900	0.33	214 000	0.16	430 000	21 270 000
Orthophosphate	81 000	0.18	450 000	0.18	450 000	24 300 000

At the end of this time the paper is dried and the band of ATP is located by viewing the paper in ultraviolet light. The strip of paper containing the ATP is cut free from the rest of the paper leaving a narrow border about 2 mm. wide on each side of the band. The remaining portion of the paper is sprayed with the molybdate spray of Hanes & Isherwood (1949) to locate and confirm the separation of the inorganic P from the ATP. The paper strip bearing the ATP is cut so that one end tapers to a point and the ATP is eluted from the paper by downward chromatography with water as a solvent. Progress of the elution is followed by viewing in the ultraviolet light at intervals. Virtually the whole of the ATP is eluted in a volume of 1–2 ml., the radioactivity left on the paper being negligible. The solution is neutralized with 0.1 M-NaHCO₃ and is stored in the deep-freeze until required. The solution, about 1.5 ml., is approx. 0.004 M with respect to ATP and contains about 0.2 mc of activity: it is diluted with non-isotopic ATP for use. An analysis of the phosphate compounds in solution by a modification of the paper-chromatographic method of Eggleston & Hems (1952) and the distribution of radioactivity in the fractions is shown in Table 1. ATP, ADP and orthophosphate which had arisen by hydrolysis subsequent to the chromatographic separation, were the only phosphate-containing compounds detected, the phosphorus being distributed as follows: ATP 80, ADP 13, orthophosphate 7%. Of the total radioactivity, ATP contained 77, ADP 11, and orthophosphate 12%.

COMMENTS

(1) The analysis of the ATP used as the starting material gave the following distribution of phosphorus: ATP 68.9, ADP 20.4, pyrophosphate 4.2, orthophosphate 1.5%. The end product was considerably purer; it was free from pyrophosphate. Although it contains only 80% ATP it is purer than the best commercially available preparations tested by us, including samples marketed as 'ATP chromatographically pure'. Further purification by paper chromatography is not difficult, but is usually unnecessary in experiments on tissues or enzyme preparations.

(2) Sheep heart rather than liver or kidney was chosen as tissue because it contains less organic phosphates and other interfering substances such as flavins.

(3) To obtain good yields no large excess of the Lohmann reagent should be used and storage of the mercury precipitate should be avoided.

(4) The chromatographic separation of ATP can be carried out directly on the suspensions acidified with HCl, omitting deproteinization and the initial Ba and Hg treatment. The product obtained, however, is contaminated by salts, and probably by added substrate. This may be irrelevant in some experiments.

(5) The procedure described here may be used for the preparation of other labelled phosphate esters whose phosphate groups exchange with ATP.

(6) Solutions should be kept cold throughout the preparation (approx. 0°) and the pH carefully controlled, especially before the addition of barium acetate.

(7) The highest activity of ATP prepared enzymically depends on the resistance of the enzyme system to the radiation from the isotope. The dose emitted by 10 mc of ³²P is approximately 885 R/hr. and as estimated by Dale, Gray & Meridith (1949) a dose of 986 R will inactivate a low concentration of carboxypeptidase. By analogy it may be expected that much higher activity than that used above would damage the phosphorylating systems.

SUMMARY

1. A description is given of a simple laboratory method of preparing ³²P-labelled adenosine triphosphate ([β:γ-³²P₂] ATP). For nomenclature see introductory paragraph of this paper. It rests on the rapid exchange between inorganic phosphate and the β and γ phosphate of adenosine triphosphate by respiring heart-muscle preparations. Non-isotopic adenosine triphosphate and KH₂³²PO₄ are incubated with a heart-muscle suspension. The labelled adenosine triphosphate is successively precipitated as the barium and mercury salt and finally purified by paper chromatography.

2. The product (3.5 mg. adenosine triphosphate) contained between 0.1 and 0.2 mc radioactivity. Of the total activity 77% was in adenosine triphosphate, 11% in adenosine diphosphate and 12% in orthophosphate; of the total phosphorus 80% was in adenosine triphosphate, 13% in adenosine diphosphate and 7% in orthophosphate.

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