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A Simple Method for the Preparation of ^{32}P -Labelled Adenosine Triphosphate of High Specific Activity

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^{32}P -labelled ATP of high specific activity is not available commercially. Chemical methods for preparing it (see, for example, Lowenstein & Metzenberg, 1960) are tedious and yield a product with the ^{32}P distributed between the β - and γ -phosphate groups in uncertain proportions. Several enzymic methods have been described (see, for example, Racker, 1961), but most of them require the use of biological materials such as mitochondria or chloroplasts, which are not readily available in many laboratories and cannot be stored. An exception is the method developed by Revel and described by Penefsky, Pullman, Datta & Racker (1960), but, though this is moderately satisfactory, in our hands the yields have not been high. A combined chemical and enzymic method described by Tanaka, Mano & Shimazono (1958) is said to give a product with 92% of the radioactivity in the γ -phosphate group, but the method is elaborate and, according to its authors, unsuitable for preparing a product of high specific activity. For these reasons, we thought it worth attempting to develop a method for the preparation of ^{32}P -labelled ATP which, at the same time, was simple, required only enzymes and substrates readily available commercially, and gave a good yield of product labelled in the γ -phosphate group.

The method finally adopted makes use of the exchange reaction between inorganic phosphate and the terminal phosphate group of ATP that occurs in the presence of phosphoglycerate kinase (reaction 1), glyceraldehyde 3-phosphate dehydrogenase (reaction 2) and suitable substrates.

3-Phosphoglycerate + ATP

1,3-Diphosphoglycerate + glyceraldehyde

The equilibrium position of reaction (1) is far to the left, so that ADP will be present only in catalytic amounts. It follows that, even if the enzyme preparations contain a trace of adenylate kinase, there should be little exchange of ^{32}P between inorganic phosphate and the β -phosphate group of ATP.

METHODS

Preparation of labelled adenosine triphosphate. The following were added, in the order given, to a 10 ml. glass-stoppered tube: 5 ml. of water; 0.5 ml. of *m*-tris ('121 grade' from Sigma Chemical Co., St Louis, Mo., U.S.A.) adjusted to pH 8.0 with HCl; 0.06 ml. of *m*- MgCl_2 ; 1.02 ml. of 0.1 *N*-NaOH; 20 μmoles of cysteine (Sigma) as the free base; 60 μmoles of crystalline ATP as the disodium salt

(Sigma); 10 μ moles of 3-phosphoglycerate as the tricyclohexylammonium salt (Boehringer und Soehne G.m.b.H., Mannheim, Germany); 1 mg. of muscle glyceraldehyde 3-phosphate dehydrogenase suspended in 0.1 ml. of 2.5 M-(NH₄)₂SO₄; 100 μ g. of yeast phosphoglycerate kinase suspended in 0.05 ml. of a solution of 2.4 M-(NH₄)₂SO₄ in 0.04 M-Na₄P₂O₇, pH approx. 7. The two enzyme suspensions were supplied by Boehringer und Soehne. (The source of the glyceraldehyde 3-phosphate dehydrogenase is important, because NAD, which is present in bound form in the enzyme prepared from muscle, is necessary for the reaction.) Water was added so that the volume after the subsequent addition of ³²P would be 10 ml. The ³²P was added as 'carrier-free' inorganic phosphate in 0.01 N-HCl, as supplied by The Radiochemical Centre, Amersham, Bucks. When only a small quantity of radioactivity was required, the [³²P]phosphate was added together with about 3 μ moles of unlabelled phosphate, pH 7.4.

After incubation at 26° for 1 hr., the contents of the tube were poured into 5 vol. of ethanol. Then 10 ml. of 17% (v/v) ethanol was used to wash the tube and the washings were added to the bulk mixture. The ethanol-treated mixture, which at this stage was cloudy, was allowed to stand for a few minutes and then clarified by the addition of about 15 ml. of water. A small sample (0.1 ml.) was withdrawn so that the total radioactivity present could be determined. The mixture was transferred to a 300 ml. round-bottomed flask, and the volume reduced to 5–8 ml. on a rotary evaporator at 40°.

Separation of the labelled adenosine triphosphate. After concentration, the solution was filtered through Whatman no. 1 paper directly on to a column of approx. 1 g. of Dowex 1 resin, the column having a diameter such that its height was about 3½ cm. The filter paper was washed with 10 ml. of water and the washings were added to the column. The column was then treated with: (1) 40 ml. of a solution of 20 mM-NH₄Cl in 0.02 N-HCl; this removed traces of AMP, ADP and inorganic phosphate. (2) 40 ml. of water, to remove NH₄⁺ ions. (3) 0.25 N-HCl; this eluted the ATP. The first 14 ml. of eluate was collected in a flask immersed in ice, and M-tris base was added to give a final pH of 7.4. About 3.4 ml. of the tris was needed, and this volume was added in small measured portions as the eluate was collected. With the column that was used in these experiments, 14 ml. of 0.25 N-HCl eluted 85–90% of the total ATP present. A higher yield could have been obtained by further treatment, but only at the cost of greater dilution. After use the column was washed with 20 ml. of N-HCl, followed immediately by water until the pH of the eluate was 5.

Reagents. Unless otherwise stated, all reagents used in the present investigation were of 'analytical reagent grade'.

Assay of radioactivity. Radioactivity was measured with a conventional skirted liquid Geiger-Müller counter.

Assay of adenosine triphosphate. The ATP concentration in the final eluate was estimated from the extinction at 259 m μ ; the molecular extinction coefficient was taken to be 15.4×10^3 at pH 7.

Precautions against radiation. With small quantities of radioactivity, screens of ½ in. Perspex gave adequate protection. With more than a few millicuries (preparation 3 in Table 1), additional lead screens were necessary to decrease secondary radiation.

RESULTS AND DISCUSSION

The synthesis has been carried out three times, twice with relatively small quantities of [³²P]-phosphate and once with 100 mc. The yields are shown in Table 1. The very large quantity of radioactivity used on the third occasion was sufficient to darken the resin column and the glass vessel in which the ATP stood overnight, but it did not interfere with the enzymic reactions.

To determine the distribution of the ³²P within the ATP molecule, the γ -phosphate group was split off with 'heavy' meromyosin; the phosphate liberated was converted into phosphomolybdate and extracted into isobutanol. If all the label had been in the γ -phosphate group no radioactivity would have been left in the aqueous phase. As shown in Table 2, after a 20 min. incubation with the adenosine triphosphatase, only 2.68% of the radioactivity was left in the aqueous phase, but even after prolonged incubation between 1 and 2% was not extracted. It is not known whether this residual activity represented labelling in the β - or perhaps even the α -phosphate group, or came from an impurity that travelled with ATP on the column. The 98% of the radioactivity that was extracted must have come from the γ -phosphate group of the ATP.

Although this suggestion has not been tested, there appears to be no reason why the method should not be extended to prepare ATP labelled in

Table 1. *Preparation of ³²P-labelled adenosine triphosphate*

Experimental details are given in the text.

Preparation no.	Total ATP present (μ moles)	Total radioactivity present (mc)	Recovery of ATP (%)	Fraction of total activity in ATP (%)	Specific activity of ATP (mc/m-mole)
1	60	0.2	87.3	82.2	3.14
2	60	0.12	88.8	86.5	1.96
3	60	100	85.5	78 $\pm$ 8*	1520

* To avoid unnecessary exposure to radiation the withdrawal of a sample of the reaction mixture for the determination of total radioactivity was omitted. The value giving the percentage of total radioactivity in the ATP fraction is therefore slightly uncertain.

Table 2. *Splitting of radioactive adenosine triphosphate with 'heavy' meromyosin to determine the fraction of ^{32}P that is in the γ -phosphate group*

The incubation mixture contained in 2.1 ml.: 1 μ mole of ATP; 5 μ moles of Ca^{2+} ion; 47 μ moles of tris buffer, pH 7.8; 0.05 ml. of a solution of 'heavy' meromyosin ($E_{280\text{m}\mu}^{1\text{cm.}} = 7.6$; see Mueller & Perry, 1962). Incubation was at 25°. After the incubation, 0.5 ml. of ammonium molybdate [2.5% (v/v) in 3 N- H_2SO_4] was added and the mixture was extracted three times with 2.5 ml. portions of isobutanol.

Sample	Fraction of radioactivity left in aqueous phase after extraction with isobutanol (%)
Labelled inorganic phosphate	0.02
ATP incubated for 20 min. with 'heavy' meromyosin	2.68
ATP incubated for 60 min. with 'heavy' meromyosin	1.77
ATP incubated for 25 min. with 'heavy' meromyosin, then 1 μ mole of unlabelled ATP added and the incubation continued for a further 65 min.	1.34

both β - and γ -phosphate groups, merely by the addition of adenylate kinase, and perhaps ADP, to the basic reaction mixture described above. On

treatment with myosin the doubly-labelled ATP should yield ADP of high specific activity labelled in the β -phosphate group.

SUMMARY

1. A method is described for preparing ^{32}P -labelled ATP of high specific activity by an exchange reaction.

2. The method is simple and requires only substrates and enzymes that are available commercially.

3. Hydrolysis of the labelled ATP with 'heavy' meromyosin indicates that 98–99% of the ^{32}P is in the γ -phosphate group.

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Inhibition of Gluconeogenesis by α -Oxo Acids

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During tests for the formation of glucose from DL-methionine in kidney-cortex slices it was noted that the addition of this amino acid to the medium inhibited the formation of glucose from endogenous substrates and from added lactate or fumarate. No inhibition was observed with L-methionine and the inhibitory effect was traced to the α -oxo derivative of methionine formed in the kidney from the D-isomer by D-amino acid oxidase. The further investigation of this effect is reported in the present paper. Several but not all α -oxo acids were found to inhibit gluconeogenesis.

EXPERIMENTAL

The general procedure and the analytical methods were as described by Krebs, Bennett, de Gasquet, Gascoyne & Yoshida (1963). Checks were made to test whether the

added α -oxo acids interfered with the determination of glucose by the glucose-oxidase method. When the samples contained 4 μ moles of α -oxo acid and about 0.1–0.2 μ mole of glucose, inhibitions of colour formation of up to 20% were observed, with the standard extinction curve remaining a straight line. As the quantity of α -oxo acid present was usually much smaller the error arising from this inhibition could be neglected in most experiments. In some cases the standard sample included approximately the same amount of α -oxo acid as the unknown sample. The glucose-oxidase method was checked in several cases by the hexokinase method as described by Slein (1963) but no major difference was found.

Some α -oxo acids were prepared with the help of the D-amino acid oxidase described by Sentheshanmuganathan & Nickerson (1962). The organism was grown according to the instructions of these authors in 500 ml. of medium in 2 l. flasks. These were inoculated with two 5 ml.-test-tube cultures and incubated on a rotary shaker for 3 days at 30°. The centrifuged and washed cells (yield about 15 g.