A simple enzymic method for the synthesis of [^{32}P]phosphoenolpyruvate

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A rapid and simple enzymic method is described for the synthesis of [^{32}P]phosphoenolpyruvate from [^{32}P]P_i, with a reproducible yield of 74%. The final product was shown to be a good substrate for pyruvate kinase (EC 2.7.1.40).

For the study of metabolic reactions in which phosphoenolpyruvate acts as donor of the phosphate group, including the bacterial phosphoenolpyruvate-dependent phosphotransferase system (Kundig *et al.*, 1964), it is essential to have available [^{32}P]phosphoenolpyruvate. However, this material is not commercially available. Chemical methods such as that described by Lauppe *et al.* (1972) gave variable and low yields in my hands. The enzymic procedure of Kundig & Roseman (1971) is more reproducible, but it requires the use of biological materials, such as coupled mitochondria, which are not always readily available and which cannot be stored without loss of activity.

The method described in the present paper has the advantages that all reactions can be performed in one test tube and avoids isolation of intermediates. The enzymes used are either commercially available or are easily prepared, and can be stored for months. The method results in the conversion of [^{32}P]P_i into [^{32}P]phosphoenolpyruvate in high yields.

The overall procedure involves two stages, as follows.

(i) Synthesis of [γ - ^{32}P]GTP by an exchange reaction between P_i and the terminal phosphate group of GTP. This is a modification of the methods of Glynn & Chappell (1964) and Martin & Voorheis (1977) for the synthesis of [γ - ^{32}P]ATP. The reaction requires glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) (eqn. 1), phosphoglycerate kinase (EC 2.7.2.10) (eqn. 2) and the appropriate substrates:

(ii) Synthesis of [^{32}P]phosphoenolpyruvate by decarboxylation of oxaloacetate, catalysed by the

GTP-dependent phosphoenolpyruvate carboxykinase (EC 4.1.1.32) (eqn. 3) present in acetone-dried powder of pig liver mitochondria:

Experimental

Materials

GTP, pyruvate kinase, phosphoglycerate kinase, 3-phosphoglycerate (trisodium salt), glyceraldehyde 3-phosphate dehydrogenase and oxaloacetic acid were obtained from Boehringer (Lewes, E. Sussex, U.K.). All other reagents were of analytical grade. The [^{32}P]P_i was obtained from Amersham International (Amersham, Bucks., U.K.).

Preparation of acetone-dried powder of pig liver mitochondria

The procedure is a modification of the methods of Chang & Lane (1966) and Lane *et al.* (1969). Fresh pig liver was homogenized in cold 250 mM-sucrose/0.5 mM-EDTA (2 litres/kg of liver). The homogenate was centrifuged at 1500 *g* for 15 min in a Sorvall RC-5B centrifuge; the supernatant solution was filtered through a few layers of muslin and was then centrifuged again at 28 000 *g* for 15 min. The sedimented materials were pooled, resuspended in the above sucrose/EDTA solution and centrifuged at 28 000 *g* for 30 min. All procedures were performed at 4°C.

The pellet, containing mostly mitochondria, was

resuspended in the sucrose/EDTA solution and then added slowly, with rapid stirring, to 20 vol. of dry acetone at -5°C. The resulting precipitate was allowed to settle for a few minutes, and was then filtered on Whatman no. 1 paper. The precipitate

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was washed with dry acetone, followed by peroxide-free ether, and was dried under vacuum.

The yield of acetone-dried powder was 42 g/kg of liver. This crude source of phosphoenolpyruvate carboxykinase was stable for months at -20°C .

Synthesis of [^{32}P]phosphoenolpyruvate

(i) *Preparation of [γ - ^{32}P]GTP.* The procedure for the synthesis of [γ - ^{32}P]ATP by Martin & Voorheis (1977) was followed, but with GTP instead of ATP. The reaction was performed in a final volume of 0.5 ml. in 50 mM-Tris/HCl buffer, pH 8.0, containing dithiothreitol (1 mM), MgCl_2 (6 mM) and GTP (1 mM), with 3-phosphoglyceric acid (0.5 mM), NAD^+ (0.1 mM), glyceraldehyde 3-phosphate dehydrogenase (0.02 mg), 3-phosphoglycerate kinase (0.1 mg) and 2.0 mCi of [^{32}P]P_i. The reaction proceeded at 30°C for 15 min, after which it was stopped by boiling the mixture for 3 min.

(ii) *Preparation of [^{32}P]phosphoenolpyruvate.* The reaction mixture was cooled in ice. To it were added, to a final volume of 1 ml, Tris/HCl buffer, pH 7.5, to a final concentration of 100 mM, MnCl_2 to 1 mM, crude phosphoenolpyruvate carboxykinase preparation (0.1 ml of a suspension of 10 mg of the acetone-dried powder/ml of 50 mM-Tris/HCl buffer, pH 7.5, clarified by centrifugation at 10000 g for 5 min), oxaloacetate to 5 mM and water. The mixture was incubated at 30°C for 15 min with gentle stirring. The proteins were denatured by boiling the mixture for 3 min and removed by centrifugation at 1000 g for 10 min.

Results and discussion

The overall reaction was conducted over a range of quantities of [^{32}P]P_i and GTP, and of volumes of incubation mixture, to optimize the yield of labelled phosphoenolpyruvate. Although the fraction of the added [^{32}P]P_i that appeared in the intermediate GTP (82%) and in the final end product phosphoenolpyruvate (74%) was independent of these variables, it is clear that some balance must be struck between the objectives of maximum yield of phosphoenolpyruvate and maximum specific radioactivity. Phosphoenolpyruvate can only be formed in stage (ii) in amounts stoichiometric with the amounts of GTP present; however, the more GTP is added in stage (i), the lower will be the final specific radioactivity of the phosphoenolpyruvate. The quantities of reagents used in the procedure described in the present paper yielded 450 nmol of phosphoenolpyruvate at a specific radioactivity of 3240 Ci/mol when 2 mCi of [^{32}P]P_i and 500 nmol of GTP were used.

The incorporation of [^{32}P]P_i initially into [γ - ^{32}P]GTP and finally into [^{32}P]phosphoenolpyruvate was followed by analysing samples from the incu-

bation mixture, by t.l.c. on PEI-cellulose (polyethyleneimine-impregnated cellulose) plates, at various times throughout the process. The plates were developed in 2 M-sodium formate buffer, pH 3.4, for 2 h and radioautographed. Fig. 1 shows that 5 min after the addition of glyceraldehyde 3-phosphate dehydrogenase and 3-phosphoglycerate kinase approx. 80% of the radioactivity initially in P_i (R_F 0.6) was incorporated into GTP (R_F 0.06). Longer incubation times did not improve the yield of [γ - ^{32}P]GTP.

In the second step 90% of the radioactivity in [γ - ^{32}P]GTP was transferred to phosphoenolpyruvate (R_F 0.2) catalysed by the GTP-dependent phosphoenolpyruvate carboxykinase present in the acetone-dried powder. Fig. 1 shows that the process was completed after 10 min incubation at 30°C (lane 7). Longer incubation periods (results not shown) had no effect on the final yield.

After removal of the denatured proteins, at the end of the synthetic process, a sample of the final mixture was incubated with pyruvate kinase in the presence of ADP. The formation of [γ - ^{32}P]ATP (lane 8) positively identified the radiolabelled compound, R_F 0.2, as [^{32}P]phosphoenolpyruvate.

Fig. 1. PEI-cellulose t.l.c. chromatogram developed in 2 M-sodium formate buffer, pH 3.4, showing the incorporation of [^{32}P]P_i initially into [γ - ^{32}P]GTP and then into [^{32}P]phosphoenolpyruvate, (1) 0 min, (2) 5 min, (3) 10 min and (4) 15 min after addition of glyceraldehyde 3-phosphate dehydrogenase/3-phosphoglycerate kinase, (5) 2 min, (6) 5 min and (7) 10 min after the addition of phosphoenolpyruvate carboxykinase preparation, and (8) production of [γ - ^{32}P]ATP after the incubation of [^{32}P]phosphoenolpyruvate (see lane 7) with pyruvate kinase. For full experimental details see the text. Abbreviation: PEP, phosphoenolpyruvate.

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