Short Communications

Preparation of [β-^{32}P] ATP and [γ-^{32}P] ATP*, **

It has been shown that, in general, the phosphorus atoms at the β- and γ-positions of ^{32}P-labeled ATP are labeled equally when the latter is synthesized by oxidative phosphorylation from AMP[1,2]. "Asymmetrically labeled" ATP, in which phosphorus atoms at the β- and γ-positions have different radioactivity, is required to elucidate the mechanism of some transphosphorylation reactions. KORNBERG *et al.*[3] reported the enzymic synthesis of radioactive ATP, without giving any details of the analysis of the product. LOWENSTEIN[4] tried without satisfactory results the chemical synthesis of asymmetrically labeled ATP by the use of DCC. The present paper describes a convenient method of preparation of asymmetrically labeled ATP, in which both chemical and enzymic steps are used, and the separate determination of the specific activity of each phosphorus in the product.

Preparation of [β-^{32}P] ATP

The scheme of synthesis is as follows:

$$\text{AMP} \xrightarrow[i^{32}P]{\text{DCC}} [\beta\text{-}^{32}\text{P}]\ \text{ADP} \xrightarrow[\text{PEP}]{\text{Pyruvate kinase}} [\beta\text{-}^{32}\text{P}]\ \text{ATP}$$

The first step was carried out by LOWENSTEIN's method[4]. Various ratios of AMP, i^{32}P (diluted with carrier iP) and DCC were tested, and the best results were obtained using the ratio 1:0.5:9. The product, [β-^{32}P] ATP, was purified by paper chromatography according to ZETTERSTRÖM AND LJUNGGREN[5], and the eluate from the paper was further purified by use of a column of Dowex-1 as described by COHN AND CARTER[6]. The eluate from the column was concentrated by passage through another small column, using 0.1 N HCl as eluting solution. The ADP was precipitated as the barium salt by addition of excess of barium acetate and 2 volumes of ethanol at pH 8.0.

In the second step, a reaction mixture with the following composition was incubated at 37° for 30 min: 0.004 M potassium PEP, 0.002 M sodium [β-^{32}P] ADP, 0.005 M $MgSO_4$, 0.05 M KCl, 0.01 M KF, 0.005 M potassium EDTA, 0.04 M tris(hydroxymethyl)aminomethane, pH 7.5 and "crystalline" pyruvate kinase prepared from rabbit muscle by the method of BÜCHER AND PFLEIDERER[7]. KF and EDTA were added to prevent the randomization of the labeling pattern due to the actions of ATPase and adenylate kinase which might contaminate the enzyme preparation. After 30 min, the reaction mixture was diluted to 5-fold with water, the pH adjusted to 8.0, and the [β-^{32}P] ATP isolated and concentrated by the two chromatographic separations on Dowex-1 columns described for the first step. The sodium salt of [β-^{32}P] ATP was crystallized at pH 3.5 by addition of 2 volumes of ethanol under the conditions reported by BERGER[8].

With this procedure a nearly quantitative yield of radioactive ATP was obtained, which showed high specific activity and negligible randomization of the labeling pattern. In an experiment which started from 2.5 mg i^{32}P (2.5 mc) and 15 mg AMP, 14 mg and 15 mg of radioactive ADP and ATP were obtained, respectively. The specific activity of each phosphorus in the ATP was as follows:

Adenine – Ribose	– P –	P –	P	
	4,960	207,700	2,790	counts/min/μatom P

Preparation of [γ-^{32}P] ATP

The synthesis was brought about according to the following steps.

$$i^{32}P \longrightarrow {}^{32}POCl_3 \xrightarrow[\beta\text{-Chlorolactic acid}]{} [^{32}\text{P}]\ \text{PEP} \xrightarrow[\text{ADP}]{\text{Pyruvate kinase}} [\gamma\text{-}^{32}\text{P}]\ \text{ATP}$$

The first two steps were carried out by the methods of AXELROD[9] and BAER[10] respectively. The final step followed the method used in the preparation of the [β-^{32}P] ATP. Starting from 130 mg

* The following abbreviations are used: ATP, adenosine-5-triphosphate; ADP, adenosine-5-diphosphate; AMP, adenosine-5-monophosphate; iP, inorganic orthophosphate; PEP, phosphoenol-pyruvate; DCC, di(cyclohexyl)carbodiimide; EDTA, ethylenediamine tetraacetate.

** The three P atoms of ATP are distinguished as α, β and γ, thus: adenosine $-P^{\alpha}-P^{\beta}-P^{\gamma}$.

i^{32}P (10 mc), 86 mg of radioactive ATP from 80 mg of ADP was obtained. The specific activity of each phosphorus was

Adenine – Ribose	–	P	–	P	–	P	
		490		1,480		22,010	counts/min/μatom P

Thus, remarkable differences in radioactivity between each phosphorus atom in ATP were obtained by this method, but it was difficult to obtain [γ-^{32}P] ATP of high specific activity because the fractional distillation of $^{32}POCl_3$ could not be carried out on a small scale.

*Method of analysis**

γ-Phosphorus was split from the ATP with ATPase, and the quantity and the radioactivity were evaluated after addition of molybdate and extraction by isobutanol[11,12]. The β- and γ-phosphorus atoms were split off by the combined action of ATPase and adenylate kinase, and the specific activity of the β-phosphorus was obtained by subtracting A from 2C. The specific activity of the α-phosphorus was calculated as 3D–(A + B). Total phosphorus was determined by KING's method[13] chemically, or, if necessary, enzymically. The latter method consisted of successive splitting with ATPase, adenylate kinase and 5'-nucleotidase, and this method is recommended in case of the presence of other phosphorus compounds. The conditions for the enzymic reactions were based on the method of BOWEN AND KERWIN[14], except that the enzyme source employed was rat skeletal muscle, and that the 5'-nucleotidase used was prepared by the simplified method of HEPPEL AND HILMOE[15]. The determination of iP in the reaction mixture was carried out by TAKAHASHI's modification[16] of the method of BERENBLUM AND CHAIN[17]. With this method well-reproducible and satisfactory results are easily obtained.

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* A, B, C and D represent the specific activities of phosphorus at the following positions: A, γ-position; B, β-position; C, β- and γ-positions; D, α-, β-, and γ-positions.

The conversion of ^{14}C-deoxynucleoside-5'-monophosphates to the corresponding di- and triphosphates by soluble mammalian enzymes

Experimental results obtained in this laboratory have indicated that the dialyzed soluble supernatant fraction prepared by centrifugation of rat-liver homogenates at 106,000 × *g* for 90 min is capable of converting uracil, uridine and uridine-5'-monophosphate to uridine di- and triphosphates[1]. In the present communication it will be shown that the deoxy-5'-mononucleotides of adenine, guanine, cytosine and thymine are converted to the corresponding di- and triphos-