A simple assay method for determination of the specific radioactivity of the γ -phosphate group of ^{32}P -labelled ATP

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A simple and rapid assay method is described for determining the specific radioactivity of the γ -phosphate group of ^{32}P -labelled ATP. Labelled ATP is incubated with cyclic AMP, cyclic AMP-dependent protein kinase and histone H2A under conditions leading to maximum phosphorylation of the histone. The specific radioactivity of the γ -phosphate group of the ^{32}P -labelled ATP is calculated from the total amount of $[\text{}^{32}\text{P}]\text{P}_i$ incorporated into a standard amount of histone. This assay method uses inexpensive commercially available materials, and it yields an accurate specific radioactivity with as little as 0.25 nmol of ^{32}P -labelled ATP.

There are widespread applications for a simple and quick assay method that determines the specific radioactivity of the γ -phosphate group of ^{32}P -labelled ATP. These include both kinetic measurements of the turnover of the γ -phosphate group and use of the steady-state specific radioactivity of the γ -phosphate group, together with the known concentration of a cellular constituent, to determine the stoichiometry of the phosphorylation of that constituent. Alternatively, knowledge of the steady-state specific radioactivity of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and the total radioactivity of a compound in rapid equilibrium with the γ -phosphate group of ATP may be used to obtain an indirect estimate of the concentration of that compound.

For many purposes the most convenient available assay method is that developed by England & Walsh (1976). This relies on measurements of the maximum amount of ^{32}P that can be enzymically incorporated into a standard amount of glycogen phosphorylase. A disadvantage of this assay method for many laboratories is that commercially available phosphorylase and phosphorylase kinase preparations may require further purification before use. They are also expensive.

A second assay method, which relies on protein phosphorylation by $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ present in simple tissue extracts, is that of Cogoli & Dobson (1981). This measures phosphorylation of casein by cyclic AMP-dependent protein kinase at ATP concentrations approaching those needed to saturate the enzyme, i.e. at or near zero-order kinetics. However, it yields direct estimates of specific radioactivity only from tissue extracts containing at least

0.4 mM-ATP. For extracts containing a lower ATP concentration, an adjustment must be made so that all samples are incubated at the same ATP concentration. Thus the ATP concentration of each radioactive tissue extract is assayed fluorimetrically, and unlabelled ATP is added so as to bring $[\text{ATP}]$ to a standard value (usually $33\text{ }\mu\text{M}$) before assay of the specific radioactivity of the γ -phosphate group. This latter procedure has two important disadvantages. First, it expends the time and materials needed to measure the ATP concentration in every labelled tissue extract, and the ultimate accuracy of the method depends on the accuracy of this estimate and of the subsequent adjustment of the sample with unlabelled ATP. Secondly, it involves an undesirable and variable isotope dilution of the high-specific-radioactivity $[\text{}^{32}\text{P}]\text{ATP}$ present in the initial tissue extracts.

The assay method described in the present paper, like that of Cogoli & Dobson (1981), employs cyclic AMP-dependent protein kinase, but it returns to the principle of phosphorylation of a substrate protein to saturation, which was the important innovation in the procedure of England & Walsh (1976). It retains the simplicity of the England & Walsh (1976) assay method and does not require a precise determination of ATP concentration to be made on each tissue extract: it therefore allows the speedy handling of many samples. Moreover, it can be performed with inexpensive commercially available reagents without their further purification, and will yield an accurate value for specific radioactivity from a much smaller sample of labelled ATP than will either of the previous assay methods.

Materials and methods

[γ - ^{32}P]ATP was obtained from Amersham International. Histone H2A ('subgroup f_{2a} ', type H-6881) and bovine heart cyclic AMP-dependent protein kinase (type P-5511) were obtained from the Sigma Chemical Co.

ATP concentrations were determined by a linked assay coupled to NADPH production as described by Lamprecht & Trautschold (1974).

Hepatocytes were isolated and incubated with [^{32}P]P_i as described by Kirk *et al.* (1981). ATP was extracted from a suspension of ^{32}P -labelled hepatocytes as follows. A 50 μl volume of 20% (v/v) HClO_4 was added to 0.5 ml of cell suspension (approx. 26 mg dry wt./ml). The sample was mixed vigorously, placed on ice for 5 min and centrifuged at 5000 g for 30 s in a Beckman Microfuge. Then 250 μl of the supernatant was taken and neutralized by using a mixture of 0.5 M-triethanolamine, 2 M-KOH and BDH Universal indicator, the mixture being kept ice-cold throughout. KClO_4 was removed after 10 min or more by brief centrifugation, and portions of the supernatant were stored at -20°C .

The specific radioactivity of the γ -phosphate group of [^{32}P]ATP was determined as follows. A 20 μl portion of a sample containing [^{32}P]ATP was added to 25 μl of an incubation medium containing 0.4 mg of histone H2A/ml (approx. 26 μM), 10 μM -cyclic AMP, 10 mM-magnesium acetate, 20 mM-dithiothreitol and 20 mM-NaF in 24 mM-potassium phosphate buffer, pH 6.5. The fluoride was essential to inhibit contaminating ATPase activity in the cyclic AMP-dependent protein kinase preparation, and the dithiothreitol was needed to sustain the kinase activity during a long incubation. The reaction was started by the addition of 5 μl of cyclic AMP-dependent protein kinase in 0.5 mM-sodium citrate buffer, pH 6.5 (total activity towards casein added: 2.7 pmol of phosphate transferred/min), and incubations were maintained at 30°C for 140 min. Reactions were terminated by transferring a 45 μl portion of the incubation mixture to a 4 cm^2 piece of Whatman 3MM filter paper, and this was immediately placed in ice-cold 10% (w/v) trichloroacetic acid. The ^{32}P incorporated into the histone precipitate was determined as described by Corbin & Reiman (1974). Briefly, the filter squares were washed four times for 15 min each in 10% trichloroacetic acid and once in ethanol. They were then dried, and their radioactivities were determined by liquid-scintillation spectrometry.

Results and discussion

Prolonged incubation of cyclic AMP-dependent protein kinase with histone H2A and a large molar excess of [γ - ^{32}P]ATP (10.1 d.p.m./pmol) resulted in the phosphorylation of histone to a stable plateau

Fig. 1. Incorporation of ^{32}P from [γ - ^{32}P]ATP into histone. Samples of [γ - ^{32}P]ATP or of ^{32}P -labelled cell extract were incubated with histone and cyclic-AMP-dependent protein kinase. At various times samples of the incubations were taken and the radioactivity incorporated into histone was determined as described in the Materials and methods section. [γ - ^{32}P]ATP concentrations were: O, 50 μM ; Δ , 100 μM ; $\bullet$, 150 μM ; $\blacktriangle$, 200 μM . The data designated $\blacksquare$ were obtained with a ^{32}P -labelled cell extract prepared from hepatocytes incubated with [^{32}P]P_i for 70 min as described in the text. Each point represents the mean of duplicate incubations.

(Fig. 1). Hence, provided that there is sufficient [^{32}P]ATP to achieve phosphorylation of all histone molecules, the final amount of incorporated ^{32}P will be a function only of the specific radioactivity of the γ -phosphate group: it should be independent of the ATP concentration. This was demonstrated with histones at approx. 13 μM and concentrations of [γ - ^{32}P]ATP between 50 and 200 μM (Fig. 2a).

If the histone concentration is lowered, then it is possible to use the assay method with lower concentrations of ATP. The limiting factor in the assay method of England & Walsh (1976) is the relatively high K_m of phosphorylase kinase for ATP (approx. 200 μM ; Krebs *et al.*, 1964). At concentrations of ATP below 50 μM , the phosphorylation of phosphorylase to completion cannot be achieved, because during the very long incubations required there would be significant contributions from contaminating ATPase and phosphatase activities. However, cyclic AMP-dependent protein kinase has a much lower K_m for ATP (approx. 10 μM ; Walsh & Krebs, 1973), and it can therefore

Fig. 2. Relationship between $[^{32}\text{P}]\text{P}_i$ incorporation into histone and the specific radioactivity of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (a) Samples of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ of known specific radioactivity were incubated with histone and cyclic AMP-dependent protein kinase under the assay conditions described in the Materials and methods section. ATP concentrations were: $\circ$, 50 μM ; $\blacktriangle$, 200 μM . Each point represents the mean of duplicate incubations. (b) A ^{32}P -labelled cell extract was obtained from hepatocytes incubated with $[^{32}\text{P}]\text{P}_i$ for 70 min, as described in the Materials and methods section. The ATP concentration of this extract was determined, and unlabelled ATP of the same concentration was added in the proportions indicated. These mixtures were then incubated with histone and cyclic AMP-dependent protein kinase under the standard assay conditions (see the Materials and methods section). Each point represents the mean of duplicate determinations.

lengthened, the assay method can be used successfully with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ concentrations as low as 5 μM (i.e. 0.25 nmol of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in a 50 μl incubation mixture).

Validation of the application of the specific radioactivity assay to $[^{32}\text{P}]\text{ATP}$ present in cell extracts

$[^{32}\text{P}]\text{ATP}$, together with other ^{32}P -labelled water-soluble compounds, was extracted from a suspension of ^{32}P -labelled hepatocytes as described in the Materials and methods section. ATP is present in such extracts at approx. 150–200 μM . When the ^{32}P -labelled cell extract was incubated with histone and cyclic AMP-dependent protein kinase, the time course of histone phosphorylation was similar to that for pure $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (see Fig. 1). The concentration of ATP in a ^{32}P -labelled cell extract was determined, and samples of the cell extract were mixed with various volumes of an unlabelled ATP solution of the same concentration. These samples were then incubated with histone and the cyclic AMP-dependent kinase (Fig. 2b). The total ^{32}P incorporated into the histone was directly proportional to the specific radioactivity of the $[^{32}\text{P}]\text{ATP}$ in the diluted cell extracts, showing that there was no significant contribution from the other nucleotides present; this is in keeping with the known high specificity of cyclic AMP-dependent protein kinase for ATP.

The routine use of this assay for assessing the specific radioactivity of cellular $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ requires that a sample of pure $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ of known specific radioactivity should be included with every experiment for calibration purposes. The assay method has been used successfully to follow the labelling with ^{32}P of the γ -phosphate group of ATP by hepatocytes and foetal rat heart slices *in vitro* (P. T. Hawkins & D. Heeley, unpublished work). With hepatocytes it yielded specific radioactivities for the γ -phosphate group that were similar to those obtained by a much more tedious high-pressure liquid chromatography procedure (Garrison *et al.*, 1979).

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catalyse the rapid phosphorylation of histones at much lower ATP concentrations. We have found that, provided that the $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ is in 5-fold molar excess over the histones and the incubation period is

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