A POSSIBLE ERROR DURING ASSAYS FOR THE ENZYMIC PHOSPHORYLATION OF PROTEINS AND NUCLEIC ACIDS*

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Summary: The formation of an insoluble complex between Mg^{++}, sodium fluoride and ATP was studied. The precipitation of ATP was dependent on the time of incubation and on the concentration of ATP, Mg^{++} and sodium fluoride. The requirement for sodium fluoride was not replaced by sodium or potassium chloride but the requirement for Mg^{++} was replaced by La^{+++} and Sn^{++}. Precipitation was stimulated by cAMP but was inhibited at high ionic strength and by EDTA, dATP, ADP and sodium pyrophosphate. The complex was slightly soluble in acid and completely soluble in alkali. Formation of this complex could result in errors in *in vitro* assays for enzymic phosphorylation of proteins and nucleic acids by acid precipitation.

Several enzymes which transfer the γ-phosphate of ATP onto proteins and nucleic acids (ATP:protein and nucleotidyl phosphotransferases) have been isolated from a variety of organisms (1-5). These enzymes fulfill many functions including the activation of certain hormone-sensitive enzymes (6) and the inactivation of ribosomes during protein synthesis (7). The phosphorylation of proteins or nucleic acids is readily assayed by following the incorporation of the γ-phosphate of ATP into an acid-precipitable product. Mg^{++} is normally an absolute requirement for this reaction and there have been occasional reports on the use of sodium fluoride in *in vitro* assays as an inhibitor of possible phosphatase activity (7,8). This paper describes some of the parameters involved in the precipitation of ATP by Mg^{++} and sodium fluoride in an attempt to emphasize the care required when undertaking assays of enzymic phosphorylations by acid precipitation.

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MATERIALS AND METHODS. ATP-^{3}H(G) and ATP-γ-^{32}P were obtained from New England Nuclear, Boston, Massachusetts. Adenosine-3',5'-cyclic monophosphate (cAMP) was obtained from Calbiochem, Los Angeles, California. All other chemicals were analytical grade reagents.

Typical assays for investigating the precipitation of ATP contained 5 μmole of Tris-HCl, pH 8, 2 μmole of dithiothreitol (DTT), 2 μmole of $MgCl_2$, 2 μmole of NaF and 10 nmole of ATP (^{3}H and ^{32}P labeled) in 0.3 ml. The reactions were incubated at 37°C for 30 min and were terminated by the addition of 1% (W/V) bovine serum albumin (50 μl) and 100% (W/V) trichloroacetic acid (TCA) (50 μl). After 30 min at 0°C the precipitated material was collected on discs of Whatman GF/C glass fiber paper (2.4 cm diameter); each disc was washed with 20 ml of 20% TCA, 20 ml of 5% TCA containing 0.2 M sodium pyrophosphate and with absolute ethanol. The discs were dried and counted [0.4% (W/V) 2,5-Diphenyloxazole and 0.005% (W/V) 1,4,bis-2-(4-methyl-5-phenyloxazolyl)-benzene in toluene] in an Intertechnique liquid scintillation counter.

TABLE 1

THE RECOVERY OF ATP AS AN ACID INSOLUBLE COMPLEX

	nmole ^{3}H-ATP	nmole ^{32}P-γ-ATP
Full reaction mix	0.80	0.95
Full reaction mix - DTT	0.65	0.71
Full reaction mix - $MgCl_2$	0.007	0.008
Full reaction mix - NaF	0.005	0.004
Full reaction mix + 1 mM EDTA	0.33	0.29
Full reaction mix + 0.1 M NaCl	0.008	0.006
Full reaction mix + 10 nmole dATP	0.048	0.019
Full reaction mix + 10 nmole ADP	0.28	0.22
Full reaction mix + 10 nmole sodium pyrophosphate	0.048	0.023

RESULTS AND DISCUSSION. Table 1 shows the results of a typical assay for investigating the precipitation of ATP. Almost 10% of the input ATP was recovered as an acid-precipitable complex. The precipitation of ATP was dependent on the time of incubation at 37°C (Fig. 1a), slightly dependent on the presence of DTT (Table 1) and totally dependent on the concentration of

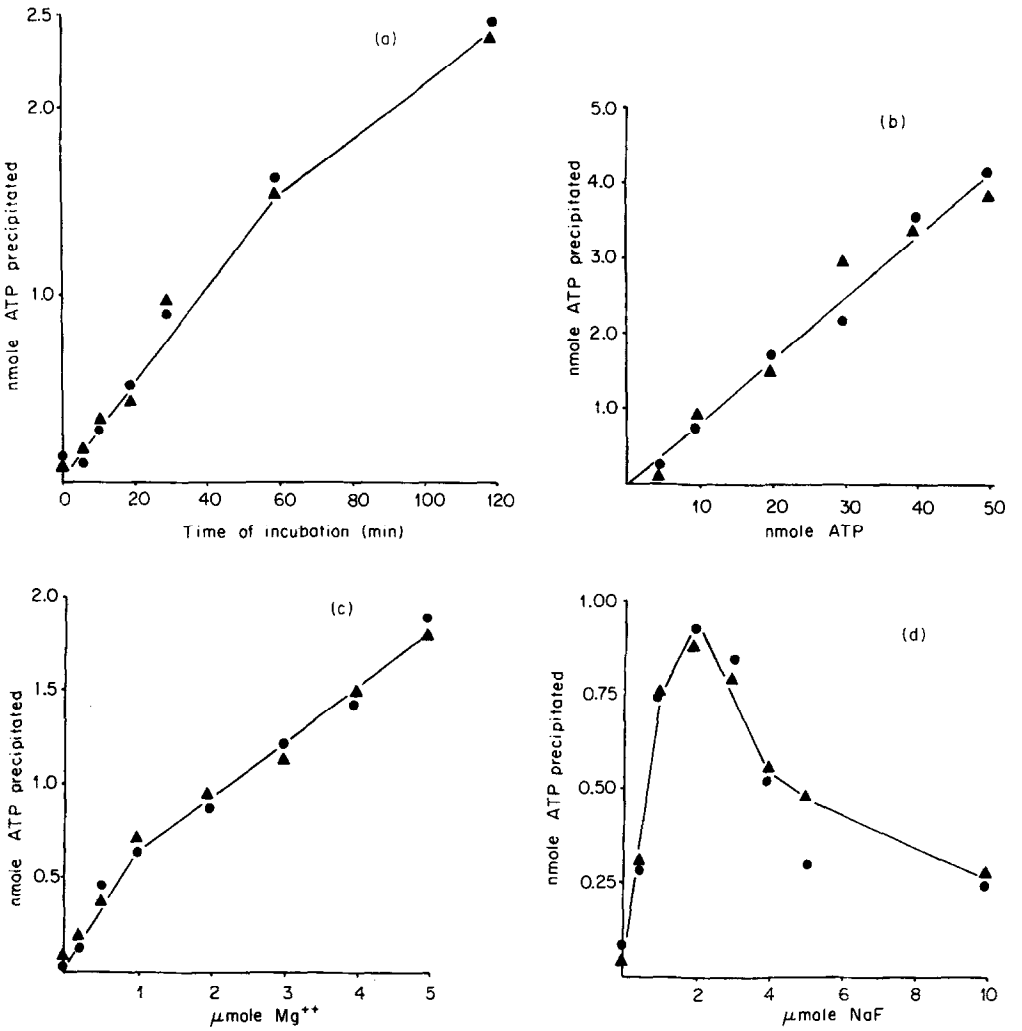

Fig. 1. The effect of (a) time of incubation at 37°C, (b) concentration of ATP, (c) concentration of Mg^{++} and (d) concentration of NaF, on the precipitation of ATP.

●—● Precipitation of ATP-γ-^{32}P.

▲—▲ Precipitation of ATP-^{3}H(G).

ATP, Mg^{++} and NaF (Table 1 and Fig. 1b-1d). In these reactions $MgCl_2$ was used; magnesium acetate did not efficiently replace $MgCl_2$ presumably due to the lower ionization constant of this compound. Other metals, with the exception of La^{+++} and Sn^{++}, did not efficiently replace the requirement for Mg^{++}. The requirement for NaF was not replaced by NaCl or KCl.

The precipitation of ATP was prevented by EDTA and at high ionic strength and was competitively inhibited by dATP, ADP and sodium pyrophosphate (Table 1). Proteins which had no phosphorylating activity were also slightly inhibitory. cAMP, which has been shown to stimulate some phosphorylating enzymes (9), also stimulated the precipitation of ATP. This stimulation was dependent on the concentration of cAMP (Fig. 2); at low concentrations there was almost a fivefold stimulation of precipitation.

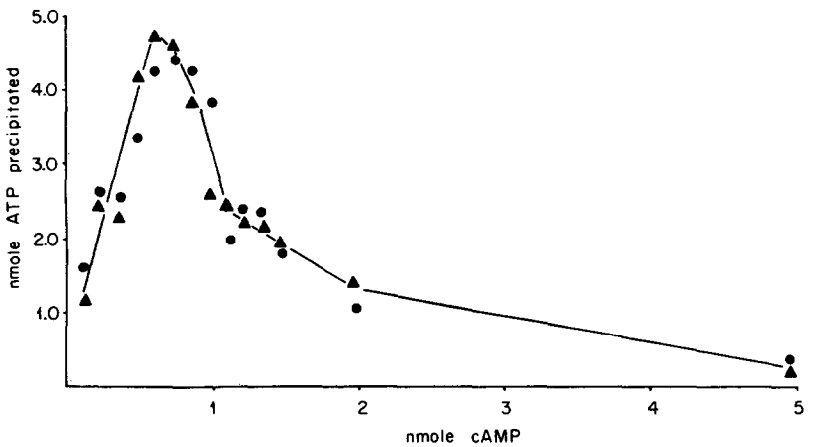

Fig. 2. The effect of the concentration of cAMP on the precipitation of ATP.

●—● Precipitation of ATP-γ-^{32}P.

▲—▲ Precipitation of ATP-^{3}H(G).

The material precipitated during these reactions was analyzed by thin-layer chromatography on polyethyleneimine-cellulose (10) and by ionophoresis on DEAE paper (11) to ensure that the results of these assays were not due to

the breakdown of ATP. These results indicated that there was only negligible breakdown of ATP under the assay conditions described. Ninety per cent of the precipitated ATP was removed by dialysis of the insoluble complex for 24 hr. The remaining ATP was acid soluble but upon re-reaction this ATP became insoluble once more. The ATP precipitated during these reactions was insoluble without prior treatment with TCA; treatment with TCA solubilized 25% of the ATP. The precipitated ATP was completely soluble in 0.1 N NaOH, addition of 20% (W/V) TCA reprecipitated approximately 10% of the ATP originally present in the complex. If dissolution and reprecipitation was repeated then the remaining ATP was completely acid soluble.

The results reported in this paper give some of the parameters involved in the precipitation of ATP by Mg^{++} and NaF. Mg^{++} is known to interact with the β,γ-pyrophosphate of ATP to form an insoluble complex and the competitive inhibition of precipitation by dATP, ADP and pyrophosphate indicates that this interaction plays a part in the precipitation of ATP described in this paper. However, this interaction cannot fully explain these results as NaF is an absolute requirement for the precipitation reaction.

These results indicate that errors may occur during assays for phosphorylating enzymes by acid precipitation of the phosphorylated products. These errors may be avoided if the acid-precipitated product is dissolved in 0.1 N NaOH and then reprecipitated; this procedure should be repeated at least twice. This paper emphasizes the absolute necessity for this procedure during all assays for ATP:protein and nucleotidyl phosphotransferases by acid precipitation.

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