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# BOVINE BRAIN $Na^+,K^+$-STIMULATED ATP PHOSPHOHYDROLASE STUDIED BY A RAPID—MIXING TECHNIQUE. $K^+$-STIMULATED LIBERATION OF [$^{32}$P]ORTHOPHOSPHATE FROM [$^{32}$P]PHOSPHOENZYME AND RESOLUTION OF THE DEPHOSPHORYLATION INTO TWO PHASES

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## Summary

Dephosphorylation of [$^{32}$P]phosphoenzyme of bovine brain $Na^+,K^+$-stimulated ATP phosphohydrolase (EC 3.6.1.3), labelled by [$\gamma$-$^{32}$P]ATP, was investigated at 21 °C by means of a rapid-mixing technique. On addition of a high concentration of KCl (10 mM) to [$^{32}$P]phosphoenzyme at steady state in the presence of $Mg^{2+}$ and $Na^+$, very rapid dephosphorylation was obtained. Simultaneously, the amount of [$^{32}$P]orthophosphate increased at about the same rate. It was concluded that this $K^+$-stimulated dephosphorylation and liberation of [$^{32}$P]orthophosphate from the [$^{32}$P]phosphoenzyme was rapid enough to participate in the $Na^+,K^+$-stimulated hydrolysis of ATP.

In order to study the dephosphorylation in absence of continuing $^{32}$P-labelling, excess unlabelled ATP or a chelator of $Mg^{2+}$ was added. Simultaneous addition of a high concentration of KCl to the [$^{32}$P]phosphoenzyme formed in the presence of $Mg^{2+}$ and $Na^+$ but in the absence of $K^+$, resulted in an initial very rapid phase and a subsequent slower phase of dephosphorylation. With KCl also initially present in the incubation medium, only the slow phase was observed. The slow phase of dephosphorylation also seemed to be sufficiently rapid to participate in the $Na^+,K^+$-stimulated ATPase reaction.

On addition of a high concentration of ADP (5 mM) to [$^{32}$P]phosphoenzyme formed in the presence of $Mg^{2+}$ and $Na^+$, an initial comparatively rapid,

Abbreviation: CDTA, (1,2-cyclohexylenedinitrilo)tetraacetic acid.

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and later slow phase of dephosphorylation were detected. This gave further support for different forms of phosphoenzyme. Approximate concentrations of these forms, in the absence and presence of KCl, were estimated by extrapolation and the turnover of these forms was calculated. The nature of the kinetically different components of phosphoenzyme and their role in the $Na^+,K^+$-stimulated ATPase reaction is discussed.

## Introduction

It is well established that preparations of $Na^+,K^+$-stimulated ATPase (EC 3.6.1.3) are phosphorylated by ATP in the presence of $Mg^{2+}$ and $Na^+$ and that the phosphoenzyme is dephosphorylated by $K^+$ [1–5]. By means of rapid-mixing techniques, both the $Na^+$-stimulated phosphorylation and the $K^+$-stimulated dephosphorylation were found to be sufficiently rapid to account for the $Na^+,K^+$-stimulated hydrolysis of ATP [6,7].

The existence of at least two kinetically different forms of phosphoenzyme was suggested previously [7], the more abundant form being insensitive to $K^+$. This suggestion was based on the very high rate constant of $K^+$-stimulated dephosphorylation and a comparatively high steady-state level of phosphoenzyme in the presence of 10 mM KCl.

One prerequisite for the very rapid $K^+$-stimulated dephosphorylation to be an intermediate step in the overall hydrolysis of ATP is that the decrease of phosphoenzyme upon addition of $K^+$ corresponds to an equally rapid liberation of orthophosphate and not to a reversal of the phosphorylation step [8].

In this paper, experiments on [$^{32}$P]orthophosphate liberation and studies on $K^+$-stimulated and ADP-stimulated dephosphorylation of [$^{32}$P]phosphoenzyme are presented.

## Experimental Procedure

### *Materials*

Millipore filters, type SSWP (pore size 3 μm), 25 mm in diameter, were used. The disodium salt and Tris salt of ATP of the highest purity available were obtained from Sigma. Stock solutions of ATP, ADP and of (1,2-cyclohexylenedinitrilo)tetraacetic acid (CDTA) were adjusted to the appropriate pH with Tris. The DEAE-cellulose was a Whatman product, type DE-52; all other chemicals were of reagent grade. Double quartz-distilled water was used throughout the experiments.

### *Preparation of [γ-$^{32}$P]ATP*

Tris-[γ-$^{32}$P]ATP was synthesized essentially as described by Engström [9] except that the incubation mixture was chromatographed on a DEAE-cellulose column (1.1 × 20 cm). The column was eluted at room temperature with a linear gradient of Tris-HCl buffer, pH 7.4 (0.1—0.25 M). In the [$^{32}$P]orthophosphate liberation experiments the [γ-$^{32}$P]ATP solution, after dilution with unlabelled ATP to obtain the appropriate specific radioactivity (10—30 Ci/mol), had to be further purified from [$^{32}$P]orthophosphate.

The second step of purification was performed by chromatography at 4°C on a small DEAE-cellulose column (0.9 × 4 cm) with stepwise elution; [$^{32}$P]orthophosphate was eluted with 100 mM Tris-HCl buffer (pH 7.4) and [$\gamma$-$^{32}$P]ATP was eluted with 200 mM Tris-HCl buffer. 0.1–0.3% of the radioactivity of the [$\gamma$-$^{32}$P]ATP fraction obtained by this procedure was [$^{32}$P]orthophosphate.

*Analytical methods*

Radioactivity was determined by measuring the Čerenkov radiation [10] in an Intertechnique Scintillation Spectrometer, Model SL 30. The efficiency of detection was about 47% [10].

Concentration of ATP was estimated at 260 nm at pH 7.0 ($\epsilon_{260\,nm}$ = 15 700; ref. 11).

Protein was assayed by the method of Lowry et al. [12] with human serum albumin as a standard. All enzyme solutions used in rapid-mixing experiments were assayed for protein.

[$^{32}$P]phosphoenzyme was determined as protein-bound acyl-[$^{32}$P]phosphate as described previously [6,7].

Figures represent results which have been reproduced at least three times. Each experimental point indicating the amount of $^{32}$P incorporated into the enzyme preparation corresponds to one sample. Background $^{32}$P-labelling was estimated either by addition of [$\gamma$-$^{32}$P]ATP to $HClO_4$-denaturated enzyme or by addition of native enzyme to a solution of 10% $HClO_4$ (w/w) containing the same amount of [$\gamma$-$^{32}$P]ATP as was used in each experiment. This background was reproducible and did not exceed 10% of the maximal amount of [$^{32}$P]phosphoenzyme and was therefore not subtracted from the experimental points. The background $^{32}$P-labelling was probably due to unspecifically adsorbed [$^{32}$P]orthophosphate to the filter and to the precipitated protein, since addition of 10 mM nonradioactive orthophosphate to the solution of $HClO_4$ used for quenching (as tried in subsequent experiments) reduced this background to less than 5%. A more pure [$\gamma$-$^{32}$P]ATP with less contamination of $^{32}P_i$ also reduced the background.

*Enzyme preparation*

$Na^+$,$K^+$-stimulated ATPase was prepared from bovine brain cortex as described by Skou and Hilberg [13]. The $Na^+$,$K^+$-stimulated and ouabain-sensitive ATPase activity was assayed as described previously [7]. Two different batches of enzyme preparation, with approximately similar activities, were used. One of them, which has been characterized in a previous report [7], was used for the [$^{32}$P]orthophosphate liberation experiments. The other preparation was used for all other experiments. Its properties are given in Table I.

*Rapid-mixing apparatus*

The design and operation of the rapid-mixing apparatus have been described previously [7,14]. One major modification was made when [$^{32}$P]orthophosphate liberation from the [$^{32}$P]phosphoenzyme was measured (Fig. 1). Two jet mixers were each coupled to two syringes. Each outlet tubing of these jet mixers was coupled to a Y-connection, which split the flow from the mixer

TABLE I

THE EFFECTS OF IONS, OUABAIN AND CONCENTRATION OF ATP ON THE HYDROLYSIS OF ATP BY THE MICROSOMAL ATPASE

The ATPase activity was assayed as described under Experimental procedure. 3 mM $MgCl_2$, 120 mM NaCl, 10 mM KCl and 0.1 mM ouabain were added as indicated in the table. In those experiments which included ouabain, the enzyme was preincubated at pH 7.4 for 1 h at 21°C before addition of ATP.

| Ions and ouabain | Hydrolysis of ATP (nmol · $mg^{-1}$ · $min^{-1}$) | |
|---|---|---|
| [$^{32}$P]ATP concn: | 0.1 mM | 1.0 mM |
| $Mg^{2+}$ | 54 | 92 |
| $Mg^{2+}$, ouabain | 40 | |
| $Mg^{2+}$, $Na^+$ | 81 | 115 |
| $Mg^{2+}$, $Na^+$, cuabain | 44 | |
| $Mg^{2+}$, $Na^+$, $K^+$ | 333 | 500 |
| $Mg^{2+}$, $Na^+$, $K^+$, ouabain | 45 | |

into two streams. The streams from one Y-connection were led to inlets 1 and 3 of a third jet mixer. The two streams from the other Y-connection were led to inlets 2 and 4 of the same (third) mixer and the dephosphorylation was carried out in the outlet tubing of this mixer. The outlet tubing ended in a quenching solution of 10% (w/w) $HClO_4$. This arrangement with four syringes was important in order to have a symmetrical flow in all parts of the mixers and thus avoid uncontrolled enzymatic hydrolysis of a small fraction of the added [$\gamma$-$^{32}$P]ATP. This hydrolysis amounted to only 0.1—0.5% of the total [$\gamma$-$^{32}$P]ATP and was therefore considered to be negligible in three-syringe experiments in which only [$^{32}$P]phosphoenzyme was measured (see ref. 7 and below). After each incubation the tubings of the rapid-mixing apparatus were carefully rinsed with distilled water.

The solutions used in the rapid-mixing experiments were kept in a ther-

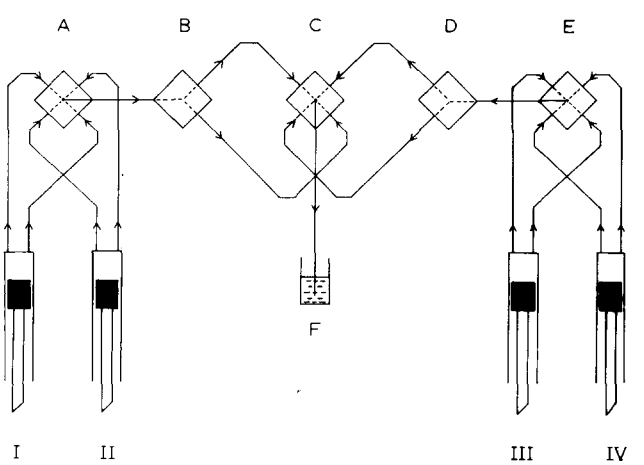


Fig. 1. Diagram showing the connections of the components of the rapid-mixing apparatus used for dephosphorylation and for [$^{32}$P]orthophosphate liberation. (A) jet mixer; (B) Y-connection; (C) jet mixer; (D) Y-connection; (E) jet mixer; (F) vial containing 10% cold $HClO_4$ for quenching. Syringes I, II, III and IV contain the solutions indicated in the text. For further details, see Experimental procedure.

mostated waterbath (21 °C) on the rear of the rapid-mixing apparatus. The pump of the waterbath was used for circulation of water through chambers in the blocks of plexiglass which constituted the barrels of the syringes.

*Assay of liberation of [$^{32}$P] orthophosphate from [$^{32}$P] phosphoenzyme*

In the study of [$^{32}$P] orthophosphate liberation from $^{32}$P-labelled enzyme, Syringe I contained 1.3 ml of 20 μM [γ-$^{32}$P] ATP and Syringe II contained 1.3 ml of enzyme solution (0.25 mg/ml). Both syringes also contained 3 mM $MgCl_2$ and 120 mM NaCl in 30 mM Tris-HCl buffer, pH 7.4. The solutions were mixed in the first jet mixer and were led through one of the Y-connections to the final jet mixer. Simultaneously the same volume, total 2.6 ml, of the solutions of Syringes III and IV, was forced to the final jet mixer via the same arrangements of jet mixer and Y-connection as for Syringes I and II (Fig. 1). Syringes III and IV contained the same solution of 3 mM $MgCl_2$ and 120 mM NaCl in 30 mM Tris-HCl buffer, with the additions given in the figures. The outlet tubing of the last mixer ended in 5 ml of 10% (w/w) $HClO_4$. Immediately after quenching the solution was filtered through a Millipore filter and duplicates of 0.5 ml of the filtrate were analysed for [$^{32}$P] orthophosphate by a modification of the method of Martin and Doty [15]. The samples were transferred to tubes containing 2—3 μmol of carrier $P_i$ in 0.5 ml of 1 M Tris-acetate buffer, pH 7.4, 1.5 ml distilled water and 2.5 ml of isobutanol/benzene, 1 : 1 (v/v). 0.5 ml of 5% ammonium molybdate in 2 M $H_2SO_4$ was added. The tubes were shaken vigorously for 20 s and the phases were separated by a short centrifugation. A 2.0 ml sample of the organic phase was transferred to a scintillation vial and 10 ml of 0.5 M NaOH was added. The yellowish colour of [$^{32}$P] phosphomolybdate in the organic phase, which may decrease the counting efficiency of the Čerenkov radiation [16,17], disappeared after the addition of NaOH. The solution of NaOH made the [$^{32}$P] phosphomolybdate enter the water phase, which was important since the counting efficiency differs in samples with different refractive indices [17,18]. By this procedure the counting efficiency of the organic phase plus the solution of NaOH was the same as in 10 ml of water (about 47%). The filtrate was also analysed for total radioactivity in order to estimate the fractional increase of [$^{32}$P] orthophosphate in each sample. The protein precipitates on the filters were washed 7 times with 10 ml of 5% (w/w) $HClO_4$ containing 10 mM orthophosphate before analysis for protein-bound acyl-[$^{32}$P] phosphate [7].

The [$^{32}$P] orthophosphate content of the [γ-$^{32}$P] ATP solution was measured every 30—40 min. The spontaneous hydrolysis of [γ-$^{32}$P] ATP under the conditions of a series of rapid-mixing experiments amounted to about 0.1% per h. The total amount of [γ-$^{32}$P] ATP used at each incubation was about 80 nmol per mg of protein, while the maximal amount of [$^{32}$P] phosphoenzyme that could be hydrolysed was about 0.47 nmol · $mg^{-1}$. Thus, to detect an increase in [$^{32}$P] orthophosphate corresponding to a decrease in [$^{32}$P] phosphoenzyme, an increase in [$^{32}$P] orthophosphate of about 0.1 nmol had to be significantly detectable. The importance of a [γ-$^{32}$P] ATP preparation with low contamination with [$^{32}$P] orthophosphate and corrections for the spontaneous hydrolysis during the experiments is thus apparent.

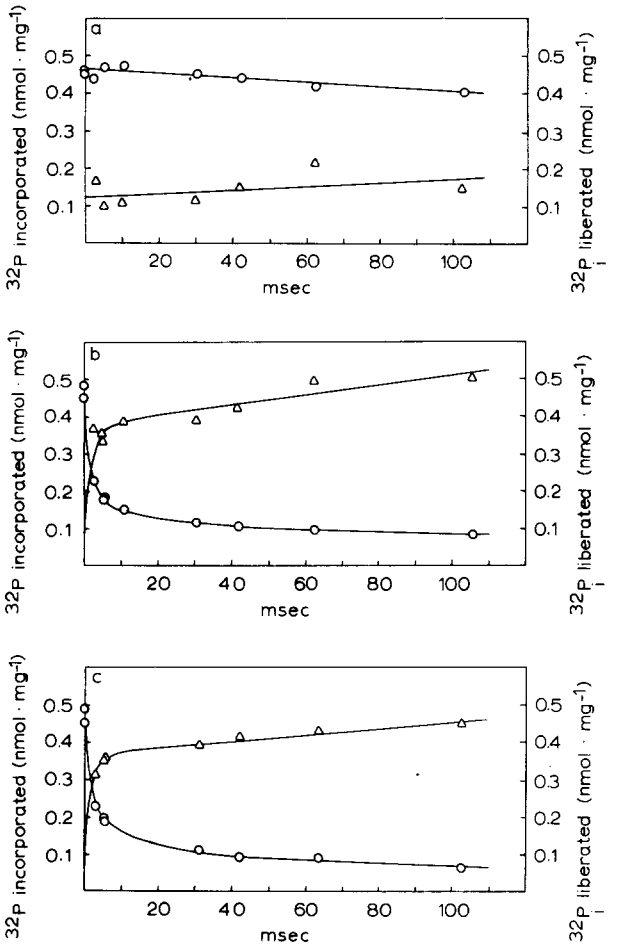


Fig. 2. Dephosphorylation of [$^{32}$P]phosphoenzyme and liberation of [$^{32}$P]orthophosphate. By means of Syringes I and II the enzyme was phosphorylated at 21° C and at 10 $\mu$M [$\gamma$-$^{32}$P] ATP, 3 mM $MgCl_2$, 120 mM NaCl and 30 mM Tris-HCl buffer (pH 7.4) for about 70 ms. At this time the contents of Syringes III and IV were added. (a) no extra additions; (b) addition of KCl, final concentration 10 mM; (c) addition of KCl plus CDTA, final concentrations 10 mM and 50 mM respectively. ○———○, [$^{32}$P]phosphoenzyme; △———△, [$^{32}$P]orthophosphate liberated. The amount of [$^{32}$P]orthophosphate liberated was corrected for spontaneous hydrolysis of [$\gamma$-$^{32}$P] ATP and mean values of duplicates are shown.

*Assay of the rate of decrease of [$^{32}$P]phosphoenzyme*

The principles of the experiments designed to assay the decrease in [$^{32}$P]-phosphoenzyme were described in a previous report (ref. 7, Fig. 1). Three syringes of the rapid-mixing apparatus were used. The volume of solution in each syringe was 0.7 ml. The temperature in all experiments was 21° C. By means of Syringes I and II, [$\gamma$-$^{32}$P] ATP was mixed in the first jet mixer with the enzyme solution (0.5 mg of protein per ml before mixing) to give a final concentration of 100 $\mu$M [$\gamma$-$^{32}$P] ATP. Both solutions contained 3 mM $MgCl_2$, 120 mM NaCl and 30 mM Tris-HCl buffer, pH 7.4. The outlet tubing of the first jet mixer was connected to a second jet mixer by a Y-connection. In addition to 30 mM Tris-HCl buffer, pH 7.4, 3 mM $MgCl_2$ and 120 mM NaCl,

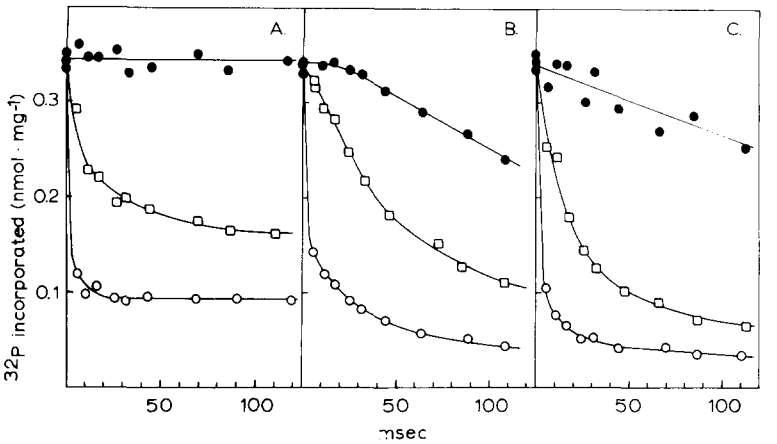


Fig. 3. Dephosphorylation of [$^{32}$P]phosphoenzyme formed at 100 $\mu$M [$\gamma$-$^{32}$P]ATP, 3 mM $MgCl_2$ and 120 mM NaCl for about 70 ms. (A) without extra additions; (B) addition of 50 mM CDTA; (C) addition of 5 mM unlabelled ATP and 5 mM extra $MgCl_2$. ●———●, dephosphorylation in the presence of 3 mM $MgCl_2$ and 120 mM NaCl; □———□, dephosphorylation in the presence of 3 mM $MgCl_2$, 120 mM NaCl and 1 mM KCl; ——— , dephosphorylation in the presence of 3 mM $MgCl_2$, 120 mM NaCl and 10 mM KCl. In dephosphorylation experiments with CDTA Syringe III did not contain any $MgCl_2$. For further details see Experimental procedure.

Syringe III also contained KCl to give a final concentration of either 1 mM or 10 mM after mixing. In some experiments, Syringe III also contained CDTA to give a final concentration of 50 mM (Figs 2C, 3B, 4, 5 and 7), or unlabelled ATP to give a final concentration of 5 mM (Figs 3C, 4 and 5). When unlabelled ATP was included, sufficient extra $MgCl_2$ was always added to give the same molar excess of free $Mg^{2+}$. In some experiments Syringe III also contained ADP (Figs 6 and 7).

In one type of experiment, 10 mM KCl was included also in Syringes I and II (Figs 4, 5B, 7 and 8B). After a phosphorylation period of about 70 ms, CDTA, unlabelled ATP or ADP was added by means of Syringe III.

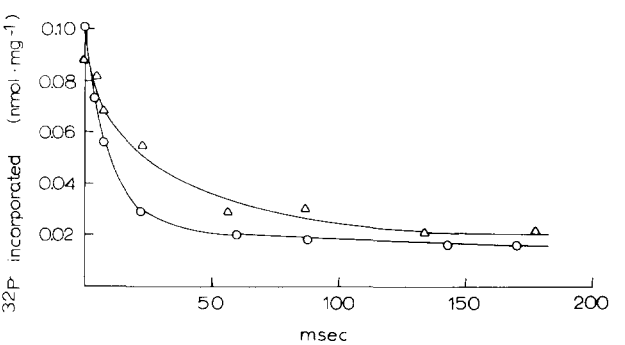


Fig. 4. Dephosphorylation of [$^{32}$P]phosphoenzyme formed at 100 $\mu$M [$\gamma$-$^{32}$P]ATP, 3 mM $MgCl_2$, 120 mM NaCl and 10 mM KCl. After about 70 ms of phosphorylation, CDTA (△———△) or unlabelled ATP plus extra $MgCl_2$ (○———○) were added to final concentrations of 50 mM, 5 mM and 8 mM, respectively. In the experiment with CDTA, Syringe III did not contain any $MgCl_2$.

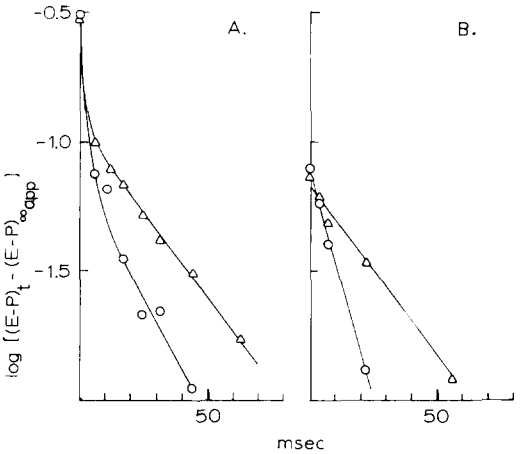


Fig. 5. First-order treatment applied to experiments presented in Figs 3 and 4. (A) From Fig. 3; Dephosphorylation of [$^{32}$P]phosphoenzyme formed in the absence of KCl. Dephosphorylation was started by the addition of KCl plus CDTA (○——△) or KCl plus unlabelled ATP and extra $MgCl_2$ (○——○). (B) From Fig. 4: Dephosphorylation of [$^{32}$P]phosphoenzyme formed in the presence of 10 mM KCl. Dephosphorylation was started by the addition of CDTA (○——○) or unlabelled ATP and extra $MgCl_2$ (○——○). $[E\text{-}P]_t$ represents the amount of $^{32}$P incorporated at different times. $[E\text{-}P]_{\infty app}$ represents the amount of $^{32}$P incorporated at the steady-state level that was obtained in the different experiments.

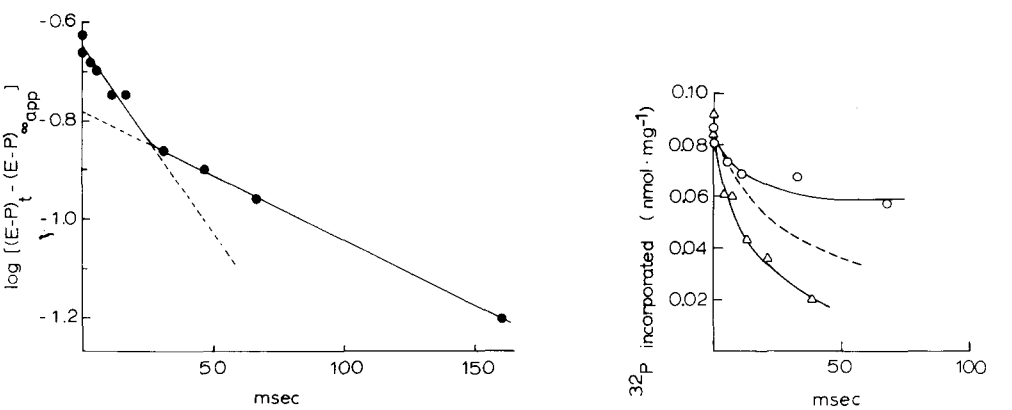


Fig. 6. Dephosphorylation of [$^{32}$P]phosphoenzyme by ADP. [$^{32}$P]phosphoenzyme was formed at 3 mM $MgCl_2$, 120 mM NaCl and 100 $\mu$M [$\gamma$-$^{32}$P]ATP. After about 70 ms of phosphorylation, ADP was added to a final concentration of 5 mM. $[E\text{-}P]_t$ represents the amount of $^{32}$P incorporated at different times. $[E\text{-}P]_{\infty app}$ represents the amount of $^{32}$P incorporated at steady-state 450 ms after the addition of ADP (0.12 nmol · mg$^{-1}$ of protein).

Fig. 7. Dephosphorylation of [$^{32}$P]phosphoenzyme by ADP. [$^{32}$P]phosphoenzyme was formed at 3 mM $MgCl_2$, 120 mM NaCl, 10 mM KCl and 100 $\mu$M [$\gamma$-$^{32}$P]ATP. After about 70 ms of phosphorylation, ADP was added to a final concentration of 0.1 mM. ○——○, addition of ADP alone; △——△, addition of ADP plus CDTA (final concentration 50 mM). The broken line represents dephosphorylation by addition of CDTA alone as described in Fig. 4.

## Results

### *Liberation of [$^{32}$P]orthophosphate from [$^{32}$P]phosphoenzyme*

The enzyme was phosphorylated at 10 $\mu$M [$\gamma$-$^{32}$P]ATP in the presence of

3 mM $MgCl_2$ and 120 mM NaCl. On addition of buffer alone to the $^{32}$P-labelled enzyme a small decrease of [$^{32}$P]phosphoenzyme occurred (Fig. 2A). The steady-state level of phosphoenzyme is rather sensitive to changes of the concentration of ATP in this concentration range [7]. The decrease was therefore probably due to a drop from 10 to 5 $\mu$M [$\gamma$-$^{32}$P]ATP. The rate of formation of [$^{32}$P]orthophosphate, as estimated from the slope of the curve, was low, but closely agreed with the rate of the overall hydrolysis of ATP, which was assayed separately at 3 mM $MgCl_3$, 120 mM NaCl and 5 $\mu$M [$\gamma$-$^{32}$P]ATP [7]. On addition of 10 mM KCl to the [$^{32}$P]phosphoenzyme at least 80% of the rapid decrease of [$^{32}$P]phosphoenzyme was accompanied by a simultaneous and equally rapid release of [$^{32}$P]orthophosphate (Fig. 2B). The rate of appearance of [$^{32}$P]orthophosphate then slowed down and proceeded not significantly different from that of the overall hydrolysis. A comparison of the dephosphorylation in the presence (Fig. 2B) and absence (by the addition of CDTA; Fig. 2C) of continuing phosphorylation showed no apparent difference either of the decrease of [$^{32}$P]phosphoenzyme or of the initial rapid release of [$^{32}$P]orthophosphate.

*Decrease of [$^{32}$P]phosphoenzyme measured in the presence of CDTA or unlabelled ATP*

The addition of chelators of $Mg^{2+}$ such as CDTA and EDTA or the addition of excess of unlabelled ATP are frequently used methods for blocking the $^{32}$P-labelling of the enzyme by [$\gamma$-$^{32}$P]ATP [8,19,20]. With these techniques the level of [$^{32}$P]phosphoenzyme would approach zero, provided the conditions used permitted a turnover of the [$^{32}$P]phosphoenzyme.

It was found in introductory experiments that if the phosphorylation was started by the addition of [$\gamma$-$^{32}$P]ATP to an enzyme with $MgCl_2$ and NaCl already present, addition of 10 mM CDTA simultaneously to the addition of [$\gamma$-$^{32}$P]ATP was not sufficient to rapidly prevent $^{32}$P-labelling. An increase of the CDTA concentration to 50 mM appeared to give a complete blocking in less than 5 ms. This concentration was therefore used in following experiments.

In order to compare rates of dephosphorylation in the presence and absence of continuing $^{32}$P-labelling, dephosphorylation was measured after addition of different concentrations of KCl without and with CDTA or excess of unlabelled ATP. On addition of 10 mM KCl to the [$^{32}$P]phosphoenzyme an apparent first-order rate constant of at least 14 000 $min^{-1}$ (cf. ref. 7) was obtained (Fig.3A). A new lower steady-state level of the $^{32}$P-labelled enzyme was reached. Apparently the same very rapid, initial dephosphorylation was obtained on simultaneous addition of 10 mM KCl plus CDTA or 10 mM KCl plus excess unlabelled ATP (Figs 3B and C). Since the $^{32}$P-labelling essentially ceased on addition of CDTA or unlabelled ATP, the decrease of [$^{32}$P]phosphoenzyme continued below the steady-state level shown in Fig. 3A. This second phase was slower than the initial very rapid phase.

It should be pointed out that the rapid-mixing apparatus does not permit detection of changes of rates of reactions with half lifes below 3 ms [7]. Thus, the possibility cannot be excluded that excess ATP and/or CDTA affects even the rapid $K^+$-stimulated decrease of [$^{32}$P]phosphoenzyme. Dephosphorylation experiments were therefore performed in the absence of KCl and at 1 mM KCl

at which conditions the rate of dephosphorylation is much slower than at 10 mM KCl. The level of [$^{32}$P]phosphoenzyme did not change on addition of buffer alone (Fig. 3A). On addition of CDTA alone, the $^{32}$P-label decreased slowly after an apparent lag period of about 20 ms (Fig. 3B). A slow rate of dephosphorylation was also seen on addition of excess ATP alone (Fig. 3C). The initial rate of dephosphorylation at 1 mM KCl was slower in the presence than in the absence of CDTA (Figs 3A and B). Addition of 1 mM KCl plus excess ATP dephosphorylated [$^{32}$P]phosphoenzyme at approximately the same rate as addition of 1 mM KCl alone (Figs 3A and C), but continuing $^{32}$P-labelling was prevented and therefore the amount of [$^{32}$P]phosphoenzyme decreased below the steady-state level obtained with 1 mM KCl alone.

On addition of either CDTA or of excess unlabelled ATP to a [$^{32}$P]phosphoenzyme formed with 10 mM KCl present in the incubation medium, only a slow rate of dephosphorylation was observed (Fig. 4). In the presence of CDTA the rate of dephosphorylation was slightly slower than in the presence of excess unlabelled ATP which was in agreement with results presented in Fig. 3.

In order to demonstrate rates of dephosphorylation of phosphoenzyme formed in the absence and presence of 10 mM KCl, results in Figs 3 and 4 are presented as first-order plots (Figs 5A and B). When the [$^{32}$P]phosphoenzyme was labelled in the absence of KCl and then KCl plus CDTA or KCl plus unlabelled ATP were added, two distinct phases were observed (Fig. 5A). The rate constant of the initial rapid dephosphorylation seemed to be at least 14 000 $min^{-1}$, irrespective of whether CDTA or unlabelled ATP was added simultaneously to KCl or not. The slow, second phase of dephosphorylation showed first-order kinetics both in the presence of CDTA and of unlabelled ATP. The rate constant of the slow phase was about 1700 $min^{-1}$ in the presence of CDTA and 2800 $min^{-1}$ in the presence of ATP.

Rate constants obtained from first-order plots of dephosphorylation of phosphoenzyme formed at 10 mM KCl were about 1700 $min^{-1}$ in the presence of CDTA and 4600 $min^{-1}$ in the presence of unlabelled ATP (Fig. 5B).

The approximate amounts of the $K^+$-insensitive and $K^+$-sensitive components of the [$^{32}$P]phosphoenzyme were obtained by extrapolation. The amount of the $K^+$-insensitive component seemed to be slightly higher in the absence of KCl than in its presence (range 0.08—0.12 nmol · $mg^{-1}$). The amount of the $K^+$-sensitive component was in the order of 0.23—0.26 nmol · $mg^{-1}$ in the absence of KCl but it was reduced to a very low concentration at 10 mM KCl. Due to the limit of resolution of the present technique a precise estimate of the concentration of this component was not possible in the presence of KCl. However, from the lower limit of the rate constant of the $K^+$-stimulated dephosphorylation (14 000 $min^{-1}$), it was calculated that not more than about 0.029 nmol · $mg^{-1}$ of the $K^+$-sensitive component would be in agreement with the overall $Na^+$, $K^+$-stimulated hydrolysis of ATP at the same conditions (288 nmol · $mg^{-1}$ · $min^{-1}$; Table I).

Dephosphorylation of both the $K^+$-sensitive and $K^+$-insensitive components seemed to occur on addition of 10 mM KCl plus CDTA or unlabelled ATP to a phosphoenzyme formed in the absence of KCl (Fig. 5A). It was therefore difficult to estimate precisely the rate of the second phase from these replots. At steady state in the presence of 10 mM KCl, the phosphoenzyme

essentially consisted of the $K^+$-insensitive component (Fig. 5B). From Fig. 5B a rate of dephosphorylation of the $K^+$-insensitive component could thus be estimated to be about $0.08 \times 1700 = 136$ nmol · $mg^{-1}$ · $min^{-1}$ with added CDTA and about $0.08 \times 4600 = 368$ nmol · $mg^{-1}$ · $min^{-1}$ with added 5 mM unlabelled ATP. The overall $Na^+$,$K^+$-stimulated hydrolysis of ATP in the presence of CDTA equals zero. However, in the absence of CDTA at a saturating concentration of ATP, i.e. 1 mM, the overall $Na^+$,$K^+$-stimulated hydrolysis of ATP amounted to 408 nmol · $mg^{-1}$ · $min^{-1}$, which is close to the rate of dephosphorylation of the $K^+$-insensitive component measured at 5 mM ATP.

*Dephosphorylation of [$^{32}$P]phosphoenzyme by ADP*

Addition of ADP to a final concentration of 5 mM to a phosphoenzyme formed in the presence of 3 mM $MgCl_2$ and 120 mM NaCl at 100 $\mu$M [$\gamma$-$^{32}$P]-ATP gave an initial comparatively rapid and a later slow phase of dephosphorylation (Fig. 6). A steady-state level of about 0.12 nmol · $mg^{-1}$ was obtained after about 450 ms. The same level was also achieved when the enzyme was $^{32}$P-labelled in the same medium as in the experiment presented in Fig. 6 but with the initial addition of 5 mM ADP (not shown). When the logarithms of the experimental points of Fig. 6 were calculated, this steady-state concentration was taken as $[E\text{-}P]_{\infty_{app}}$. The high concentration of ADP used in this experiment probably competes with [$\gamma$-$^{32}$P]ATP for the phosphorylation site of the enzyme and thus prevents new $^{32}$P-labelling [21]. The slow rate of dephosphorylation was similar to the rates obtained by addition of CDTA or unlabelled ATP to phosphoenzyme formed in the absence of KCl (Figs 3B and C). It is therefore likely that the second phase corresponds mainly to spontaneous hydrolysis of [$^{32}$P]phosphoenzyme but the initial, more rapid phase results from a dephosphorylation of an ADP-sensitive component. By extrapolation the amount of this component was estimated at about 0.07 nmol · $mg^{-1}$ of protein. Addition of 5 mM ADP to a phosphoenzyme $^{32}$P-labelled with 10 mM KCl present in the incubation medium caused a very rapid and nearly complete dephosphorylation (not shown). At steady-state in the presence of 10 mM KCl the phosphoenzyme consisted mainly of the $K^+$-insensitive component , the amount of which was about 0.08 nmol · $mg^{-1}$ (Fig. 5B). The amounts of the ADP-sensitive and $K^+$-insensitive components seem thus to be of the same magnitude.

The concentration of ADP in the above experiments was very high. The effect of a low concentration was therefore also investigated. Addition of ADP to a final concentration of 0.1 mM to a phosphoenzyme formed in the presence of 3 mM $MgCl_2$ and 120 mM NaCl at 100 $\mu$M [$\gamma$-$^{32}$P]ATP gave only a small decrease of [$^{32}$P]phosphoenzyme (less than 10% of maximal amount of phosphoenzyme; data not shown). When 0.1 mM ADP was added to [$^{32}$P]phosphoenzyme formed in the same medium but with 10 mM KCl in addition, a small decrease in [$^{32}$P]phosphoenzyme was observed (Fig. 7). By a simultaneous addition of 0.1 mM ADP and CDTA in order to block continuing $^{32}$P-labelling, a considerable increase of the rate, as well as the extent, of dephosphorylation was obtained (Fig. 7). The half life was about 9 ms compared with a half life of about 24 ms on addition of CDTA alone (Fig. 4). Due to the close similarity of

the structures of ADP and ATP and the possibility of an inhibition of ADP binding by ATP, CDTA was chosen as a blocking agent.

*Rate of hydrolysis of ATP*

The rates of overall hydrolysis of ATP at 0.1 mM and 1 mM ATP in different ionic environments are presented in Table I. The $Na^+$,$K^+$-stimulated hydrolysis of ATP at 3 mM $MgCl_2$, 120 mM NaCl and 10 mM KCl was 288 and 408 nmol · $mg^{-1}$ · $min^{-1}$, respectively.

## Discussion

In a previous report [7] it was suggested from the comparatively high steady-state level of phosphoenzyme in the presence of 10 mM KCl and from the high rate constant of the $K^+$-stimulated dephosphorylation that at steady state only a small fraction of total phosphoenzyme consists of a $K^+$-sensitive form. The present investigation demonstrated that addition of 10 mM KCl to a [$^{32}$P]phosphoenzyme labelled at 10 $\mu$M [$\gamma$-$^{32}$P]ATP, 3 mM $MgCl_2$ and 120 mM NaCl caused a rapid, initial release of [$^{32}$P]orthophosphate, which occurred concomitantly with and at the same rate as the very rapid $K^+$-stimulated disappearance of phosphoenzyme previously described [7]. Thus, it is concluded that the $K^+$-stimulated dephosphorylation, as well as the $Na^+$-stimulated phosphorylation [6,7], fulfills an important criterion of an intermediary step in the $Na^+$,$K^+$-stimulated hydrolysis of ATP, i.e. to be at least as rapid as the overall reaction. This mechanism has been postulated by several investigators but it is only in the present and a previous study [7] that the rates of the partial reactions have been determined under conditions giving measurable stimulation of the hydrolysis by $Na^+$ and $K^+$.

The conclusion that the rapid dephosphorylation of [$^{32}$P]phosphoenzyme is essentially due to a release of [$^{32}$P]orthophosphate on addition of KCl seems to be in disagreement with a report by Fukushima and Tonomura [8]. On adding $K^+$ to the [$^{32}$P]phosphoenzyme, they found a rapid decomposition of the [$^{32}$P]phosphoenzyme, which was accompanied by the formation of an equal amount of [$\gamma$-$^{32}$P]ATP. They used extremely low concentrations of [$\gamma$-$^{32}$P]ATP (0.1 $\mu$M or less) in their experiments. The amount of [$\gamma$-$^{32}$P]ATP was in fact less than the total amount of active sites of the enzyme. Furthermore, a high concentration of EDTA was added, which would enhance reversal of the phosphorylation by chelating $Mg^{2+}$ (compare Fig. 7 in the present study). As shown in previous reports from this laboratory [7,22] and by other authors [21,23], the phosphoenzyme is probably preceded by an enzyme · ATP complex. It is also known that the binding of ATP to the enzyme is antagonized by $K^+$ [21,23]. Addition of $K^+$ to the [$^{32}$P]phosphoenzyme may therefore release bound [$\gamma$-$^{32}$P]ATP as well as stimulate the liberation of [$^{32}$P]orthophosphate. For this reason the findings of Fukushima and Tonomura [8] do not necessarily contradict the interpretation of the present results. The possibility of ATP resynthesis was not investigated in the present study, since a release of bound [$\gamma$-$^{32}$P]ATP would have been undetectable at the high concentration of [$\gamma$-$^{32}$P]ATP used.

No apparent difference was observed between the rapid rates of dephos-

phorylation by the addition of 10 mM KCl alone or 10 mM KCl plus CDTA to the phosphoenzyme irrespective of whether a high or a low concentration of [$\gamma$-$^{32}$P] ATP was used (Figs 2B and C, 3A and B). At the high concentration, in the absence of CDTA, a new comparatively high steady-state level of $^{32}$P-labelled enzyme was obtained while in the presence of CDTA a second slow phase of dephosphorylation was detected. At the low concentration of [$\gamma$-$^{32}$P] ATP a slow phase of dephosphorylation seemed to be present even in the absence of CDTA. This is explained by a $K^+$-stabilization of a dephosphoform of the enzyme [20,22]. This $K^+$-effect is most apparent at low concentrations of ATP [22]. Since $K^+$ stabilizes a dephosphoform and thus partly inhibits continuing $^{32}$P-labelling, the difference between the experiments without and with CDTA at the low concentration of [$\gamma$-$^{32}$P] ATP (Figs 2B andC) was less than in similar experiments at the higher concentration of [$\gamma$-$^{32}$P] ATP (Figs 3A and B).

When 10 mM KCl ± CDTA was added to the phosphoenzyme formed in the absence of KCl (Figs 3A and B) the rates of the initial $K^+$-stimulated dephosphorylation seemed to be too rapid to detect any significant difference. However, by the addition of 1 mM KCl plus CDTA (Fig. 3B) to the [$^{32}$P] phosphoenzyme the rate of the $K^+$-stimulated dephosphorylation was slow enough to observe a decreased rate of dephosphorylation as compared with the rate of dephosphorylation obtained by addition of 1 mM KCl alone (Fig. 3A). In experiments performed at 1 mM KCl plus low concentrations of CDTA (5 and 10 mM) an immediate increase in the rate of $K^+$-stimulated dephosphorylation was observed (not shown); this would be the result if CDTA mainly inhibits the phosphorylation step. Addition of CDTA or unlabelled ATP alone to a phosphoenzyme formed in the absence of KCl (Figs 3B and C) resulted in almost the same rates of dephosphorylation. CDTA may enhance a reversal of the phosphorylation step even if no extra ADP or $K^+$ is added; a decreased rate of the forward reaction may thus be neglected.

The rate constants of the slow phase of dephosphorylation in the presence of 10 mM KCl were similar in the different experiments (Figs 3B, C and 4). However, the first-order rate constant obtained in the presence of CDTA was a little lower than that obtained in the presence of unlabelled ATP. Since CDTA does not form tight complexes with monovalent cations [24] it is not likely that the decrease of the rate constant in the presence of CDTA is due to a binding of $K^+$ by CDTA. In order to test this hypothesis the concentration of $K^+$ was increased from 10 to 30 mM simultaneously to the addition of CDTA; no significant change was obtained indicating that already 10 mM $K^+$ was sufficient to saturate the enzyme (results not shown).

The present results may suggest that CDTA at high concentrations chelates $Mg^{2+}$ which is originally bound to the phosphoenzyme and that the $Mg^{2+}$-depleted enzyme is more slowly dephosphorylated by $K^+$ than the normal $Mg^{2+}$-containing phosphoenzyme. An $Mg^{2+}$ bound to the normal phosphoenzyme is also indicated by the requirement of $Mg^{2+}$ in the phosphorylation by $P_i$ [25].

The enzyme preparation used in the present investigation contained about 10—15% $Mg^{2+}$-dependent, ouabain-insensitive ATPase. The possibility that part of the $^{32}$P-labelling of the membranes, e.g. the $K^+$-insensitive and ADP-sensitive

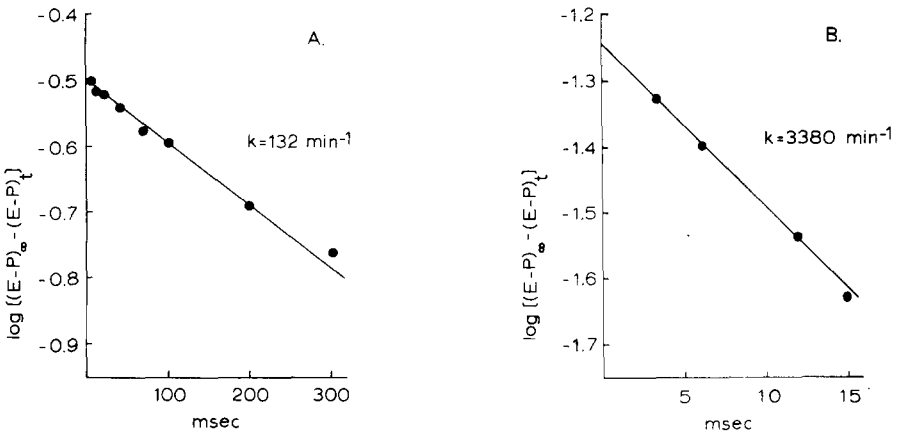


Fig. 8. $^{32}$P-labelling from [$\gamma$-$^{32}$P] ATP of nonradioactive phosphoenzyme. Phosphoenzyme was formed at 5 $\mu$M unlabelled ATP, 3 mM $MgCl_2$ and 120 mM NaCl in the absence (A) and presence (B) of 10 mM KCl. After about 70 ms, [$\gamma$-$^{32}$P] ATP was added to a final concentration of 100 $\mu$M. $[E\text{-}P]_\infty$ represents the steady-state level of $^{32}$P incorporated. $[E\text{-}P]_t$ represents the amount of $^{32}$P incorporated at different times. Correction was made for the change in specific radioactivity of [$\gamma$-$^{32}$P] ATP.

component, constitutes a $^{32}$P-labelling of the $Mg^{2+}$-dependent ATPase, can be excluded since the $^{32}$P-labelling with stability properties of acyl-[$^{32}$P] phosphate is formed only in the presence of $Mg^{2+}$ plus $Na^+$ and this $^{32}$P-labelling is completely inhibited by ouabain which is in contrast to the $Mg^{2+}$-dependent ATPase activity [6]. Nor do any of the kinetically different components consist of an enzyme · [$^{32}$P] ATP complex or unspecifically bound [$\gamma$-$^{32}$P] ATP [7] nor esterbound [$^{32}$P] phosphate or [$^{32}$P] phospholipids [6].

A more likely explanation of the two components would be two populations of vesicles with restricted accessibility for $K^+$ and ADP respectively. However, the same steady-state level of phosphoenzyme was obtained when KCl was added either before or after the addition of [$\gamma$-$^{32}$P] ATP to start phosphorylation (compare Figs 3A and 4). These results seem to exclude the existence of two populations of vesicles. A homogeneous enzyme preparation was also indicated by the observation of monophasic phosphorylation either [$\gamma$-$^{32}$P] ATP was added before or simultaneously with $Na^+$ [7,22]. Thus, it is concluded that the kinetically different forms of the phosphoenzyme represent two predominant forms of the phosphoenzyme itself.

Several facts favor a reaction model of $Na^+$,$K^+$-stimulated ATPase which includes a sequential formation of the two forms of [$^{32}$P] phosphoenzyme, i.e. the ADP/ATP exchange reaction is enhanced by inhibition of the formation of the $K^+$-sensitive form by *N*-ethylmaleimide [26] but this inhibition does not reduce the formation of [$^{32}$P] phosphoenzyme [8]. Inhibition by *N*-ethylmaleimide gives a continuous increase of the ADP-sensitive form [8] which corresponds to the $K^+$-insensitive form. A transient formation of [$^{32}$P] phosphoenzyme formed from [$\gamma$-$^{32}$P] ATP in the presence of $Mg^{2+}$, $Na^+$ and $K^+$, suggests a sequence of two kinetically different forms of the [$^{32}$P] phosphoenzyme, a $K^+$-insensitive form preceding a $K^+$-sensitive one [22]. Perhaps one of the strongest demonstrations of a sequence of different forms of the phospho-

enzyme is obtained by $^{32}$P-labelling from $^{32}P_i$ [25]. Under suitable conditions this $^{32}$P-labelling gives a $K^+$-sensitive form which is not sensitive to ADP, but which can be made ADP-sensitive and $K^+$-insensitive by the addition of $Na^+$. Addition of ADP to the latter form of [$^{32}$P]phosphoenzyme yields free [$\gamma$-$^{32}$P] ATP, i.e. a reversal of the ATPase reaction.

The dephosphorylation of both components of [$^{32}$P]phosphoenzyme found in the present investigation was rapid enough to permit these to participate in the overall hydrolysis of ATP. The components seem to correspond to the previously described $E_1$-P and $E_2$-P [2—5] in the sense that $E_1$-P is $K^+$-insensitive but ADP-sensitive and $E_2$-P is $K^+$-sensitive but ADP-insensitive. It is suggested that the former component ($E_1$-P) is dephosphorylated via the latter component ($E_2$-P) and probably not dephosphorylated in parallel or separate reactions [1,27]. If this suggestion is true the turnover of each component at steady state would be the same and it would equal the overall hydrolysis at any condition used for the experiments. This hypothesis was tested by addition of 100 $\mu$M [$\gamma$-$^{32}$P] ATP to a phosphoenzyme preformed at 5 $\mu$M unlabelled ATP in the presence of $Mg^{2+}$ and $Na^+$ without and with $K^+$. A concentration of 5 $\mu$M [$\gamma$-$^{32}$P] ATP is not sufficient to give maximal $^{32}$P-labelling of the enzyme [7]. Yet, at this low concentration an amount of [$^{32}$P]phosphoenzyme is obtained which is fairly close to the amount obtained at 100 $\mu$M [$\gamma$-$^{32}$P] ATP. Only a slow rate of $^{32}$P-labelling of about 46 nmol $\cdot$ mg$^{-1}$ $\cdot$ min$^{-1}$ was obtained in the absence of KCl (Fig. 8A). This was in contrast to the rapid $^{32}$P-labelling of about 270 nmol $\cdot$ mg$^{-1}$ $\cdot$ min$^{-1}$ observed in the presence of 10 mM KCl (Fig. 8B). In both experiments the rates were in good agreement with that of the overall reaction at 100 $\mu$M ATP, which was about 40 and 288 nmol $\cdot$ mg$^{-1}$ $\cdot$ min$^{-1}$ in the absence and presence of $K^+$, respectively (Table I). The curves seem to indicate first-order kinetics which should be the case if one of the components was a precursor for the other. These results are also in agreement with the finding that CDTA at high concentrations decreases the rates of both the initial rapid phase and the later slow phase of dephosphorylation (Figs 3B, 4 and 5).

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