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FRUCTOKINASE FROM RAT LIVER
II. THE ROLE OF K^+ ON THE ENZYME ACTIVITY

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SUMMARY

The effect of K^+ on the activation of fructokinase (ATP: D-fructose-1-phosphate transferase EC 2.7.1.3) from rat liver has been studied. Substrate (fructose) kinetics are Michaelian in the presence of K^+, but K^+ stimulates the reaction in a cooperative manner. ADP is a noncompetitive inhibitor towards fructose and competitive with respect to MgATP. K^+ partially reverts ADP inhibition. In a purely kinetic sense, ADP and K^+ are competitive antagonists. Fructokinase activity is dependent on the ratios $ATP^{4-}/MgATP^{2-}$ and $KATP^{3-}/MgATP^{2-}$. The optimal ratio varies depending on the enzyme saturation and the K^+ concentration. Slight modification in the level of ATP^{4-} or Mg^{2+} alter the optimal ratio, provoking inhibition by either ATP^{4-} or Mg^{2+}. K^+ can partially revert this inhibition.

INTRODUCTION

Fructokinase (ATP: D-fructose-1-phosphate transferase, EC 2.7.1.3) presents an interesting behaviour. It is strongly and noncompetitively inhibited by ADP and also inhibited by ATP in excess of equimolarity with Mg^{2+} (ref. 1). However, according to PARKS *et al.*[1] the important aspect of the inhibition by ATP is the fact that in the Michaelis region slight alteration of ATP or Mg^{2+} concentration may have relatively great effects on reaction velocity. Fructokinase is distinguished from other hexokinases by an absolute requirement for K^+. In light of this information it would seem appropriate to study if K^+ could modify the inhibition produced by ADP and ATP. This paper reports such a study carried out with the purified enzyme described in the preceding paper[2].

MATERIAL AND METHODS

All chemical and analytical procedures were the same as in the preceding communication[2], except for those mentioned below.

Solutions of ATP (disodium salt) were transformed into the Tris salt by passage through 10 cm × 0.5 cm column of Dowex-50 (H^+ form) and adjusted to pH 7.2 by addition of Tris in the free base form.

Enzyme determination

Assay d. In order to measure fructokinase under conditions which were not convenient for fructose or ADP measurement, a radiochemical test was carried out as follows: 0.25 μmole of [$^{14}C_6$]fructose ($2.4 \cdot 10^5$ counts/min per μmole), 0.3 μmole of ATP, 0.3 μmole of $MgCl_2$, 25 μmoles of KCl, 5 μmoles of Tris buffer (pH 7.4), and enzyme in a total volume of 0.05 ml. Incubation was carried out for 10 min at 37° and the reaction was stopped by adding 10 μl of 10% trichloroacetic acid or 10 μl of 0.2 M EDTA. Aliquots of the solution were spotted on 1 cm × 30 cm strips of Whatman No. 3 MM filter paper. The strips were chromatographed with butanol–pyridine–water (6:4:3, by vol.) for 15 h (ref. 3) or with ethylacetate–pyridine–water (12:5:4, by vol.) for 3–4 h (ref. 4). The area corresponding to fructose 1-phosphate was cut and counted in a Packard or Beckman scintillator with a toluene phosphor. A similar assay was developed by ADELMAN *et al.*[5] but using Whatman DEAE-cellulose paper. Attempts to use anion-exchange paper did not give reproducible results, presumably on account of the higher concentration of KCl used in the assay.

Calculations of concentrations of complex ions

The concentrations of the complex ion $MgATP^{2-}$ at each experimental point was calculated by employing a stability constant for $MgATP^{2-}$ of 20 000 M^{-1} at pH 8.0 in Tris–HCl buffer[6]. The quoted value of the stability constant for $MgATP^{2-}$ was chosen because the determinations were made in the presence of Tris–HCl buffer. The concentrations of the molecular species present in the Mg^{2+}–ATP system were calculated employing the equation given by MELCHIOR AND MELCHIOR[8] which can be used for pH 7.

The ionic species $HATP^{3-}$ was neglected on the assumption that its concentration was small under the experimental conditions used. On the other hand, since MELCHIOR[7] has demonstrated that K^+ forms complexes with ATP^{4-} and affects the kind and concentration of the molecular species present in the Mg^{2+}–ATP system, the concentration of $KATP^{3-}$ was calculated according to MELCHIOR AND MELCHIOR[8] who solve their equation assuming that Na^+ (or K^+) is approximately equal to total Na^+ (K^+). Parentheses enclosing a molecular formula with the subscript T, *e.g.* $(ATP)_T$, indicate the sum of the concentrations of all the molecular species.

RESULTS

K^+ effect

When ATP used in the reaction was neutralized with Tris, the enzyme was without activity in the absence of K^+, as can be seen in Fig. 1. Maximal activation was effected by 150 mM K^+, and the apparent Michaelis constant for this cation was $2 \cdot 10^{-2}$ M (inset Fig. 1). Expansion of the plot of v against K^+ concentration for values less than 40 mM reveal a sigmoid curve (Fig. 2). Such a relationship could be indicative of cooperativity between K^+ and a site on the enzyme.

ADP inhibition

Studies on ADP inhibition of fructokinase present the additional complication that ADP is produced as the reaction proceeds. However, this difficulty could be overcome by estimating enzyme activity at short intervals. Under this condition there is

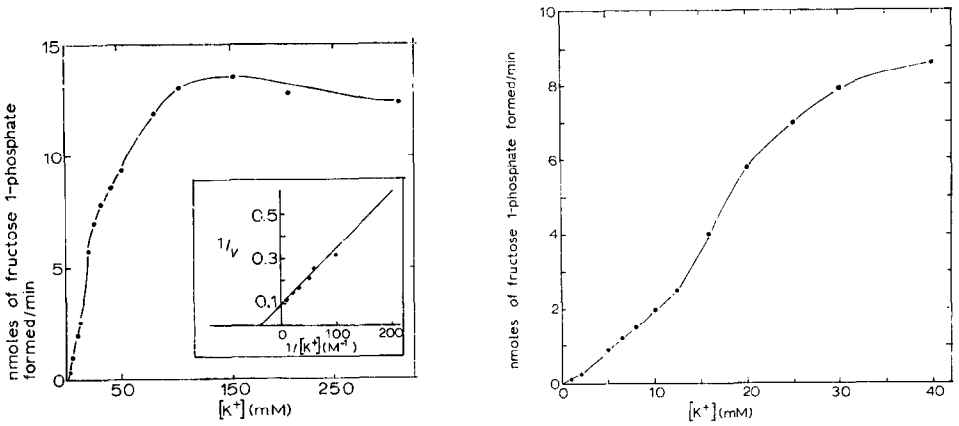

Fig. 1. Effect of K^+ concentration on the rate of fructose 1-phosphate synthesis and (inset) double-reciprocal plot for K^+ with fructose, 5 mM; $(Mg^{2+})_T$ and $(ATP)_T$, 10 mM.

Fig. 2. Expansion of the plot *v versus* K^+ concentration for values less than 40 mM. Condition as in Fig. 1.

no difference between determinations carried out in the absence or presence of an ATP-regenerating system.

The kinetics of ADP inhibition towards fructose is of the classical noncompetitive inhibitor type, *i.e.* reciprocal plots for fructose are linear and each inhibitor concentration increases the slopes changing the y intercept ($1/V$). However, ADP inhibition is modified by the K^+ concentration. The data illustrated in Fig. 3 show

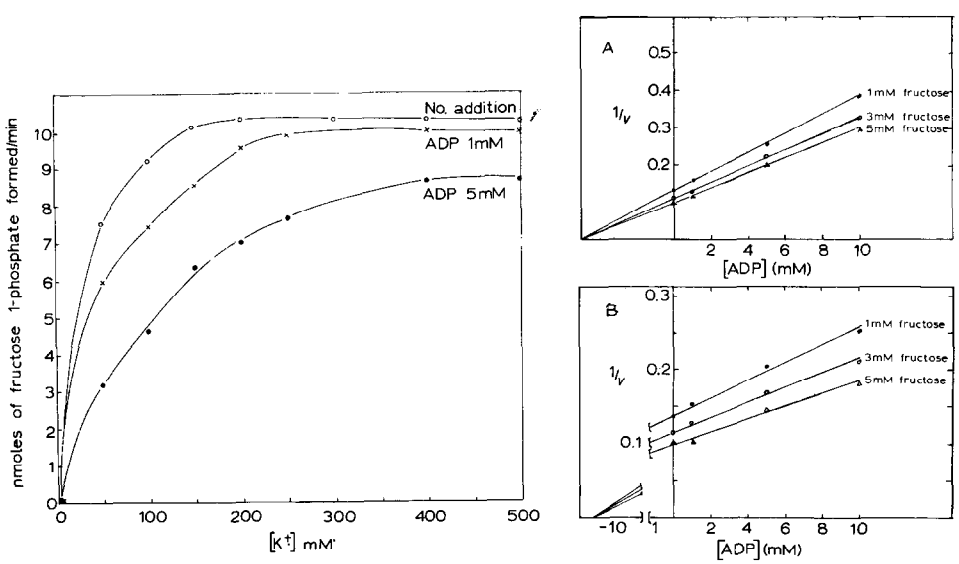

Fig. 3. Effect of ADP on the rate of fructose 1-phosphate synthesis at different K^+ concentrations. Fructose, 5 mM; $(Mg^{2+})_T$, 10 mM; $(ATP)_T$, 10 mM.

Fig. 4. Determination of the inhibition constant for ADP at different fructose concentrations. $(Mg^{2+})_T$, 10 mM; $(ATP)_T$, 10 mM. (A) 0.1 M K^+. (B) 0.4 M K^+.

TABLE I

INHIBITION CONSTANTS (K_i) FOR ADP (mM)

	0.1 M K+	*0.4 M K+*
Fructose, [$MgATP^{2-}$] = constant	6	12
$MgATP^{2-}$, [Fructose] = constant	1.7	2

that K^+ can partially revert the inhibition. Plots of $1/v$ against ADP at various fructose concentrations and holding $MgATP^{2-}$ concentration constant, at two different levels of K^+, show that K_i varies with the K^+ concentration (Fig. 4).

In order to determine the relationship between ADP, $MgATP^{2-}$ and K^+, experiments were carried out varying ADP and $MgATP^{2-}$ concentrations at two different levels of K^+, holding fructose concentration constant. The kinetics of ADP towards $MgATP^{2-}$ is of the competitive type (Fig. 5). The results of these experiments are summarized in Table I. It can be seen that different levels of K^+ modify the inhibition constant of ADP towards fructose without affecting that of ADP towards $MgATP^{2-}$.

The interaction of K^+ and ADP was studied further by means of experiments in which fructose and $MgATP^{2-}$ concentrations were held constant and both K^+ and ADP concentrations were varied. "Inhibition" plots of $1/v$ *versus* ADP concentration

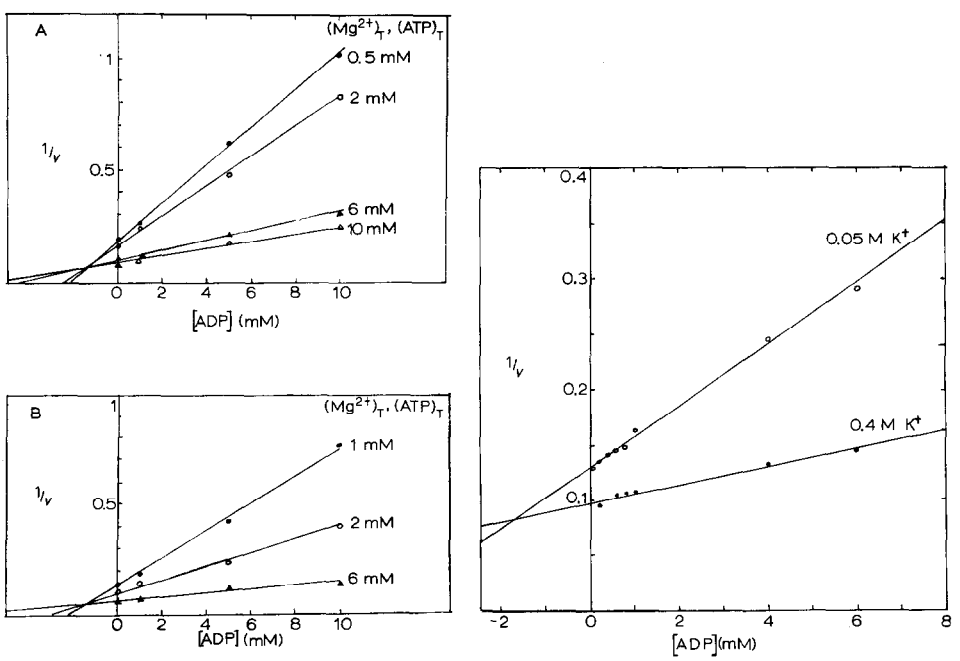

Fig. 5. Determination of the inhibition constant for ADP at different $(Mg^{2+})_T$ and $(ATP)_T$ concentrations. Fructose, 5 mM. (A) 0.1 M K^+. (B) 0.4 M K^+.

Fig. 6. Plots of $1/v$ *versus* ADP concentration at different K^+ concentration. Fructose, 5 mM; $(Mg^{2+})_T$ and $(ATP)_T$, 10 mM.

are rectilinear (Fig. 6), indicating that the interaction between K^+ and ADP is of competitive type with an apparent "K_i" of 1.7 mM.

Mg^{2+}–ATP interrelationship

Fructokinase activity is strikingly dependent on the ratio of $(Mg^{2+})_T$ to $(ATP)_T$. The curves in Fig. 7 illustrate the change in enzyme activity at various $(ATP)_T$, $(Mg^{2+})_T$ and $(K^+)_T$ concentrations. The figure also shows the variation of the ionic species $MgATP^{2-}$, $KATP^{3-}$, and Mg^{2+}.

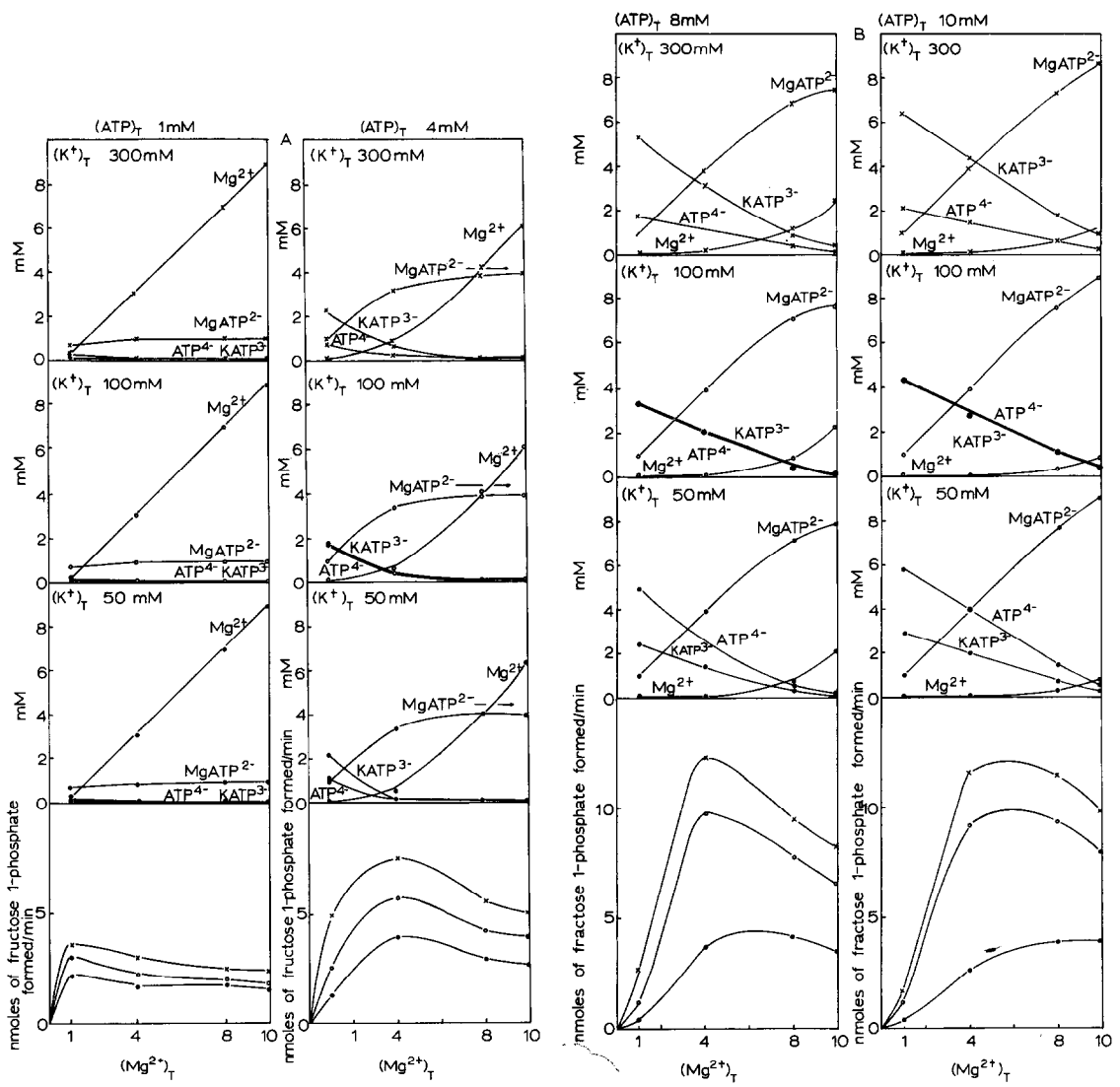

Fig. 7. $(Mg^{2+})_T$ velocity plots at different $(ATP)_T$ concentrations. Upper curves show the variation of the different ionic species in the Mg^{2+}–ATP system. (A) No saturated enzyme. (B) Saturated enzyme. Fructose, 5 mM. ●—●, 0.05 M K^+; ○—○, 0.1 M K^+; ×—×, 0.3 M K^+.

It can be seen that inhibition of the reaction occurs whenever ATP^{4-} or Mg^{2+} are in excess with respect to $MgATP^{2-}$. This inhibition can be partially reverted by increasing $(K^+)_T$ concentration. However, once the enzyme is saturated (Fig. 7B), the increase of activity that can be observed when $(K^+)_T$ concentration varies from 50 to 100 mM is parallel to the increase in the concentration of the ionic species $KATP^{3-}$ and a diminution of ATP^{4-}. On the other hand, there seems to be no variation in $MgATP^{2-}$. Optimal activity at $(K^+)_T$ above 50 mM is obtained when the relation $KATP^{3-}/MgATP^{2-}$ approaches unity while the relation $ATP^{4-}/MgATP^{2-}$ changes to approx. 0.4. It should be noticed that even under this condition slight modifications in the concentration of ATP^{4-} or Mg^{2+} would alter these ratios, provoking inhibition by either ATP^{4-} or Mg^{2+}.

DISCUSSION

The data concerning the effect of K^+ on the fructokinase activity does not permit a decision on the mechanism of its action. However, the kinetics effects of K^+ are apparently those expected of an allosteric activator; the velocity of the reaction is increased in a cooperative manner by K^+ (Fig. 2), whereas velocity is directly proportional to substrate (fructose) concentration. The activation process is such that K^+ increases the K_m for fructose, thus affecting the affinity of the enzyme for fructose. It should be noted that no significant change in the K_m for $MgATP^{2-}$ is brought by K^+ (see preceding paper)[2].

It has been shown[7] that both K^+ and Na^+ can form the complex ions $KATP^{3-}$ and $NaATP^{3-}$. Similar complexes with ADP^{3-} can also be formed but are far less stable[7]. It is difficult then to explain the interaction between K^+ and ADP^{3-} as purely a consequence of the formation of complex ions. K^+ counteracts the inhibition of ADP^{3-}, even if not completely. In the purely kinetic sense, ADP^{3-} and K^+ are competitive antagonists (Fig. 5). However, this does not mean that they compete for the same site, but rather and only, that their effect towards the catalytic site is competitive. It should be noted that the interaction between ADP and $MgATP^{2-}$ is of the competitive type, and that no significant change in the K_m for $MgATP^{2-}$ is brought by K^+. These facts, taken together, argue for the existence in the structure of the enzyme of two sites: one for binding ADP and $MgATP^{2-}$ and a second for fructose and K^+.

The effect of K^+ should be considered also in relation to the dependence of enzyme activity on the concentrations of ATP^{4-}, $MgATP^{2-}$, $KATP^{3-}$ and Mg^{2+}. The results presented here show that K^+ can counteract the inhibition produced by an excess of ATP^{4-} or Mg^{2+} (Figs. 7A and 7B). Furthermore, the data seem to indicate that the increase in activity is related to that of $(K^+)_T$ and not only to the increase in the formation of $KATP^{3-}$ (Fig. 7). Thus K^+ appears not only as an activator of the enzyme activity but also as a modifier. However, we are reluctant to classify fructokinase as an allosteric enzyme, as the data presented are not conclusive as to the existence of a binding site which differs from the catalytic site or sites.

The most important implications of the data presented in this paper refer to the interaction found for K^+, Mg^{2+}, ADP, and ATP which occurs at physiological concentrations[9]. From the results presented here it is tempting to speculate that coordinate changes of these metabolites would control the fructokinase activity *in*

vivo. If this were the case, one may ask about the sense of such regulation for fructokinase.

Two possibilities could be discussed. First, one might speculate that regulation of fructokinase could be connected with the conversion of fructose 1-phosphate to a nucleoside diphosphate derivative which might then be isomerized and finally hydrolyzed to give glucose, as MUNTZ[10] has suggested. This pathway would provide an alternative way of converting fructose to glucose[10].

On the other hand, more recent data seem to explain fructose utilization in liver after phosphorylation solely through the action of aldolase B (ref. 11). However, aldolase B activity is inadequate to keep up with fructokinase when large concentrations of fructose are administered[12]. Regulation of fructokinase activity could provide a way to correct this situation.

Finally, as a second possibility, it could be mentioned that the same metabolites that modify fructokinase activity also affect the activity of pyruvate kinase. In this connection EGGLESTON AND WOODS[13] have suggested that the concentration of fructose 1-phosphate could play a very important role in the activation of pyruvate kinase for attaining maximal rate of fructolysis.

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