Biochem. J. (1966) **101**, 70

Kinetic Studies with Liver Galactokinase

By F. J. BALLARD

Fels Research Institute, Temple University School of Medicine, Philadelphia 40, Pa., U.S.A.

(*Received* 3 *March* 1966)

1. Kinetic measurements of the forward reaction catalysed by ATP–galactose phosphotransferase were carried out with a purified preparation from pig liver. 2. The rate of reaction at pH 7·8 is dependent on the concentration of $MgATP^{2-}$ rather than total ATP or magnesium chloride concentrations. 3. The effect of changes in pH on K_m (galactose), K_m ($MgATP^{2-}$) and $V_{max.}$ was studied. 4. Of several possible nucleotide substrates only ATP and deoxyATP were effective. 5. The initial-velocity patterns both in the absence and presence of products were determined. 6. Galactose 1-phosphate is a non-competitive inhibitor when either galactose or $MgATP^{2-}$ was the variable substrate. 7. $MgADP^{-}$ was a non-competitive inhibitor with galactose and a competitive inhibitor with $MgATP^{2-}$ as variable substrate. 8. These results are consistent with an ordered reaction pathway in which galactose combines with an initial enzyme–$MgATP^{2-}$ complex.

Kinetic experiments, utilizing initial-velocity measurements both in the absence and presence of a reaction product, have been used to investigate the reaction mechanisms of many enzymes. Such studies have shown that even within a single group of enzymes, such as ATP-phosphotransferases, there does not seem to be any uniformity with regard to mechanism. Recent studies with creatine kinase (EC 2.7.3.2) (Morrison & James, 1965), yeast hexokinase (EC 2.7.1.1) (Fromm & Zewe, 1962*b*) and pyruvate kinase (EC 2.7.1.40) (Reynard, Hass, Jacobsen & Boyer, 1961) suggest that substrates combine with these enzymes in a random manner, although differences occur in the kinetics of product inhibition. Investigations of the reaction mechanism of brain hexokinase (Fromm & Zewe, 1962*a*) give evidence of a compulsory sequence of substrate addition, with one substrate combining with the enzyme and releasing a product before the addition of the second substrate. Similar results have been found with muscle hexokinase (Hanson & Fromm, 1965).

Wong & Hanes (1962) and Cleland (1963) have proposed simple rules for the prediction of enzyme mechanisms from initial-velocity and product-inhibition studies without analysing a large number of possible rate equations.

Although a bivalent metal cation such as Mg^{2+} or Mn^{2+} is generally considered essential for catalysis by ATP-phosphotransferases, conflicting evidence has been obtained with regard to the kinetic role of these cations. Proposals have been presented for an enzyme–metal ion complex for arginine kinase (EC 2.7.3.3) (Griffiths, Morrison & Ennor, 1957) and for the combination of lombricine kinase (EC 2.7.3.*b*) (Gaffney & O'Sullivan, 1964) or creatine kinase (Morrison & O'Sullivan, 1965) with either a metal ion–nucleotide complex or with free nucleotide.

In this present work with purified pig-liver galactokinase (EC 2.7.1.6), the effects of metal ions, changes in pH and nucleotide specificity are reported. Initial-velocity experiments were carried out without products and with either ADP or galactose 1-phosphate present. The results of these studies suggest a compulsory pathway in which the combination of $MgATP^{2-}$ with enzyme occurs before the binding of galactose.

MATERIALS AND METHODS

Chemicals. ATP, ADP, CTP, GTP, ITP, thymidine triphosphate, UTP, deoxyATP, deoxyCTP and deoxyGTP (all sodium salts) were obtained from P-L Biochemicals Inc., Milwaukee, Wis., U.S.A. Aqueous solutions of these compounds were adjusted to pH 7·8 with NaOH and standardized against known extinction peaks. Chelex-100 was purchased from Bio-Rad Laboratories, Richmond, Calif., U.S.A., and phosphoenolpyruvate, lactate dehydrogenase (EC 1.1.1.27), NADH and pyruvate kinase from Calbiochem, Los Angeles, Calif., U.S.A. [1-^{14}C]Galactose was obtained either from Nuclear–Chicago Corp., Des Plaines, Ill., U.S.A., or from International Chemical and Nuclear Corp., City of Industry, Calif., U.S.A. Chromatography on Whatman DEAE-cellulose paper with water as solvent showed 1–10% of a radioactive impurity that did not migrate near the solvent front. The impurity was removed by this chromatographic technique and the galactose eluted with water.

Assay of galactokinase. (1) ADP production. ADP formed in the galactokinase reaction from $MgATP^{2-}$ and galactose was measured spectrophotometrically at 340mμ with phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase and NADH by the rate of oxidation of NADH (Ballard, 1966). This assay was used during the enzyme purification and for some preliminary studies of the properties.

(2) Radioactive assay. [^{14}C]Galactose was converted into [^{14}C]galactose 1-phosphate by ATP in the presence of Mg^{2+}. The assay is similar to that used by Sherman & Adler (1963) for the *Escherichia coli* galactokinase. Unless otherwise stated, the assay mixture contained 0·2μmole of [^{14}C]galactose (2×10^5 counts/min.), 100μmoles of tris buffer (adjusted to pH 7·8 with HCl), 5μmoles of β-mercaptoethanol, ATP, $MgCl_2$ and enzyme in a total volume of 0·5ml. ATP and $MgCl_2$ were added at the concentrations indicated in each experiment. After addition of the reaction components, the tubes were preincubated at 37° for 5min. before the addition of enzyme. Samples (30μl.) of the reaction mixture were removed at three time-periods (usually 5, 10 and 15min.) and spotted on DEAE-cellulose paper strips. These strips were chromatographed, cut, and the radioactivity was counted in the galactose and galactose 1-phosphate areas as described by Sherman & Adler (1963). The maximum percentage conversion of galactose into galactose 1-phosphate was 20%. Under these conditions, the agreement between portions withdrawn from the reaction mixture after correction for the different incubation times was within ±5%. Velocity in all experiments was expressed in mμmoles of galactose 1-phosphate formed/min./μg. of enzyme at 37°.

Enzyme preparation. Galactokinase was purified from pig liver as previously described (Ballard, 1966) and stored at 0–4° as an ammonium sulphate suspension. The stability of each preparation was not completely reproducible but usually 80% of the activity remained after 2 weeks. In all experiments except those on the effects of metal ions the enzyme was diluted 1:50 with water and 50μl. (0·4μg. of enzyme protein) used for each enzyme assay.

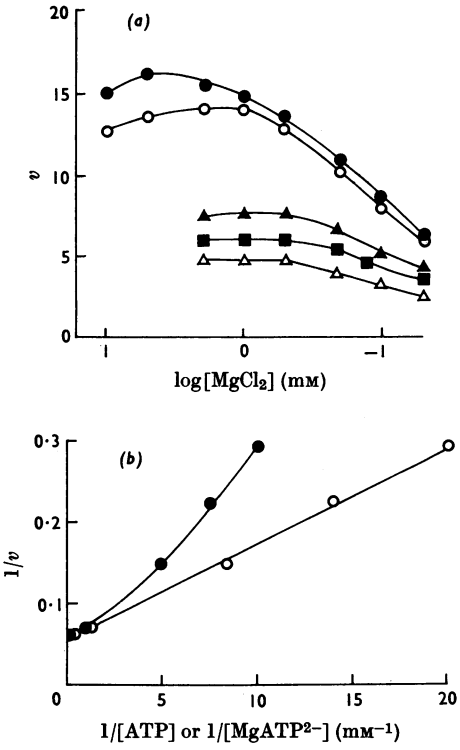


Fig. 1. (*a*) Effect of varying the concentration of total magnesium on the velocity of galactokinase at different concentrations of ATP added to the reaction mixture. The concentrations of ATP were: ●, 5mM; ○, 1mM; ▲, 0·2mM; ■, 0·13mM; △, 0·1mM. (*b*) Reciprocal plots of $1/v$ against 1/[ATP] (●) or 1/[$MgATP^{2-}$] (○). The points in this Figure are those values in Fig. 1(*a*) in which the concentrations of added magnesium and ATP were the same. The concentration of $MgATP^{2-}$ was calculated by using the stability constant of this ion (O'Sullivan & Perrin, 1961).

RESULTS

Effect of metal ions. Galactokinase in ammonium sulphate suspension was dissolved in 50mM-tris–hydrochloric acid buffer, pH 7·5, and dialysed for 24hr. against three changes of the same buffer. Samples of this solution were assayed at different concentrations of magnesium chloride and ATP. In the absence of added magnesium chloride the reaction rate of the undialysed preparation was approx. 10% of the maximum velocity. This rate was not significantly changed by dialysis of the enzyme but was decreased by treatment of the components of the reaction mixture with chelating resin (Chelex-100).

In the presence of magnesium chloride (Fig. 1*a*) maximum activity was obtained at an ATP/Mg^{2+} ratio 1 when the ATP concentration was high, whereas at lower ATP concentrations more magnesium chloride was required for optimum activity.

By using the stability constant of $MgATP^{2-}$ for the reaction:

$$MgATP^{2-} \rightleftharpoons Mg^{2+} + ATP^{4-}$$

as $20mM^{-1}$ in tris buffer (O'Sullivan & Perrin, 1961), the concentrations of $MgATP^{2-}$, ATP^{4-} and Mg^{2+} can be calculated in each experiment (Melchior & Melchior, 1958). The stability constant of $NaATP^{3-}$ is low ($0{\cdot}01\,mM^{-1}$; Melchior & Melchior, 1958) and would have a negligible effect on the concentration of $MgATP^{2-}$ at the concentration of Na^+ present in the reaction mixture.

A Lineweaver & Burk (1934) plot of $1/v$ against 1/[ATP] for the data in Fig. 1(*a*), where ATP and magnesium chloride were added at equimolar concentrations, is shown in Fig. 1(*b*). This plot is not linear, and only becomes linear when the variable

substrate is considered as $MgATP^{2-}$ rather than ATP. To avoid the calculation of the $MgATP^{2-}$ concentration for each assay of galactokinase, especially when other components are added to the reaction mixture, a large excess of magnesium chloride over ATP would be desirable. However, a large excess of magnesium chloride is inhibitory at high ATP concentrations (Fig. 1*a*) and consequently cannot be used.

In all later experiments a 1mM excess of magnesium chloride with respect to ATP was used. Under these conditions no inhibition by Mg^{2+} occurs, and the ATP present may be considered to exist as $MgATP^{2-}$ at the relatively high pH (7·8) used in the enzyme assays.

To investigate further the inhibition at high magnesium chloride concentrations, Dixon plots (Dixon & Webb, 1964) of $1/v$ against the calculated value of $[Mg^{2+}]$ were constructed at two concentrations of $MgATP^{2-}$ (Fig. 2). These data show that Mg^{2+} is a competitive inhibitor of $MgATP^{2-}$ with K_i 25mM. Small changes in the stability constant of $MgATP^{2-}$, caused by changes in ionic strength or by using a temperature of 37° compared with the 30° used by O'Sullivan & Perrin (1961), would have little effect on the Dixon plot or the conclusions drawn from these results.

Comparative effects of activating metal ions. Manganous chloride, ferrous chloride and calcium chloride in addition to magnesium chloride increase the galactokinase activity above that found in the absence of metal ion. The relative maximum activities of the metal ions are: Mn^{2+}, 100; Mg^{2+}, 77; Ca^{2+}, 18; Fe^{2+}, 18. With Mn^{2+}, Mg^{2+} and Ca^{2+}, the maximum activity was found at concentrations near 5mM, which was the concentration of ATP added to the reaction mixture. Metal ion concentrations above this level inhibited the reaction. Concentrations of ferrous chloride greater than 2mM or of manganous chloride greater than 10mM could not be used as precipitation occurred.

Addition of zinc chloride, cobalt chloride, cadmium chloride or nickel chloride did not stimulate galactokinase above the low endogenous level.

Nucleotide specificity. At concentrations of 5mM and 1mM, ITP, UTP, CTP, GTP, thymidine triphosphate, deoxyCTP and deoxyGTP were inactive as substrates for galactokinase. In addition to ATP only deoxyATP gave significant activity. The K_m for deoxyATP was 0·8mM and $V_{max.}$ was 66% of that found for ATP. To check that the deoxyATP did not contain a small ATP contaminant, a sample was chromatographed in a

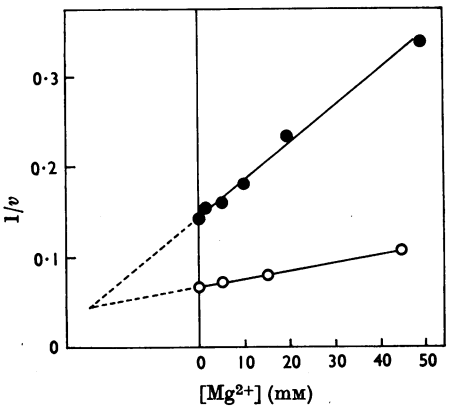


Fig. 2. Dixon plots of inhibition of galactokinase by excess of Mg^{2+}. The concentration of Mg^{2+} was calculated by using the stability constant of $MgATP^{2-}$ (O'Sullivan & Perrin, 1961). The $MgATP^{2-}$ concentrations were: ●, 0·1mM; ○, 5mM. This experiment gives K_i 25mM for Mg^{2+}.

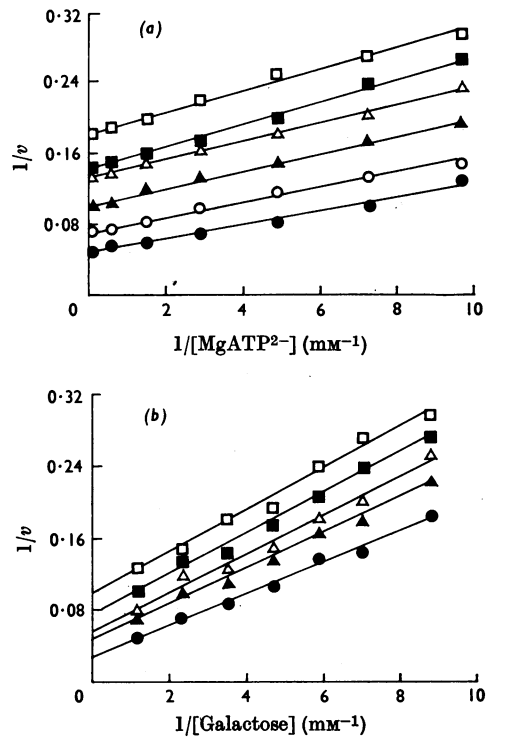


Fig. 3. (*a*) Effect of galactose concentration on the initial velocity of the galactokinase reaction with $MgATP^{2-}$ as the variable substrate. The concentrations of galactose were: □, 0·114mM; ■, 0·143mM; △, 0·171mM; ▲, 0·214mM; ○, 0·43mM; ●, 0·86mM. (*b*) Effect of $MgATP^{2-}$ concentration on the initial velocity of the galactokinase reaction with galactose as the variable substrate. The concentrations of $MgATP^{2-}$ were: □, 0·103mM; ■, 0·137mM; △, 0·206mM; ▲, 0·68mM; ●, 8·6mM.

system that separated the two compounds. Thin-layer chromatographic plates coated with MN cellulose powder 300 (Macherey, Nagel and Co., Düren, Germany) were developed in solvent isobutyric acid–aq. ammonia (sp.gr. 0·88)–water (96:1:22, by vol.) and the spots made visible in cyanogen bromide (Ziporin & Hanson, 1966). No spot with an R_F similar to that of ATP occurred in the commercial deoxyATP.

Initial-velocity experiments in the absence of products. In Figs. 3(*a*) and 3(*b*) are presented initial-velocity measurements for liver galactokinase. These Lineweaver & Burk (1934) plots are linear within the limits of experimental error and over the concentration ranges shown. The lines are not parallel, but will converge below the *x*-axis. Secondary plots of $1/V_{max.}$ against $1/s$ (not shown) from the experiment in Fig. 4 and three similar experiments give K_m (galactose) $0{\cdot}58 \pm 0{\cdot}04$mM (S.E.M.) and K_m ($MgATP^{2-}$) $0{\cdot}17 \pm 0{\cdot}02$mM.

Concentrations of galactose greater than 0·9mM were not used in these experiments as substrate inhibition occurs above this concentration.

Inhibition by ADP. Lineweaver & Burk (1934) plots of the effect of different concentrations of ADP are shown in Figs. 4(*a*) and 4(*b*). In these experiments equimolar quantities of magnesium chloride were added with the ADP. The results show that ADP (or $MgADP^-$) is a competitive inhibitor with respect to $MgATP^{2-}$, with K_i 0·20mM, and a non-competitive inhibitor with galactose.

Inhibition by galactose 1-phosphate. In Figs. 5(*a*) and 5(*b*) are shown double-reciprocal plots at different concentrations of galactose 1-phosphate. The inhibition is non-competitive when the concentration of either galactose or $MgATP^{2-}$ is varied. From secondary plots of $1/V_{max.}$ against galactose 1-phosphate concentration K_i 11·5mM is obtained.

Effect of pH. Galactokinase activities in tris buffer between pH 7 and pH 9 were used to determine the maximum velocity and the K_m values for

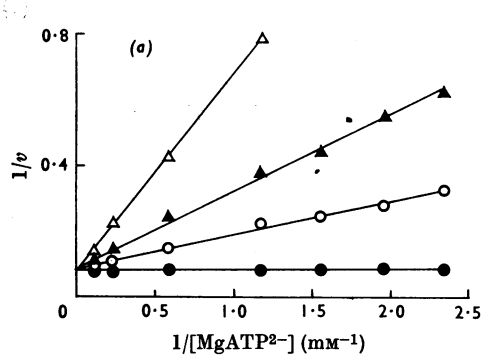


Fig. 4. (*a*) Product inhibition of galactokinase by $MgADP^-$ with $MgATP^{2-}$ as the variable substrate, with the concentration of galactose held constant at 0·4mM. The concentrations of $MgADP^-$ were: △, 10mM; ▲, 5mM; ○, 2·5mM; ●, none. (*b*) Product inhibition of galactokinase by $MgADP^-$ with galactose as the variable substrate, with the concentration of $MgATP^{2-}$ held constant at 1mM. The concentrations of $MgADP^-$ were: △, 10mM; ▲, 5mM; ○, 2·5mM; ●, none.

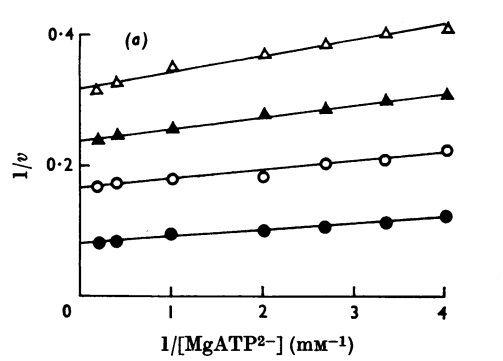


Fig. 5. (*a*) Product inhibition of galactokinase by galactose 1-phosphate with $MgATP^{2-}$ as the variable substrate, with the concentration of galactose held constant at 0·4mM. The concentrations of $MgATP^{2-}$ were: △, 30mM; ▲, 20mM; ○, 10mM; ●, none. (*b*) Product inhibition of galactokinase by galactose 1-phosphate with galactose as the variable substrate, the concentration of $MgATP^{2-}$ being held constant at 1mM. The concentrations of galactose 1-phosphate were: △, 40mM; ▲, 20mM; ○, 10mM; ●, none.

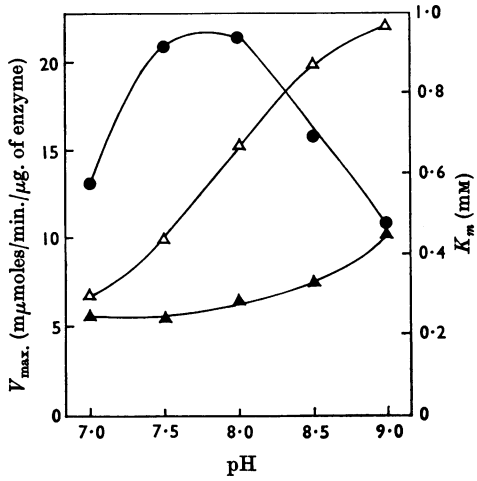


Fig. 6. Effect of pH on $V_{max.}$ (●), K_m (galactose) (△) and K_m ($MgATP^{2-}$) (▲), tris buffer being used. The $MgATP^{2-}$ concentration was held constant at 8·6mM for the determination of K_m (galactose) and the galactose concentration was held constant at 0·86mM for the determination of K_m ($MgATP^{2-}$). The $V_{max.}$ was taken as the average from reciprocal plots with varying galactose and varying $MgATP^{2-}$.

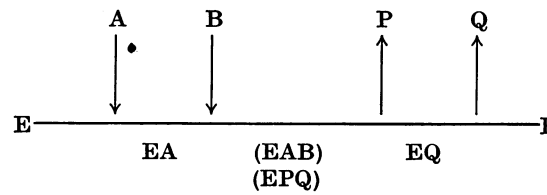
galactose and $MgATP^{2-}$ (Fig. 6). The pH optimum ($V_{max.}$) is 8, and the K_m values for both galactose and $MgATP^{2-}$ are lowest at pH 7. The interpretation of these data is complicated at low pH by the occurrence in significant amounts of $MgATP^{-}$ in addition to $MgATP^{2-}$. No allowance has been made for the concentration of $MgATP^{-}$ in these experiments.

DISCUSSION

The kinetic experiments performed in this study enable several conclusions to be drawn on the nature of the galactokinase reaction. The low activities measured in the absence of added Mg^{2+} probably do not indicate a true reaction without Mg^{2+}, but rather incomplete removal of this ion from the assay solutions. The reaction rate increased to a maximum when the concentration of Mg^{2+} was equal to or greater than that of ATP. Calculations of the concentrations of Mg^{2+}, ATP^{4-} and $MgATP^{2-}$ present indicate that activity is dependent on $MgATP^{2-}$, and provide strong evidence that this is the probable ionic form of the nucleotide substrate. This result has also been reported for creatine kinase (Morrison & James, 1965) and for muscle hexokinase (Hanson & Fromm, 1965). Inhibition at high Mg^{2+} concentrations has been found with several enzymes, including enolase (Wold & Ballou, 1957) and creatine kinase (Kuby, Noda & Lardy, 1954; Morrison & O'Sullivan, 1965). It cannot be determined at the present time whether the effect of excess of Mg^{2+} on galactokinase activity is due to its combination with the enzyme, to combination with the $MgATP^{2-}$ complex to form an inhibitory Mg_2ATP complex, or perhaps to non-specific effects such as an increase in ionic strength.

As shown originally by Segal, Kachmar & Boyer (1952) information on the mechanisms of two-substrate enzymic reactions may be obtained from analysis of experimental Lineweaver–Burk plots produced by holding the concentration of one substrate at several constant levels and determining initial velocities when the concentration of the second substrate is varied from below to well above the apparent K_m of that substrate. With galactokinase, linear plots are obtained that converge when extrapolated. Although these plots are close to parallel, the divergence can be repeated and is constant, so that secondary plots of slopes are linear with non-zero slopes. Several reaction mechanisms are compatible with these data: (1) a random interaction of substrates leading to ternary complexes, where all steps are rapid except the interconversion of the ternary complexes; (2) a compulsory pathway involving one or more ternary complexes; (3) a pathway such as that proposed by Theorell & Chance (1951) in which two binary complexes occur. Hanson & Fromm (1965) have proposed a mechanism for both muscle and brain hexokinases in which the product of the first substrate dissociates from the enzyme before the addition of the second substrate. Such a mechanism for galactokinase is unlikely as the Lineweaver–Burk plots in Figs. 3(*a*) and 3(*b*) would need to be parallel.

The non-competitive nature of the galactose 1-phosphate inhibition, when either galactose or $MgATP^{2-}$ is the variable substrate, precludes the binding of galactose and galactose 1-phosphate with the same enzyme form. This information, together with the finding that $MgADP^{-}$ is a competitive inhibitor when $MgATP^{2-}$ is varied, suggests that the initial reaction is a combination of $MgATP^{2-}$ with enzyme. These data obtained for galactokinase are very similar to the results of Wratten & Cleland (1963) with yeast or liver alcohol dehydrogenase (EC 1.1.1.1), from which the following compulsory pathway was derived:



For galactokinase, A, B, P, Q and E represent $MgATP^{2-}$, galactose, galactose 1-phosphate, $MgADP^{-}$ and enzyme respectively. Studies of isotopic exchange at equilibrium, however, have provided evidence for a random pathway for alcohol dehydrogenase (Silverstein & Boyer, 1964) rather than the ordered mechanism of Wratten & Cleland (1963). Similar studies may clarify the galactokinase mechanism.

Nucleotide substrates that differ from ATP either by removal of a phosphate (ADP) or by modification of the purine base (GTP) are not active with galactokinase. Pyrimidine nucleotides are not active. The reaction of deoxyATP with galactokinase indicates that the enzyme specificity towards the ribose portion of the molecule is not complete. Thus an alternative nucleotide substrate is reactive as well as alternative sugar substrates such as 2-deoxygalactose and galactosamine (Ballard, 1966).

The physiological significance of the results of inhibition studies with galactokinase is uncertain. The pH (7·8) used in these studies was chosen to simplify the interpretation of the metal ion–nucleotide kinetics, and is probably too far from the cell pH to permit quantitative comparisons. The high concentrations of magnesium and ADP reported in liver and other tissues (Long, 1963), together with the K_i values determined here, suggest that these inhibitors may exert a significant effect on galactokinase activity within the cell. The amounts of galactose 1-phosphate that accumulate in untreated galactosaemic infants and galactose-poisoned animals (Schwartz, Goldberg, Komrower & Holzel, 1956), although high, are probably not sufficient to exert any effective product inhibition of the galactokinase reaction.

The author is indebted to Dr Gerald Litwack and Dr Sidney Weinhouse for many helpful discussions during the course of these experiments. Financial assistance from the Commonwealth Scientific and Industrial Research Organization of Australia and from the U.S. National Institutes of Health (Grants AM5487 and CA7174) and from the American Cancer Society (Grant P202) is gratefully acknowledged.

REFERENCES

Ballard, F. J. (1966). *Biochem. J.* **98**, 347.
Cleland, W. W. (1963). *Biochim. biophys. Acta*, **67**, 188.
Dixon, M. & Webb, E. C. (1964). *Enzymes*, 2nd ed., p. 329. New York: Academic Press Inc.
Fromm, H. J. & Zewe, V. (1962*a*). *J. biol. Chem.* **237**, 1661.
Fromm, H. J. & Zewe, V. (1962*b*). *J. biol. Chem.* **237**, 3027.
Gaffney, T. J. & O'Sullivan, W. J. (1964). *Biochem. J.* **90**, 177.
Griffiths, D. E., Morrison, J. F. & Ennor, A. H. (1957). *Biochem. J.* **65**, 153.
Hanson, T. L. & Fromm, H. J. (1965). *J. biol. Chem.* **240**, 4133.
Kuby, S. A., Noda, L. & Lardy, H. A. (1954). *J. biol. Chem.* **210**, 65.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Long, C. (1963). *Biochemists' Handbook*. Princeton: D. Van Nostrand Co. Inc.
Melchior, N. C. & Melchior, J. B. (1958). *J. biol. Chem.* **231**, 609.
Morrison, J. F. & James, E. (1965). *Biochem. J.* **97**, 37.
Morrison, J. F. & O'Sullivan, W. J. (1965). *Biochem. J.* **94**, 221.
O'Sullivan, W. J. & Perrin, D. D. (1961). *Biochim. biophys. Acta*, **52**, 612.
Reynard, A. M., Hass, L. F., Jacobsen, D. D. & Boyer, P. D. (1961). *J. biol. Chem.* **236**, 2277.
Schwartz, V., Goldberg, L., Komrower, G. M. & Holzel, A. (1956). *Biochem. J.* **62**, 34.
Segal, H. G., Kachmar, J. F. & Boyer, P. D. (1952). *Enzymologia*, **15**, 187.
Sherman, J. R. & Adler, J. (1963). *J. biol. Chem.* **238**, 873.
Silverstein, E. & Boyer, P. D. (1964). *J. biol. Chem.* **239**, 3901.
Theorell, H. & Chance, B. (1951). *Acta chem. scand.* **5**, 1127.
Wold, F. & Ballou, C. E. (1957). *J. biol. Chem.* **227**, 313.
Wong, J. T.-F. & Hanes, C. S. (1962). *Canad. J. Biochem. Physiol.* **40**, 763.
Wratten, C. C. & Cleland, W. W. (1963). *Biochemistry*, **2**, 935.
Ziporin, Z. Z. & Hanson, R. W. (1966). *Analyt. Biochem.* **14**, 78.