Kinetic Studies of *Escherichia coli* Galactokinase*

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ABSTRACT: An improved method of isolation and purification has resulted in a stable preparation of galactokinase from *Escherichia coli* which is almost 85% pure. Using this preparation the forward and reverse reactions catalyzed by galactokinase have been studied kinetically at pH 8.0 in the presence and absence of products, and with the free magnesium concentration held constant at 1 mM. Isotopic exchange studies at equilibrium were also conducted. The initial velocity and product inhibition patterns are consistent with a random mechanism in which all steps are in rapid equilibrium except for the interconversion of the central ternary complexes, and in which two dead-end complexes (enzyme–galactose–MgADP and enzyme–MgATP–galactose 1-phosphate) are formed. Values have been determined for the Michaelis and dissociation constants involved in the combination of each substrate with various enzyme forms. The data from the isotopic exchange studies at equilibrium confirm the random nature of the mechanism and the existence of two dead-end complexes.

However, MgATP–MgADP exchange was found to be 2.5 times faster under a variety of conditions than galactose–galactose phosphate exchange. The mechanism of the reaction is thus truly random, and the rapid equilibrium assumption is not valid, although initial velocity, product inhibition, and isotopic exchange patterns are consistent with rate equations derived for the rapid equilibrium random model.

Galactokinase, the first enzyme in the familiar Leloir pathway (Cardini and Leloir, 1961; Kalckar, 1958), catalyzes the following reaction[1] at pH 8.0 in the presence of excess magnesium.

$$\text{galactose} + \text{MgATP}^{2-} \rightleftharpoons \text{MgADP}^{-} + \text{galactose 1-phosphate}^{2-} + \text{H}^{+}$$

Recently, galactokinase has been partially purified from *Escherichia coli* by Sherman and Adler (1963) and Wilson (1966), from yeast by Heinrick (1964), and from pig liver by Ballard (1966). The purification procedure for galactokinase from *E. coli* as devised by Sherman is relatively simple and can be completed in 24 hr resulting in a 40-fold purification with a 13% recovery. Wilson's purification procedure, on the other hand, is laborious; however, he obtains a more highly purified final fraction (100-fold purification) and a greater recovery (30%) than does Sherman. In this study a successful attempt was made to retain the simplicity of Sherman's procedure while obtaining the higher purification and greater recovery of Wilson's procedure.

To date, no thorough kinetic studies of galactokinase have appeared in the literature. Various authors (Sherman and Adler, 1963; Wilson, 1966; Heinrick, 1964, 1965; Ballard, 1966; Alvarado, 1960; Atkinson *et al.*, 1961; Cardini and Leloir, 1953) have conducted preliminary studies with their partially purified preparations; however, no attempt has been made to elucidate the mechanism of the galactokinase reaction. In this study the mechanism of the galactokinase reaction has been studied by initial velocity measurements both in the presence and absence of products and by isotopic exchange studies at equilibrium.

Experimental Procedure

Materials and Methods. The sodium salts of ADP and ATP were obtained from P. L. Biochemicals and were used without further purification. Stock solutions of both nucleotides were adjusted to pH 7.0 with 0.5 N NaOH and stored at −10°. Crystalline D-galactose was obtained from the Sigma Chemical Co. Galactose 1-phosphate dihydrate (A grade) and dithiothreitol were purchased from Calbiochem. Stock solutions of these compounds were adjusted to pH 7.0 and stored at −10°. [8-^{14}C]ATP and [8-^{14}C]ADP were purchased from Schwarz BioResearch Inc. and [1-^{14}C]-galactose from the New England Nuclear Corp. Triethanolamine was purchased from Eastman Organic Chemicals and used without further purification. A stock aqueous solution was prepared by weight and adjusted to the required pH with 5 N acetic acid. Formic acid was obtained from Mallinckrodt Chemical Works; EDTA and magnesium acetate were certified reagents from Fisher Scientific Co. Aqueous solutions of magnesium acetate were standardized by passing measured amounts through a Bio-Rad 50W-X8-H^{+}

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[1] Abbreviations used: ADP and ATP, adenosine di- and triphosphates.

TABLE I: Purification of *E. coli* Galactokinase.[a]

Fraction	Vol (ml)	Protein (mg)	Act. (units)	Sp Act. (units/mg)	Recov (%)
I. Sonic extract	103	1,620	98,010	60.4	100
II. Streptomycin sulfate supernatant	134	905	98,610	109	101
III. Ammonium sulfate (43–55%)	13.0	225	90,540	403	92.4
IV. Bio-Gel P-60 eluate	56.5	59.8	77,800	1,300	79.4
V. DEAE-cellulose eluate	16.8	7.8	51,600	6,610	52.7

[a] See Experimental Procedure for details.

column and titrating the effluent acid with standard alkali. Crystalline bovine serum albumin was a product of the Armour Pharmaceutical Co. Distilled, deionized water was used for the preparation of all reagents.

Cellex-D anion-exchange cellulose (capacity, 0.61 mequiv/g) and Bio-Rad AG 50W-X8 cation exchanger (hydrogen form) were purchased from Bio-Rad Laboratories. DEAE-cellulose paper (DE-81) was obtained from E. H. Sargent and Co. (capacity, 0.4 mequiv/g; basis weight, 85 g/m^2). Sephadex G-50 (fine grade) was a product of Pharmacia Laboratories, Inc. Bio-Gel P-60, P-100, and P-150 were obtained from Calbiochem.

Protein was determined by the Biuret method after precipitation of the protein with 10% trichloroacetic acid, or from the optical density at 279 mμ, assuming 1 mg/ml gives 1.0 OD/cm. The concentrations of the free and complexed nucleotides in reaction mixtures were calculated as described by Morrison *et al.* (1961) using the appropriate apparent stability constants of the magnesium complexes. The values for $MgATP^{2-}$ and $MgADP^{-}$ at pH 8.0, ionic strength of 0.1 M, and 30° were taken to be 70,000 and 4000 M^{-1} respectively (Morrison *et al.*, 1961; Kuby and Noltman, 1962; O'Sullivan and Perrin, 1964).

Galactokinase Assay. During the isolation procedure the assay of Sherman and Adler (1963) was used except that the buffer was 0.1 M triethanolamine–acetate (pH 8.0); the SH-protecting agent was dithiothreitol (1.5 mM); magnesium acetate replaced magnesium chloride; and the assay time was 6 min. One unit of enzyme is defined as that amount of enzyme that phosphorylates 1 μmole of D-galactose/hr under these conditions.

For kinetic studies, the reaction mixture contained in a total volume of 0.2 ml: 0.1 M triethanolamine–acetate (pH 8.0), 1 mM dithiothreitol, and substrates and Mg^{2+} at the concentrations indicated. After the addition of the components the tubes were kept in ice, but before the addition of the enzyme they were incubated for 3 min at 30°. Dilutions of the enzyme were made in 1 mM triethanolamine–acetate (pH 8.0), containing 1.3 mM dithiothreitol. The reactions were initiated by the addition of the enzyme and at least four 30-μl samples were taken at equal time intervals and spotted on DEAE-cellulose anion-exchange paper. Initial velocities were determined from the slopes of plots of radioactivity found in product *vs.* time. All studies were at 30°.

Sherman and Adler (1963) reported that the spotting of samples of the galactokinase reaction on DEAE-cellulose anion-exchange paper will stop the enzymatic reaction. This was found to be true when the reverse reaction (MgADP and galactose 1-phosphate as substrates) was studied; however, for the forward reaction this was found to vary from batch to batch of DEAE-cellulose paper. Since galactokinase is unstable in the presence of organic solvents (Trucco *et al.*, 1948) various solvents were tested for their ability to stop the galactokinase reaction by saturating the DEAE-cellulose paper with solvent after a sample of the reaction mixture had been spotted on the paper. Absolute ethanol was found to be effective in stopping the reaction; therefore, this ethanol treatment was used in all studies of the forward reaction. No treatment was used when the reverse reaction was studied.

[1-^{14}C]Galactose 1-phosphate was measured as the radioactivity remaining on DEAE-cellulose paper after elution of [1-^{14}C]galactose from the paper with water as described by Sherman and Adler (1963). Separation and counting of radioactive ATP and ADP were performed with DEAE-cellulose paper chromatography as described by Morrison and Cleland (1966).

When the effects of free Mg^{2+} on the initial velocity of the forward and reverse reaction were studied, 1 mM free Mg^{2+} was found to be the highest level that could be used without appreciable inhibition by the free Mg^{2+}. All kinetic experiments unless otherwise stated were therefore done with a free Mg^{2+} concentration of 1 mM. For the forward reaction, the pH optimum is between 8.0 and 8.5. All of the kinetic experiments both for the forward and reverse reaction have been carried out at pH 8.0 since the dissociation constants for the magnesium nucleotide complexes have been determined at that pH and are highly dependent upon pH.

Enzyme Preparation. Galactokinase was isolated from *E. coli* as described by Sherman and Adler (1963) except that a constitutive strain of *E. coli* (H-81-B_1^-, obtained from Dr. Harrison Echols) was used as the source of galactokinase and the cells were grown

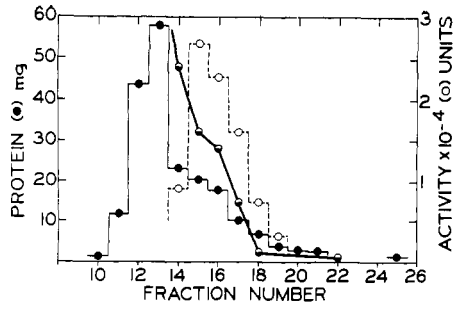


FIGURE 1: Fractionation of galactokinase on Bio-Gel P-60. The procedure for the gel filtration was the same as described by Sherman and Adler (1963) with the exceptions mentioned in the text. The column used was 50 × 3.5 cm and fractions of 10 ml were collected. (◒——◒) Isotopic exchange activity between MgATP and MgADP caused by some enzyme other than galactokinase (see text). The reaction mixture for assay of this exchange activity contained 0.1 M triethanolamine–acetate (pH 8.0), 2 mM ATP, 2 mM [^{14}C]ADP, 6 mM potassium fluoride, 3 mM dithiothreitol, and 6 mM magnesium acetate.

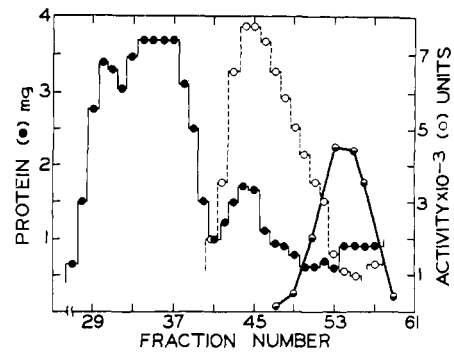


FIGURE 2: DEAE-cellulose chromatography of galactokinase. Fraction IV was chromatographed on DEAE-cellulose as described by Sherman and Adler (1963) with the exceptions given in the text. Fractions (8 ml) were collected. (◒——◒) Isotopic exchange activity between MgATP and MgADP. See Figure 1 for the reaction mixture used to measure this exchange activity.

for only 24–28 hr. The only buffer used in the isolation procedure contained 0.04 M triethanolamine–acetate (pH 7.6), 1 mM magnesium acetate, 1.5 mM EDTA, 0.1 mM galactose, 0.1 mM ATP, and 1 mM dithiothreitol. Bio-Gel P-60 replaced Sephadex G-50 in the gel filtration step, and the assay for galactokinase was changed as described above. The results obtained with this modified purification procedure are shown in Table I. The results of the P-60 gel filtration step are shown in Figure 1, and Figure 2 demonstrates the separation obtained by DEAE-cellulose chromatography.

Crude fractions of galactokinase catalyzed isotopic exchange between MgATP and MgADP in the absence of galactose and galactose 1-phosphate, but not between galactose and galactose 1-phosphate in the absence of the nucleotides. The exchange activity between MgATP and MgADP was found to be associated with protein which was eluted with the main protein peak during the Bio-Gel P-60 chromatography (Figure 1) and was eluted from the DEAE-cellulose column directly after the galactokinase peak (Figure 2). Removal of this activity was necessary in order to carry out the isotopic exchange studies described in this paper.

Polyacrylamide disc electrophoresis was performed on fraction V as described by Davis (1964) except that 1 mM dithiothreitol was present in the buffer. An optical density recording of the resulting electrophoretic pattern showed two major peaks, one accounting for 80–90% of the protein and the other for 10–20%. A few minor peaks totaled less than 1% of the protein. On the basis of the relative proportions of the bands during purification, the major peak appears to be galactokinase.

The purified enzymes obtained from various preparations (0.4–0.7 mg/ml of protein) were stored at −10° in 0.04 M triethanolamine–acetate (pH 7.6), containing 1 mM magnesium acetate, 1.5 mM EDTA, 1 mM dithiothreitol, 0.1 mM galactose, and 0.1 mM ATP. Such stock solutions are stable for at least 7 months when stored at −10°, even when thawed and refrozen several times. Elimination of the stability problems encountered by Sherman and Adler (1963) and Wilson (1966) is most probably the result of the use of dithiothreitol as the SH-protecting agent (Cleland, 1964).

Before use all enzyme preparations were shown to be free of activators and inhibitors and to be stable under the assay conditions by verifying that the enzyme times time relationship held. Most of the kinetic studies were done with an enzyme preparation of specific activity 6610 units/mg of protein, but some of the earlier studies were done with a preparation of specific activity 4109. Galactokinase was found to be stable for more than 80 min after dilution of stock solutions with 1 mM triethanolamine–acetate (pH 8.0), containing 1 mM dithiothreitol. No kinetic experiments lasted for more than 80 min.

Data Processing. Reciprocal velocities were first plotted graphically against the reciprocals of substrate concentrations. When these plots were linear, the data were fitted to eq 1 using a least-squares method and assuming equal variance for the velocities (Wilkinson, 1961).

$$v = \frac{VA}{K + A} \tag{1}$$

All least-squares fits were performed by a digital computer using the FORTRAN programs of Cleland (1963a). These programs provide values for the constants in a fitted equation, standard errors of their estimates, and

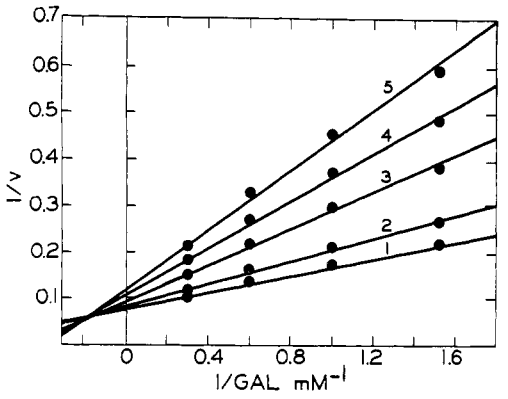


FIGURE 3: Initial velocity pattern with galactose as the varied substrate. MgATP concentrations: (1) 0.884 (2) 0.590, (3) 0.295, (4) 0.205, and (5) 0.154 mM. Initial velocities are expressed as millimicromoles of [1-^{14}C]-galactose 1-phosphate per minute. Enzyme concentration, 0.29 μg/ml.

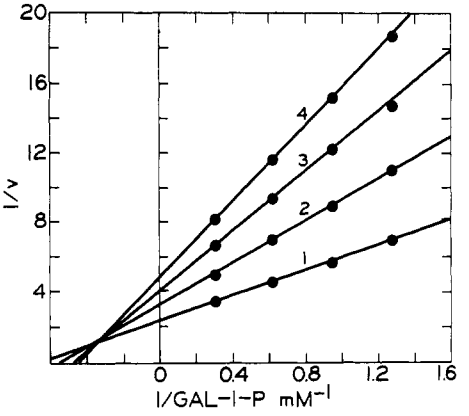


FIGURE 4: Initial velocity pattern with galactose 1-phosphate as the varied substrate. MgADP concentrations: (1) 0.502, (2) 0.250, (3) 0.166, and (4) 0.125 mM. Initial velocities are expressed as millimicromoles of [8-^{14}C]ATP per minute. Enzyme concentration, 2.9 μg/ml.

weighting factors for further analysis. Slopes (K/V) and intercepts ($1/V$) obtained from fits to eq 1 were then plotted graphically against either the inhibitor concentration (for inhibition experiments) or the reciprocal of the nonvaried substrate concentration (for initial velocity experiments) to determine the form of the over-all rate equation. Final values for the kinetic constants were obtained by fitting all data points used in the first analysis to this over-all equation. Data conforming to a sequential initial velocity pattern were fitted to eq 2; data conforming to linear competitive inhibition were fitted to eq 3; data conforming to linear noncompetitive inhibition were fitted to eq 4; and data showing substrate inhibition were fitted to eq 5.

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \quad (2)$$

$$v = \frac{VA}{K(1 + I/K_{is}) + A} \quad (3)$$

$$v = \frac{VA}{K(1 + I/K_{is}) + (1 + I/K_{ii})A} \quad (4)$$

$$v = \frac{VA}{K + A + A^2/K_i} \quad (5)$$

If the original double-reciprocal plot appeared parabolic, the data were fitted to eq 6.

$$v = \frac{VA^2}{a + bA + A^2} \quad (6)$$

The points drawn in figures showing double-reciprocal plots are the experimentally determined values. The lines drawn through these points are calculated from fits of these data to eq 1 unless otherwise stated in the figure caption.

Results

Initial Velocity Patterns. When galactose and MgATP were substrates, the initial velocity pattern shown in Figure 3 was obtained. The data appear to fit eq 2, indicating a sequential mechanism (that is, both substrates must add before either product is released). An identical pattern was obtained with galactose 1-phosphate and MgADP (Figure 4). The kinetic constants from fits of these data to eq 2 are shown in Table II.

In order to determine accurately the ratio between maximum velocities in forward and reverse directions, it is necessary to determine both maximum velocities

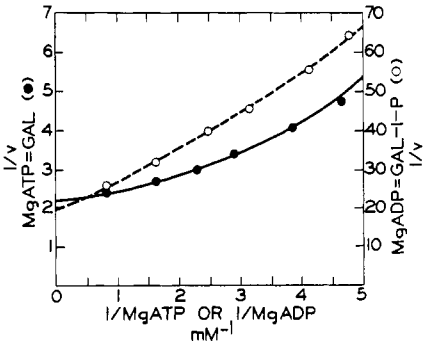


FIGURE 5: Reciprocal plots with the concentration of both substrates varied in a constant ratio. The enzyme concentration for the reverse reaction (2.58 μg/ml) was nine times that for the forward reaction. The data were fitted to eq 6.

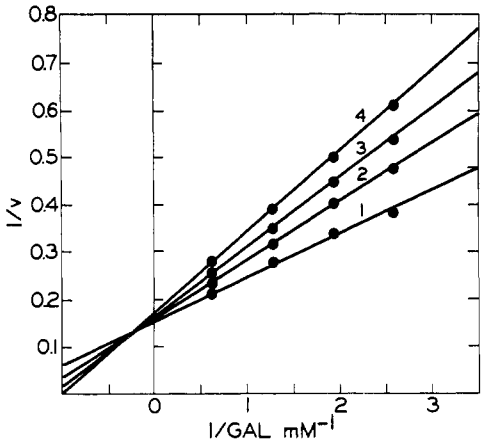


FIGURE 6: Reciprocal plots with galactose as the varied substrate and galactose 1-phosphate as inhibitor. The MgATP concentration was 0.641 mM. Galactose 1-phosphate concentrations: (1) 0, (2) 0.394, (3) 0.910, and (4) 1.51 mM. Initial velocities are expressed as millimicromoles of [1-^{14}C]galactose 1-phosphate per minute. Enzyme concentration, 0.58 μg/ml.

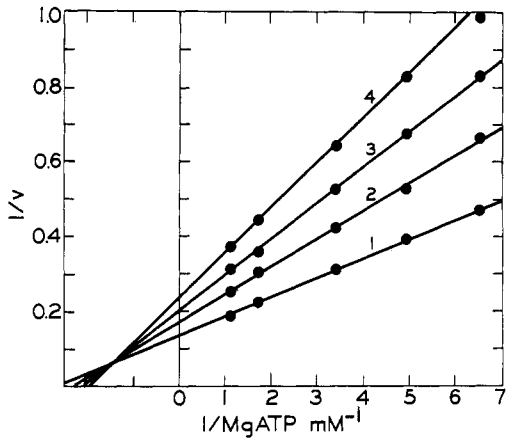


FIGURE 7: Reciprocal plots with MgATP as the varied substrate and galactose 1-phosphate as inhibitor. The galactose concentration was 0.650 mM. Galactose 1-phosphate concentrations: (1) 0, (2) 0.785, (3) 1.57, and (4) 2.36 mM. Initial velocities are expressed as millimicromoles of [1-^{14}C]galactose 1-phosphate per minute. Enzyme concentration, 0.58 μg/ml.

in the same experiment with the same enzyme solution. For this purpose, maximum velocities are most easily determined by varying the concentrations of both substrates in a constant ratio. Examination of eq 2 shows that if $A = xB$, where x is any constant, eq 2 reduces to eq 6. Thus, if the concentrations of A and B are varied together in constant ratio, $1/v$ becomes a parabolic function of $1/A$ or $1/B$, and the vertical intercept of the reciprocal plot gives $1/V$. Figure 5 shows the results of such an experiment. The ratio of maximum velocities determined from fits to eq 6 is given in Table II.

TABLE II: Kinetic Constants for Galactokinase Determined from Initial Velocity and Product Inhibition Studies.[a]

Kinetic Constant	Initial Velocity (mM)	Product Inhibn (mM)
K_a	0.64 ± 0.04	0.3 – 0.7
K_{ia}	2.16 ± 0.33	0.3 – 0.66
K_{Ia}		0.9 ± 0.1
K_b	0.066 ± 0.008	
K_{ib}	0.22 ± 0.02	<0.2
K_{Ib}		0.2
K_p	0.275 ± 0.012	
K_{ip}	0.703 ± 0.043	<0.24
K_{Ip}		0.20 ± 0.07
K_q	1.05 ± 0.06	
K_{iq}	2.97 ± 0.17	0.85 – 1.0
K_{Iq}		1.8 ± 0.2
V_1/V_2	79.5 ± 2.0	

[a] Identifying subscripts: a, galactose; b, $MgATP^{2-}$; p, $MgADP^{-}$; q, galactose 1-phosphate. The values from initial velocity studies are in each case weighted means of constants from fits to eq 2 of three sets of data, including those from Figures 3 and 4.

Product Inhibition Patterns. Galactose 1-phosphate gave linear competitive inhibition with galactose as variable substrate (Figure 6), but the apparent K_{is} values from fits to eq 3 varied with the concentration of MgATP. At 0.415 mM MgATP, values of 0.96 ± 0.12 and 1.08 ± 0.09 mM were obtained for K_{is}, while at 0.641 mM MgATP the value was 1.49 ± 0.10 mM. With MgATP as variable substrate, galactose 1-phosphate gave linear noncompetitive inhibition (Figure 7). Apparent K_{is} and K_{ii} values from fits to eq 4 varied with the concentration of galactose. At 0.65 mM galactose values of 1.74 ± 0.10 and 3.57 ± 0.26 mM were obtained for K_{is} and K_{ii}, while at 1.61 mM galactose, values of 3.0 ± 0.8 and 3.6 ± 1.2 mM were obtained for K_{is}, and 7.3 ± 1.6 and 6.0 ± 1.4 mM for K_{ii}.

MgADP gave linear competitive inhibition *vs.* MgATP (Figure 8), but again the apparent K_{is} values from fits to eq 3 seemed to vary with the concentration of galactose. Apparent K_{is} values of 0.243 ± 0.019 and 0.290 ± 0.005 mM were obtained at 0.391 and 0.782 mM galactose. With galactose as variable substrate, MgADP gave linear noncompetitive inhibition (Figure 9), with apparent K_{is} and K_{ii} values from a fit to eq 4 of 1.0 ± 0.4 and 1.4 ± 0.5 mM at 0.398 mM MgATP.

Equilibrium Constant. The equilibrium constant of the galactokinase reaction was determined at pH 8.0 and 30° by incubating 2.62 mM galactose 1-phosphate, 0.83 mM [^{14}C]MgADP, and 5 mM Mg^{2+} in the presence

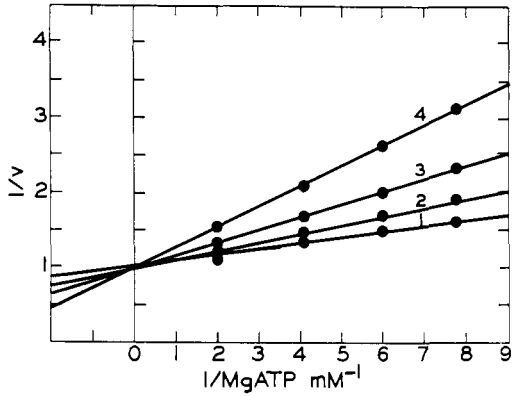


FIGURE 8: Reciprocal plots with MgATP as the varied substrate and MgADP as inhibitor. The galactose concentration was 0.391 mM. MgADP concentrations: (1) 0, (2) 0.151, (3) 0.293, and (4) 0.568 mM. Initial velocities are expressed as millimicromoles of [1-^{14}C]-galactose 1-phosphate per minute. Enzyme concentration, 0.58 μg/ml.

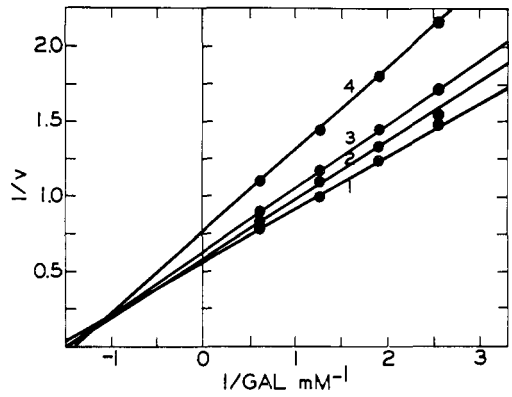


FIGURE 9: Reciprocal plots with galactose as the varied substrate and MgADP as inhibitor. MgATP concentration was 0.398 mM. MgADP concentrations: (1) 0, (2) 0.132, (3) 0.274, and (4) 0.472 mM. Initial velocities are expressed as millimicromoles of [1-^{14}C]galactose 1-phosphate per minute. Enzyme concentration, 0.39 μg/ml.

of 3.87 μg/ml of galactokinase until equilibrium was reached. In six experiments, values for K_{eq}, defined as

$$K_{eq} = \frac{[MgADP^{-}][galactose\ 1\text{-}phosphate^{-2}]}{[MgATP^{-2}][galactose]}$$

were obtained ranging from 174 to 210, with a mean of 190 ± 13. This value may be compared with a value of 235 derived from the one reported by Atkinson *et al.* (1961) by correcting for the difference in pH values.

Initial Velocities of Isotopic Exchange at Equilibrium. All experiments were carried out at pH 8.0 and 30° in the presence of 5 mM free Mg^{2+}, and the concentrations of reactants were adjusted to give a (products/reactants) ratio of 190. Trial experiments to determine reaction times and enzyme concentrations were not necessary, since an estimate of the initial exchange rate to be expected under the various equilibrium conditions could be calculated by substituting the equilibrium concentrations of reactants, and values of the kinetic constants from Table II into eq 12 (see Discussion).

The initial velocities of the MgATP–MgADP and galactose–galactose 1-phosphate exchanges were determined under three sets of equilibrium conditions (Table III), and in each case there was a 2.5-fold difference in the exchange rates with the MgATP–MgADP exchange occurring at a faster rate than the galactose–galactose 1-phosphate exchange.

When the concentrations of galactose and galactose 1-phosphate were varied with a constant ratio between them so that equilibrium was maintained, reciprocal plots of initial velocity of isotopic exchange vs. galactose concentration were linear (Figure 10), showing that the rate equation has the form of eq 1, although the rates for galactose–galactose 1-phosphate and MgATP–MgADP exchange were not identical. A linear reciprocal plot was also obtained when the concentrations of MgATP and MgADP were varied in a constant ratio and the initial velocity of galactose–galactose 1-phosphate exchange was measured (Figure 11).

When the concentrations of MgATP and galactose 1-phosphate were varied together, however, the reciprocal plot of galactose–galactose 1-phosphate-exchange rate was not linear, but appeared to have the form predicted by eq 5 (Figure 12). Similar behavior was observed when the concentrations of galactose and

TABLE III: Isotopic Exchange Rates at Equilibrium.[a]

Expt	MgATP–MgADP (mμmoles/min)	Galactose–Galactose 1-Phosphate (mμmoles/min)	Ratio
1	0.982	0.370	2.66
2	0.623	0.252	2.47
3	0.371	0.147	2.53

[a] The reaction mixtures contained in 0.2 ml the following components in 0.1 M triethanolamine–acetate (pH 8.0) at 30° in the presence of 1 mM dithiothreitol, 5 mM Mg^{2+}, and 4.64 μg/ml of enzyme: (1) 0.242 mM galactose, 0.242 mM MgATP, 3.24 mM MgADP, and 3.24 mM galactose 1-phosphate; (2) 0.1 mM galactose, 0.1 mM MgATP, 1.38 mM MgADP, and 1.38 mM galactose 1-phosphate; and (3) 0.05 mM galactose, 0.05 mM MgATP, 0.69 mM MgADP, and 0.69 mM galactose 1-phosphate.

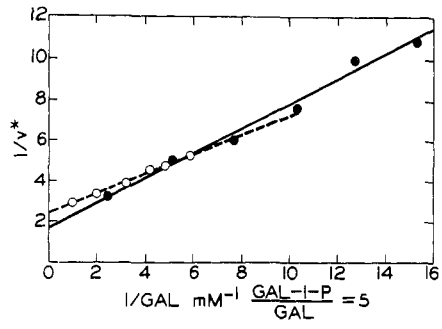


FIGURE 10: Double-reciprocal plot of the effect of raising the galactose–galactose 1-phosphate pair on the initial velocity of the galactose–galactose 1-phosphate and MgATP–MgADP exchanges. Initial velocities of isotopic exchange are expressed as millimicromoles of [1-^{14}C]galactose 1-phosphate or [8-^{14}C]MgADP per minute. Enzyme concentration, 4.64 μg/ml. (●——●) Galactose–galactose phosphate exchange with MgADP, 3.56 mM, and MgATP, 0.0935 mM. (O- - -O) MgATP–MgADP exchange with MgADP, 2.58 mM, and MgATP, 0.068 mM. The two plots show the results from separate experiments.

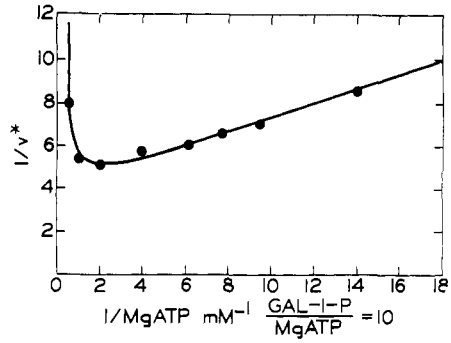


FIGURE 12: Double-reciprocal plot of the effect of raising the MgATP–galactose 1-phosphate pair on the initial velocity of the galactose–galactose 1-phosphate exchange. Initial velocities of isotopic exchange are expressed as millimicromoles of [1-^{14}C]galactose 1-phosphate per minute. Galactose, 0.10 mM; MgADP, 1.90 mM. Enzyme concentration, 4.64 μg/ml. The data were fitted to eq 5.

MgADP were varied together and the initial velocity of the MgATP–MgADP exchange was measured (Figure 13).

Discussion

The intersecting initial velocity patterns shown in Figures 3 and 4 demonstrate that galactokinase from *E. coli* has a sequential mechanism in which both substrates must add to the enzyme before either product is released. (If the lines were parallel, a Ping-Pong mechanism would be indicated, in which the first product left before the second substrate combined with the enzyme; Cleland, 1963b.) These studies do not tell, however, whether there is an obligatory order of addition or release of reactants, or whether combination in random order occurs. Until recently it was thought that eq 2 was given only by mechanisms in which ordered addition and release of reactants took place, or by a random mechanism where the rate-limiting step was the interconversion of central complexes, with all other unimolecular rate constants greatly exceeding the turnover numbers. Work in this laboratory has shown, however, that random mechanisms with other relationships between the rate constants also give eq 2 or a close approximaiton to it, and that linearity of reciprocal plots and consistency with eq 2

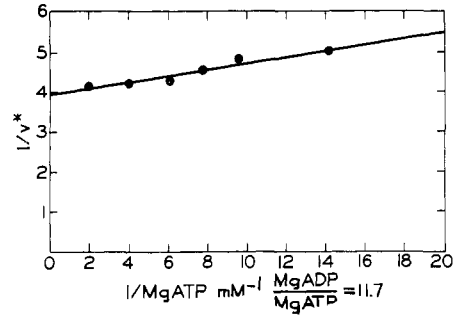


FIGURE 11: Double-reciprocal plot of the effect of raising the MgATP–MgADP pair on the initial velocity of galactose–galactose 1-phosphate exchange. Initial velocities of isotopic exchange are expressed as millimicromoles of [1-^{14}C]galactose 1-phosphate per minute. Galactose, 0.10 mM; galactose 1-phosphate, 1.625 mM. Enzyme concentration, 4.64 μg/ml.

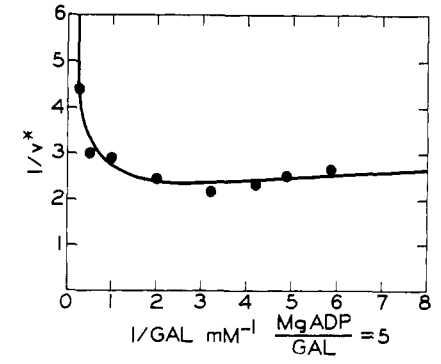
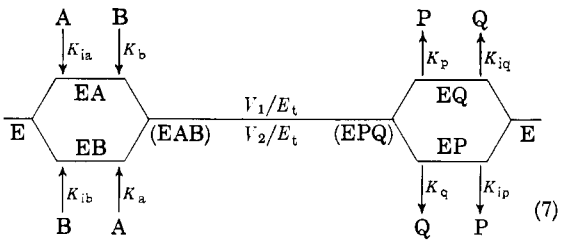


FIGURE 13: Double-reciprocal plot of the effect of raising the concentration of the galactose–MgADP pair on the initial velocity of the MgATP–MgADP exchange. Initial velocities of isotopic exchange are expressed as millimicromoles of [8-^{14}C]MgADP per minute. Galactose 1-phosphate, 2.58 mM; MgATP, 0.068 mM. Enzyme concentration, 4.64 μg/ml. The data were fitted to eq 5.

do not rule out the possibility of a mechanism being random. In fact, the unimolecular rate constants for release of substrates from the enzyme must be considerably smaller than the turnover number in order to see clearly the nonlinear (hyperbolic) reciprocal plots that have been thought to characterize random mechanisms.

Product inhibition patterns may, however, be used to distinguish between the various possible sequential mechanisms. A mechanism in which reactant addition and release is completely ordered shows only one competitive product inhibition, that between the first substrate to add to and the last product to dissociate from the enzyme. All other product inhibitions are noncompetitive. In an ordered mechanism where the rate-limiting step is solely the release of the second product and the steady-state level of the central complexes is essentially zero (Theorell–Chance mechanism), the second substrate added and first product released also show competitive inhibition. In both of these cases, however, the apparent inhibition constants for the competitive inhibitions (K_{is} in eq 3) do not vary with the concentration of the nonvaried substrate (Cleland, 1963b). The data obtained in the present study (see Results) show two competitive product inhibitions, one between the two sugars, and the other between the nucleotides, but in both cases the K_{is} values from fits to eq 3 appear to be significantly different at different levels of the nonvaried substrate.

These data suggest that the mechanism may be a random one in which the interconversion of central complexes is largely the rate-limiting step (rapid equilibrium random), and in which two dead-end complexes may form between enzyme, galactose, and MgADP, and between enzyme, galactose 1-phosphate, and MgATP. This mechanism is shown in mechanism 7, where A, B, P, and Q represent galactose, $MgATP^{2-}$, $MgADP^{-}$, and galactose 1-$phosphate^{2-}$, and the dissociation constants for the various steps are defined as indicated. The rate constants for the interconversion of the two central complexes are equal to the turnover numbers V_1/E_t and V_2/E_t. The rate equation for this



(7)

$$EA + P \xrightleftharpoons{K_{Ip}} EAP \xrightleftharpoons{K_{Ia}} EP + A$$

$$EB + Q \xrightleftharpoons{K_{Iq}} EBQ \xrightleftharpoons{K_{Ib}} EQ + B$$

mechanism is eq 8, with the kinetic constants defined as indicated in mechanism 7.

In addition, the following relationships hold for this mechanism: $K_{ia}K_b = K_aK_{ib}$, $K_{ip}K_q = K_pK_{iq}$, $K_{Ia}K_{ip} = K_{ia}K_{Ip}$, and $K_{Ib}K_{iq} = K_{Iq}K_{ib}$. The Haldane relationships are given in eq 11.

To see the effect of Q as a product inhibitor in this mechanism, P is set equal to zero in eq 8, and the resulting equation is rearranged so that either A (eq 9) or B (eq 10) is the variable substrate.

$$1/v = \frac{K_a}{V_1}\left(1 + \frac{K_{ib}}{B}\right)\left(1 + \frac{Q}{\dfrac{K_{iq}\left(1 + \dfrac{B}{K_{ib}}\right)}{(1 + B/K_{Ib})}}\right)\frac{1}{A} + \frac{1}{V_1}\left(1 + \frac{K_b}{B}\right) \quad (9)$$

$$1/v = \frac{K_b}{V_1}\left(1 + \frac{K_{ia}}{A}\right)\left(1 + \frac{Q}{K_{iq}(1 + A/K_{ia})}\right)\frac{1}{B} + \frac{1}{V_1}\left(1 + \frac{K_a}{A}\right)\left(1 + \frac{Q}{K_{Iq}(1 + A/K_a)}\right) \quad (10)$$

Equation 9 corresponds to linear competitive inhibition (eq 3) and eq 10 to linear noncompetitive inhibition (eq 4). A similar set of equations describing product inhibition by P is obtained when Q is set equal to zero in eq 8. Thus the full set of product inhibition patterns calls for competitive inhibition between A and Q, and B and P, and noncompetitive inhibition between A and P, or B and Q.

Equations 9 and 10 predict that the apparent K_{is} and K_{ii} values from fits to eq 3 or 4 will vary with the concentration of the nonvaried substrate as was observed in the present study. For example, the apparent inhibition constants from fits to eq 4 correspond in eq 10 to

$$K_{is} = K_{iq}(1 + A/K_{ia}) \qquad K_{ii} = K_{Iq}(1 + A/K_a)$$

If inhibition constants are determined at two different values of A, it is possible to get independent solutions for K_{ia}, K_a, K_{iq}, and K_{Iq} from these equations, although the values will not be very precise because the answers are ratios between fairly small differences. The data in the present study give values for K_{ia} of 0.3 and 0.66 mM, and for K_a of 0.3 and 0.73 mM, with the higher values probably more valid (certainly the true values cannot lie above 1 mM). While the value of K_a agrees with that from initial velocity studies, the value of K_{ia} does not. Assuming that K_a is 0.64 mM (the initial velocity value), K_{Iq} can now be calculated

$$v = \frac{V_1\left[AB - \dfrac{PQ}{K_{eq}}\right]}{K_{ia}K_b + K_bA + K_aB + AB + \dfrac{K_{ia}K_bP}{K_{ip}} + \dfrac{K_{ia}K_bQ}{K_{iq}} + \dfrac{K_{ia}K_bPQ}{K_pK_{iq}} + \dfrac{K_{ia}K_bAP}{K_{ip}K_{Ia}} + \dfrac{K_{ia}K_bBQ}{K_{iq}K_{Ib}}} \quad (8)$$

from the apparent constants as 1.8 ± 0.2 mM (weighted average of three values). If K_{ia} is assumed to equal K_a, values of K_{iq} ranging from 0.85 to 1.0 mM are calculated. Thus both K_{ia} and K_{iq} appear on the basis of these experiments to be lower than the values determined from initial velocity studies by a factor of 2 or 3.

Data fitting eq 4 also allow direct calculation of one other kinetic constant. The horizontal coordinate of the crossover point in this inhibition pattern (when $1/v$ is plotted against $1/B$ at different Q levels) is $-K_{is}/(K_{ii}K)$, which in eq 10 is equal to $-1/K_{Ib}$. If the apparent Michaelis constant in the absence of inhibitor (K in eq 4) seems consistent with its calculated value, this provides a good estimate of K_{Ib}. In the present case, K_{Ib} appears to be about 0.2 mM.

The apparent inhibition constant in eq 9 is a function not just of two kinetic constants, as are those in eq 10, but of three, and thus data from at least three levels of B would be needed to get independent solutions for all three constants. Data are available in the present case for only two levels of B, but these are sufficient to show that: (1) $K_{ib} < K_{Ib}$, since the apparent inhibition constant increased with the concentration of B, and (2) since $K_{ib} < K_{Ib}$, K_{iq} must be less than the observed apparent inhibition constants, and thus less than 1 mM. Thus both experiments with Q as inhibitor give values of K_{iq} considerably less than that obtained by initial velocity studies.

The data for competitive inhibition of MgATP by MgADP should fit eq 9, if A and B are interchanged and P is substituted for Q in both concentrations and subscripts of kinetic constants. Data are again available at only two A levels, and thus independent solutions for K_{ia}, K_{Ia}, and K_{ip} cannot be obtained. Since the apparent inhibition constant is higher at the higher A level, $K_{ia} < K_{Ia}$ and $K_{ip} < 0.24$ mM. Since K_{Ia} is 0.9 mM or less (see below), this calls for both K_{ia} and K_{ip} to be considerably smaller than the constants determined from initial velocity studies.

The noncompetitive inhibition of MgADP against galactose was studied at only one MgATP concentration, so independent values of K_{ib}, K_b, K_{ip}, and K_{Ip} cannot be obtained in the manner described above. If the value of K_b from initial velocity experiments is assumed correct, however, K_{Ip} is 0.2 ± 0.07 mM. If K_{ip} is taken as 0.2 mM (see above), K_{ib} would have to be 0.1 mM. A lower value of K_{ip} gives a still lower value of K_{ib}. The crossover point yields a value of K_{Ia} of 0.9 ± 0.17 mM, which is probably a maximum value since the apparent K in this experiment is somewhat higher than its calculated value.

The product inhibition experiments thus yield inhibition patterns consistent with mechanism 7, but the values of the inhibition constants in all cases are lower than those obtained from initial velocity studies. Where galactose phosphate is used as inhibitor, this might result if the rapid equilibrium assumption is invalid (see below), since the initial rate of appearance of ^{14}C in galactose phosphate may exceed the chemical reaction rate if galactose phosphate is initially present, and if isotopic exchange takes place between galactose and galactose phosphate. This will not occur if the mechanism is a rapid equilibrium one, and cannot occur in any case when MgADP is the inhibitor.

Further evidence on this point can be obtained by examination of the Haldane relationships. For mechanism 7 these are

$$K_{eq} = \frac{V_1K_{ia}K_b}{V_2K_{ip}K_q} = \frac{V_1K_aK_{ib}}{V_2K_{ip}K_q} = \frac{V_1K_{ia}K_b}{V_2K_pK_{iq}} = \frac{V_1K_aK_{ib}}{V_2K_pK_{iq}} \quad (11)$$

If the values for the various kinetic constants determined from initial velocity studies are substituted into these equations, values ranging from 414 to 461 are obtained for K_{eq}. Since the measured value is 190, it appears that some of the constants for P and Q are too large, or those for A and B are too low. This suggests that the constants determined by product inhibition may be more valid, in particular for P and Q since initial velocities in the reverse direction are difficult to measure accurately because of the short distance to equilibrium.

The most sensitive test for a rapid equilibrium random mechanism is the isotopic exchange patterns. If the interconversion of central complexes is truly rate limiting, the rates of all possible exchanges at equilibrium will be identical, since they are all limited by the flux through this step, and the initial rate of isotopic exchange is given by

$$v^* = \frac{V_1}{\left[\frac{K_{ia}K_b}{AB} + \frac{K_a}{A} + \frac{K_b}{B} + 1 + \frac{V_1}{V_2}\left(\frac{K_p}{P} + \frac{K_q}{Q} + \frac{K_qA}{K_{Ia}Q} + \frac{K_pB}{K_{Ib}P} + 1\right)\right]} \quad (12)$$

This equation predicts linear reciprocal plots when A and Q or B and P are varied in constant ratio, but total substrate inhibition (eq 6) when A and P or B and Q are varied together because of the formation of dead-end EAP and EBQ complexes. The observation of linear reciprocal plots when galactose and galactose phosphate (Figure 10) or MgATP and MgADP (Figure 11) were varied together, but of substrate inhibition when galactose and MgADP (Figure 13) or galactose phosphate and MgATP (Figure 12) were varied together is consistent with eq 12 and supports the existence of the dead-end enzyme–MgADP–galactose and enzyme–MgATP–galactose phosphate complexes which were indicated by product inhibition studies. However, as shown by Table III, the exchange rates are not equal, and it is clear that while the interconversion of central complexes may be the sole rate-limiting step for MgATP–MgADP exchange, other steps such as the dissociation of reactants are partly rate-limiting for galactose–galactose phosphate exchange. Galactokinase thus has a random, but not truly rapid equilibrium random mechanism.

The demonstration that the rapid equilibrium assumption does not hold for this mechanism raises the question whether the numerical discrepancies between

inhibition constants determined by initial velocity and product inhibition techniques could be the result of this failure. We have used simulation techniques to study in some detail the properties of mechanism 7, assuming various rate constants, and studying cases where the central step was and was not rate limiting. The approach used was to have a digital computer calculate initial velocities as a function of substrate and product concentrations, and then to fit these true velocities to eq 2-4, as appropriate, and compare the numerical values of constants from simulated initial velocity and product inhibition experiments. In no case could really significant differences be found. Further it was obvious that for a random mechanism such as mechanism 7, initial velocities are predicted very accurately by eq 2, 9, and 10, which are derived from eq 8, even though the rapid equilibrium assumption is completely invalid. The observed discrepancies are thus probably the result of experimental difficulties.

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Quantitative Distribution of Histone Components in the Pea Plant*

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ABSTRACT: Histones, prepared by acid extraction of purified chromatin from many different organs of the pea plant, are fractionated by column chromatography and by quantitative analytical disc electrophoresis. By these criteria there is no histone fraction specific to any pea tissue. The chromatins of different pea organs do, however, exhibit reproducible quantitative differences in the representation of the several histone fractions.

The histones are basic proteins which occur bound to DNA in the chromosomes of higher organisms. The concept that different molecular species of histones might be found in different types of cells of the same organism arose originally from the observation that the sperm of some fish contain an unusual sort of histone, protamine. Important to the further development of this concept were the findings that a single type of cell contains several histones and that there are reproducible differences in amino acid composition between crude histone fractions of chicken erythrocytes and of other chicken tissues (Stedman and Stedman, 1950, 1951). These observations gave rise to the theory that the main function of histones is control of gene expression. Implicit in the simplest statement of this theory is the idea of gene recognition by specific histone molecules (histone specificity) and thus great histone heterogeneity.

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