DETERMINATION OF THE EQUILIBRIUM CONSTANT FOR AN ENZYME CATALYZED REACTION

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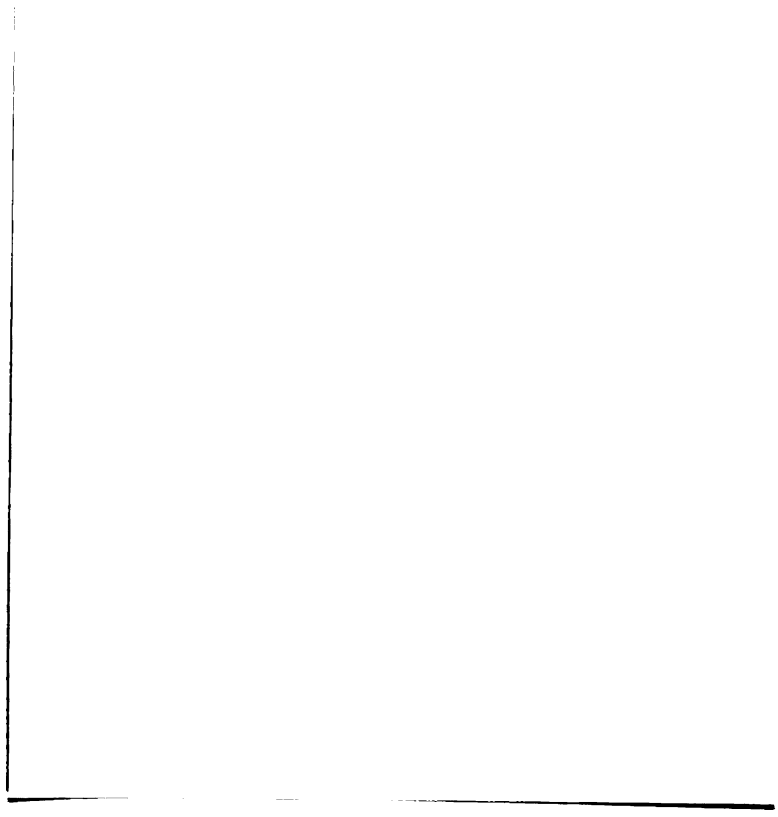

ABSTRACT

DETERMINATION OF THE EQUILIBRIUM CONSTANT FOR AN ENZYME CATALYZED REACTION

by Karen E. DeFazio

The study of energy transformations in living organisms has become an integral part of understanding the nature of many enzymatically catalyzed reactions. In isolation, these reactions will reach a point of equilibrium at which no further net chemical change takes place. This equilibrium is expressed by a thermodynamic constant.

The equilibrium constant for a reaction involving the crystalline enzyme, uridine diphosphate glucose pyrophosphorylase, was determined. This enzyme catalyzed the biosynthesis of uridine diphosphate glucose from uridine triphosphate and glucose-1-phosphate. A new spectrophotometric method was available for the determination of inorganic pyrophosphate. Thus, spectrophotometric determinations of all four components of the reaction were performed as a basis for the equilibrium calculations. Further, a new chromatographic method for the separation of all four components of the reaction was developed using polyethyleneimine-impregnated paper and a 2.0 M formic acid-0.4 M LiCl solvent. Employing radioactive substrates, the concentrations of the reactants were determined chromatographically. The equilibrium constant determined by both the spectrophotometric and the chromatographic procedures was found to be about 0.20. Thus, when

the products were allowed to accumulate, the reaction proceeded to about 30% uridine diphosphate glucose formation from equivalent amounts of the substrates uridine triphosphate and glucose-1-phosphate.

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By

Karen E. DeFazio

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VITA

Karen E. DeFazio was born on July 29, 1943, at Elyria, Ohio. She graduated from Elyria High School in 1961. She attended Baldwin-Wallace College and received the B.S. degree in Chemistry in 1965 from the Department of Chemistry. Her graduate studies were pursued at Michigan State University in the Department of Biochemistry to complete the requirements for the degree of Master of Science.

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INTRODUCTION

The study of energy-transformations in living organisms has become an integral part of understanding the nature of many enzymatically catalyzed reactions. We know that, in isolation, these reactions designated by the general equation

$$A + B \rightleftharpoons C + D \qquad (1)$$

will reach a point of equilibrium at which no further net chemical change takes place. The constant which expresses this chemical equilibrium and which, in turn, is related to the standard free energy change of the system is called the thermodynamic equilibrium constant,

$$Keq = \frac{[C]\,[D]}{[A]\,[B]} \qquad (2)$$

where the brackets express the concentration of the reactants and products at the point of equilibrium. The presence of an enzyme affects only the time and rate at which equilibrium is achieved, but should not affect the final Keq (1).

The purpose of this thesis is to determine the equilibrium constant of an enzymatically catalyzed reaction, specifically, the biosynthesis of UDP-Glc[1] from UTP and Glc-1-P

$$UTP + Glc\text{-}1\text{-}P \rightleftharpoons UDP\text{-}Glc + PPi \qquad (3)$$

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[1]The following abbreviations are used: UMP, UDP, and UTP, uridine mono-, di-, and tri-phosphate; UDP-Glc, uridine diphosphate glucose; UDP-Gal, uridine diphosphate galactose; PPi, inorganic pyrophosphate; Glc-1-P, glucose-1-phosphate; AMP, ADP, and ATP, adenosine mono-, di-, and tri-phosphate; ADP-Glc, adenosine diphosphate glucose; ADP-Man, adenosine

which involves the enzyme, uridine diphosphate glucose pyrophosphorylase (UTP:α-D-glucose-1-P uridylyl transferase). The literature reports a range of 0.1 to 1.0 for the equilibrium constant of the pyrophosphorylase reaction. In view of the fact that the Keq should be independent of the enzyme source, attempts were made to more precisely measure its value using a new spectrophotometric method for the determination of PPi and a new chromatographic method for separation of all four components of the reaction.

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diphosphate mannose; d-AMP, deoxyadenosine monophosphate; GMP, GDP, and GTP, guanosine mono-, di-, and tri-phosphate; GDP-Glc, guanosine diphosphate glucose; IMP, IDP, and ITP, inosine mono-, di-, and tri-phosphate; IDP-Glc, inosine diphosphate glucose; CMP, CDP, and CTP, cytidine mono-, di-, and tri-phosphate; CDP-Glc, cytidine diphosphate glucose; TMP, TDP, and TTP, thymidine mono-, di-, and tri-phosphate; TDP-Glc, thymidine diphosphate glucose; TPN, triphosphopyridine nucleotide; LiCl, lithium chloride; PEI, polyethyleneimine.

LITERATURE REVIEW

General History of Uridine Diphosphate Glucose Pyrophosphorylase

In 1950, Leloir and coworkers (2) isolated the nucleotide, uridine diphosphate glucose, from yeast. Shortly afterwards, Kalckar and Cutolo (3) discovered in a Zwischenferment preparation a pyrophosphorylase enzyme which could cleave UDP-Glc to bring about the formation of UTP and Glc-1-P. They also found that this reaction was reversible with the enzyme acting as a uridyl transferase. Since then the enzyme has become known as the uridine diphosphoglucose pyrophosphorylase (UTP:α-D-glucose-1-P uridylyl transferase) which catalyzes the following reaction:

$$\text{UTP} + \text{Glc-1-P} \rightleftharpoons \text{UDP-Glc} + \text{PPi} \qquad (3)$$

In 1955, Munch-Petersen (4) purified the enzyme from yeast, established a spectrophotometric assay for its activity, and reported some of its properties. She did an equilibrium study on the reaction by incubating 0.2 μmole amounts of UDP-Glc and PPi with the enzyme and assaying the Glc-1-P formed in aliquots taken out at different time intervals. Glc-1-P was analyzed by the addition of phosphoglucomutase, cysteine, TPN, and Glc-6-P dehydrogenase. When readings at 340 mμ were constant, indicating that all the Glc-1-P was used, excess UDP-glucose pyrophosphorylase and PPi were added

to measure the residual UDP-Glc. It was concluded that the reaction stopped at 45% conversion, yielding an equilibrium constant near 1.0.

Following this initial work of Munch-Petersen, a UDP-glucose pyrophosphorylase was found in liver extracts (5), in mammary glands (6), in sugar beet leaves (7), in mung bean seedlings (8, 9), and in the plant Impatiens holstii (10). In 1957, Turner and Turner (11) isolated the enzyme from pea seed extracts and attempted to determine the equilibrium constant. With a concentration of 5.0 mM magnesium and at pH 7.9 and 30°, the Keq in the direction of synthesis of UDP-Glc and PPi was 0.14 which is significantly lower than 1.0 reported by Munch-Petersen (4). They observed that an increase in the magnesium ion concentration and a decrease in the pH of the reaction mixture each depressed the value of the constant. However, they did observe that precipitates were formed in the enzymic digests where the molar concentration of magnesium ions was equal to or greater than the concentration of sodium pyrophosphate. The presence of a complex formation with the sodium pyrophosphate was suggested to have caused the depressing effect of the high magnesium ion concentration on the pyrophosphorylase reaction.

In 1959, Palasi and Larner (12) purified the UDP-glucose pyrophosphorylase from skeletal muscle, and in 1961, Basu and Bachhawat (13) purified it from human brain. However, no further equilibrium studies were reported until 1965 when Kamogawa and Kurahashi (14) preceded to purify the enzyme

from Escherichia coli K_{12}. For studying the constant they prepared an incubation mixture which contained 0.30 μmole of UDP-Glc, 0.28 μmole of PPi, 3.8 μg of enzyme, and 3.0 μmole of $MgCl_2$ in 1.0 ml of 0.5 M Tris-HCl buffer (pH 7.5). At various time intervals aliquots were withdrawn and the reaction stopped by boiling. They measured the amount of UDP-Glc by UDP-glucose dehydrogenase, Glc-1-P by phosphoglucomutase and glucose-6-P dehydrogenase (4), UTP by nucleoside-diphosphatekinase and hexokinase, and PPi by inorganic pyrophosphatase. Their results are shown in Table I. Averaging their values, Keq was calculated as 0.20 in the direction of synthesis of UDP-Glc and PPi, showing that the equilibrium was in favor of the degradation of UDP-Glc.

In 1966, UDP-glucose pyrophosphorylase was crystallized for the first time by Albrecht et al (15), and the source was calf liver. They did an equilibrium study by incubating the substrates with magnesium acetate, 2 mM, in 0.1 M Tris-acetate buffer (pH 7.8). After the addition of 0.35 μg of enzyme per ml, aliquots were removed at various time intervals and heated to boiling to stop the reaction. Equilibrium was attained in about 10 min at 30°. The concentrations of the substrates and reaction products were determined as follows: Glc-1-P by phosphoglucomutase and glucose-6-P dehydrogenase (4), UTP as described (16), UDP-glucose after hydrolysis with venom pyrophosphatase and then measuring Glc-1-P as above, and PPi by difference. Their results are shown in Table II. The equilibrium constant in

Table I. Equilibrium study of UDP-glucose pyrophosphorylase reaction in E. Coli K_{12}.

Time Minutes	UDP-Glc	PPi	Glc-1-P	UTP	Keq
0	0.28	0.26	0.02	0.02	182.00
30	0.11	0.11	0.18	0.16	0.42
60	0.10	0.08	0.20	0.19	0.21
90	0.10	0.07	0.20	0.18	0.19
120	0.11	0.08	0.20	0.19	0.23

All concentrations are micromolar.

Reference (14).

Table II. Equilibrium study of UDP-glucose pyrophosphorylase reaction in calf liver.

Experiment	Glc-1-P		UTP		UDP-Glc		PPi		Keq
	Initial	Final	Initial	Final	Initial	Final	Initial	Final	
1	0.56	0.38	0.48	0.28	0	0.19	0	0.19	0.34
2	2.82	2.50	0.48	0.14	0	0.34	0	0.34	0.33
3	0.35	0.24	0.29	0.18	0	0.11	0	0.11	0.28

All concentrations are millimolar.

Reference (15).

the direction of synthesis of UDP-Glc was determined to be between 0.28 and 0.34.

Development of Chromatographic Methods to Separate All Reaction Components

The established importance of nucleoside diphosphate sugar pyrophosphorylases and their related substrates in carbohydrate metabolism made necessary the development of chromatographic methods capable of analyzing their incubation mixtures. The procedures involved ion-exchange column chromatography, paper electrophoresis, paper chromatography, and thin-layer chromatography.

The paper chromatography method was most useful for the analysis of enzymic reaction mixtures containing nucleoside diphosphate sugars and nucleoside mono-, di-, and triphosphates. The solvent commonly used was ethanol and ammonium acetate (17, 18) and required a separation time of 24 to 64 hr. This being an extremely slow procedure brought about the investigations of more rapid and appropriate methods.

It has been known for many years that polyethyleneimine and other basic polymers could be fixed on cellulose fibers, but it has not been realized that polyethyleneimine is an effective anion-exchanger which can be used in column, thin-layer, and paper chromatography. However, in 1963 Randerath published a paper indicating ribonucleotides could be separated on PEI paper with a 1.0 M NaCl solvent in less than 1 hr (19). The same year it was reported that deoxyribonucleotides could be separated from ribonucleotides by anion-exchange

thin layer chromatography (20). The plates were coated with the PEI-cellulose and developed with a solution of LiCl in aqueous boric acid. The borate added a net negative charge to the compound, thus increasing their distribution coefficients on the anion-exchanger.

In 1963, Dietrich (21) found that phosphate derivatives of sugars could be separated from nucleotides using an ECTEOLA-cellulose powder on thin-layer plates. Randerath (22) found he could resolve complex nucleotide mixtures by two dimensional anion-exchange chromatography on PEI-cellulose thin layers. A LiCl solvent was used in one dimension and formic-NH_4formate buffer (pH 3.4) in the other to develop the chromatogram. DPN, TPN, six nucleotide sugars, and fourteen common nucleoside-5'-mono-, di-, and tri-phosphates were resolved in less than 3 hr. It was also possible to quantitatively elute small amounts of the nucleotides from the PEI plates (23). Finally, the effect of neutral and acid solvents on the development patterns of the nucleotides was compared (24). A formic acid-LiCl combination also showed some promise.

In 1965, Verachtert et al (25) published a method for characterizing nucleoside diphosphate sugars in mixtures containing nucleoside mono-, di-, and tri-phosphates. PEI paper with only LiCl as the solvent achieved a good separation in 3-4 hr. At the same time, Randerath (26) reported the separation of nucleotide sugars from nucleoside monophosphates on PEI-cellulose thin-layer plates using either an acetic acid-LiCl solvent or a sodium borate-boric acid solvent.

MATERIALS AND METHODS

Chemicals

All nucleotides and sugars were commercial products except IDP-Glc and TDP-Glc. These were synthesized by a procedure which has been described (27). ADP-Man was synthesized enzymically using a calf liver extract (28).

Polyethyleneimine was obtained as a 50% aqueous solution from Chemirad Corp. (East Brunswick, New Jersey).

The radioactive substrates U.L.-^{14}C-Glc-1-P and ^{32}PPi were obtained from New England Nuclear, and UDP-Glc-^{14}C-U.L. from ICN. UTP-β,γ-^{32}P was synthesized in the laboratory from ^{32}PPi (S.T. Bass and R.G. Hansen, 1968, unpublished data).

All enzymes were commercially prepared except for the UDP-glucose pyrophosphorylase which was crystallized in the laboratory according to the method described by Albrecht *et al* (15).

Quantitative Measurements

UDP-glucose pyrophosphorylase activity was determined by the method described by Albrecht *et al* (15). One unit of enzyme is defined as that amount required to liberate 1.0 μmole of product per min at 25°.

The chemical determination of the equilibrium constant was carried out by incubating 1.0 μmole quantities of either Glc-1-P and UTP or UDP-Glc and PPi with 1.0 μmole of magnesium

acetate in 1.0 ml of 0.1 M Tris-acetate buffer (pH 7.8). After addition of the desired amount of enzyme, aliquots were removed at various time intervals and heated to boiling to stop the reaction. Equilibrium was attained in about 15 min at 30°. The concentration of the reaction products was determined as follows: Glc-1-P by an end-point assay using phosphoglucomutase and glucose-6-P dehydrogenase (4, 15), UTP as described (16), PPi by further addition of UDP-Glc and UDP-glucose pyrophosphorylase (29), and UDP-Glc by further addition of PPi and UDP-glucose pyrophosphorylase. A typical assay for determining Glc-1-P, PPi, and UDP-Glc follows: In quartz cuvettes with 1-cm light paths, 1.0 μmole of magnesium acetate, 0.2 μmole of TPN, an aliquot of the incubation mixture and enough phosphoglucomutase and glucose-6-P dehydrogenase to complete the reaction in 15 min or less at 25° were added successively to 0.1 M Tris-acetate buffer (pH 7.8) to make a final volume of 0.5 ml. When an end-point was reached, indicating that all the Glc-1-P was used, excess UDP-glucose pyrophosphorylase and 1.0 μmole of PPi were added to determine UDP-Glc, or excess UDP-glucose pyrophosphorylase and 0.2 μmole of UDP-Glc were added to determine PPi. All reactions were followed at 340 mμ in a Beckman model DU spectrophotometer equipped with a Gilford automatic sample changer and recorder (30).

Radioactivity was measured in a Packard Instrument Company Tri-Carb liquid scintillation counter. The counting solution consisted of 770 ml of xylene, 770 ml of p-dioxane,

462 ml absolute ethanol, 0.1 g of α-N-PO, 10.0 g of PPO, and 160.0 g of napthalene (31). Samples were placed in vials containing 15 ml of the counting fluid, shaken to achieve dispersion, and counted for 10 min each.

Qualitative Measurements

Polyethyleneimine-impregnated paper was employed for all chromatographic work. This was prepared by treating Whatman No. 1 paper with PEI (25). Between 0.05 and 0.10 μmoles of nucleotides and sugars were applied as spots and 0.200 ml of each incubation mixture as streaks about 3 inches from the base of the paper, and development was achieved in a descending direction in 3 or 4 hr at room temperature, using the desired solvent. The ultraviolet-absorbing compounds were detected with a Mineralight ultraviolet lamp, and the sugar phosphates with a molybdate spray (32).

EXPERIMENTAL PROCEDURES AND RESULTS

Determination of Equilibrium Constant by Spectrophotometric Methods

The time course of the reaction was observed to determine the level of enzyme and length of time which was necessary to bring about equilibrium of the UDP-glucose pyrophosphorylase. Two mixtures were incubated, one containing 1.0 μmole quantities of the substrates Glc-1-P and UTP, the other containing 1.0 μmole quantities of UDP-Glc and PPi. After the addition of 0.053 units of enzyme recrystallized three times, aliquots of each mixture were removed at various time intervals and were assayed for the substrates and reaction products. The results are shown in Table III. One can see that equilibrium in either direction was achieved within 30 min at 30°, giving a Keq value of 0.23-0.25.

The effect of ammonium sulfate in the reaction mixture on the equilibrium was investigated next. Four mixtures were prepared, each containing a different level of $(NH_4)_2SO_4$ in the enzyme. 1.0 μmole quantities of the substrates Glc-1-P and UTP were incubated for 1 hr at 30° with 1.0 μmole of magnesium acetate in 1.0 ml of 0.1 M Tris-acetate buffer (pH 7.8). The results are shown in Table IV. No significant effect of the salt on the Keq value was observed. A test

Table III. Time course of the pyrophosphorylase reaction.

Mixture	Direction	Time Incubation	Glc-1-P	UTP[1]	UDP-Glc	PPi	Keq
1	I	15 min	0.738	---	0.370	0.325	0.22
	I	30 min	0.674	---	0.313	0.361	0.25
	I	1 hr	0.691	---	0.325	0.370	0.25
	I	2 hr	0.687	---	0.333	0.358	0.25
	I	3 hr	0.653	---	0.353	0.310	0.26
2	II	15 min	0.720	---	0.275	0.411	0.22
	II	30 min	0.708	---	0.275	0.411	0.23
	II	1 hr	0.682	---	0.259	0.386	0.21
	II	2 hr	0.727	---	0.267	0.390	0.20
	II	3 hr	0.725	---	0.275	0.444	0.23

[1]UTP was not measured.

Mixture 1 contained 1.0 μmole of Glc-1-P, UTP, and magnesium acetate and mixture 2 contained 1.0 μmole of UDP-Glc, PPi, and magnesium acetate in 1.0 ml of 0.1 M Tris-acetate buffer (pH 7.8). The reactions were each started with the addition of 0.053 units of enzyme. The direction of synthesis is indicated:

$$\text{Glc-1-P} + \text{UTP} \underset{\text{II}}{\overset{\text{I}}{\rightleftharpoons}} \text{UDP-Glc} + \text{PPi} \ .$$

Keq is defined as $\frac{[\text{UDP-Glc}]\,[\text{PPi}]}{[\text{Glc-1-P}]\,[\text{UTP}]}$. All concentrations are micromolar.

Table IV. The effect of ammonium sulfate and enzyme concentration on the equilibrium constant.

Mixture	Direction	Enzyme Units	% $(NH_4)_2SO_4$	Glc-1-P	UTP[1]	UDP-Glc	PPi	Keq
1	I	0.053	0.002	0.816	---	0.284	0.337	0.14
2	I	0.53	0.02	0.743	---	0.312	0.317	0.18
3	I	5.30	0.2	0.729	---	0.357	0.358	0.24
4	I	53.0	2.0	0.738	---	0.354	0.333	0.22
5	II	0.91	0.02	0.618	---	0.271	0.345	0.24
6	II	1.05	Dialyzed	0.625	---	0.284	0.374	0.27

[1]UTP was not measured.

1.0 µmole quantities of substrates were incubated with 1.0 µmole of magnesium acetate in 1.0 ml of 0.1 M Tris-acetate buffer (pH 7.8) for 1 hr at 30°. The direction and Keq definition are the same as defined in Table III. All concentrations are micromolar.

involving the presence of $(NH_4)_2SO_4$ in the enzyme was also performed. A portion of the enzyme crystals was dialyzed overnight against 0.01 M tricine buffer (pH 8.5). An equilibrium was then established with mixture 5 containing enzyme in $(NH_4)_2SO_4$ and mixture 6 containing the dialyzed enzyme. The results are shown in Table IV. Again no significant effect was observed. Therefore, the presence of $(NH_4)_2SO_4$ was ignored in Keq calculations.

Since Turner and Turner (11) reported that the plant enzyme was affected by the magnesium concentration, incubation mixtures were prepared with levels of magnesium acetate ranging from 0.01 mM to 5.0 mM. A magnesium level of 0.1 mM gave an equilibrium value in the same range as the 1.0 mM level (Table V). With the lower level of 0.01 mM magnesium equilibrium was not achieved in 1 hr of incubation. The enzyme turnover rate was probably inadequate due to the low level of magnesium. The high level of 5.0 mM caused a precipitation of the PPi in the mixture and could not be assayed accurately. However, the mixture in the direction of synthesis of UDP-Glc and PPi was assayed and gave a Keq value near that observed when 1.0 mM magnesium was used. Further consideration of this issue should be given.

Assuming now that the ammonium sulfate had little or no effect on the equilibrium constant and the 1.0 mM was a favorable level of magnesium concentration to use, a series of experiments were performed wherein the various substrates were incubated for 1 hr at 30°. The results are summarized in Table VI. The Keq value in direction I is 0.21 and in

Table V. The effect of the amount of magnesium on the equilibrium constant.

Mixture	Direction	µmoles/ml Magnesium	Glc-1-P	UTP	UDP-Glc	PPi	Keq
1	I	0.1	0.667	0.584	0.234	0.251	0.15
2	II	0.1	0.695	0.640	0.247	0.314	0.17
3	I	0.01	0.788	0.733	0.100	0.112	0.02
4	II	0.01	0.495	0.499	0.436	0.507	0.89
5	I	5.00	0.568	0.491	0.297	0.243	0.26
6	II	5.00	0.594	0.532	0.297	-----*	----

*The PPi precipitated with the magnesium.

All mixtures were incubated for 1 hr at 30°. 1.0 µmole quantities of substrates were used. The direction and Keq definition are the same as defined in Table III. All concentrations are micromolar. 0.14 units of enzyme were used.

Table VI. Determination of the equilibrium constant by the spectrophotometric method.

Experiment	Direction	Enzyme Units	Glc-1-P	UTP	UDP-Glc	PPi	Keq
1	I	0.37	0.575	----*	0.275	0.276	0.23
	II	0.37	0.668	----*	0.230	0.341	0.17
2	I	0.17	0.689	0.677	0.306	0.306	0.20
	II	0.17	0.719	0.697	0.314	0.285	0.18
3	I	0.17	0.697	0.653	0.310	0.289	0.20
	II	0.17	0.753	0.758	0.318	0.289	0.16
4	I	0.31	0.645	0.617	0.301	0.289	0.22
	II	0.31	0.735	0.685	0.297	0.318	0.19
5	I	0.28	0.707	0.689	0.306	0.289	0.18
	II	0.28	0.775	0.689	0.301	0.323	0.18

*was not measured.

1.0 μmole quantities of substrates were incubated with 1.0 μmole of magnesium acetate in 1.0 ml of 0.1 M Tris-acetate buffer (pH 7.8) for 1 hr at 30°. The direction and Keq definitions are the same as defined in Table III. All concentrations are micromolar.

direction II is 0.18.

Development of the Chromatographic System

For separating the reactants and products, solutions containing 10.0 μmoles each of the compounds UMP, UDP, UTP, UDP-Glc, UDP-Gal, PPi, and Glc-1-P were prepared. These were applied to PEI paper and their ion-exchange behavior as a function of decreasing formic acid concentration was studied. The results are shown in Table VII. Only the nucleotide, UMP and the sugar, Glc-1-P moved appreciably. This method can be applied to the separation of nucleoside monophosphates from other nucleotides but will not separate the desired four substrates.

A formic acid-LiCl solvent system was tested next. Concentrations of 0.5 M to 4.0 M formic acid containing 0.1 M, 0.5 M, 1.0 M, and 2.0 M LiCl were employed. Of these various mixtures the 2.0 M formic acid containing LiCl gave the best results. It was found that at salt concentrations below 0.3 M, the nucleoside di-, and tri-phosphates and PPi migrated very little. On the other hand, UMP and Glc-1-P moved appreciably. The two nucleoside diphosphate sugars moved slightly and did not separate. With concentrations of LiCl above 0.3 M, the nucleoside di-, and tri-phosphate moved appreciable with the diphosphate preceding the tri-phosphate. PPi moved between UDP and UTP, indicating for the first time the ability to separate PPi from UTP. The nucleoside diphosphate sugars migrated still further and Glc-1-P moved the farthest followed by UMP. It was observed

Table VII. R_f values for separation of UMP from other nucleotides using a formic acid solvent.

	Concentration of Formic Acid			
Compound	4.0 M	2.0 M	1.0 M	0.5 M
UMP	0.47	0.34	0.25	0.21
UDP	0.01	0.01	0.01	0.01
UTP	0.01	0.00	0.00	0.01
UDP-Glc	0.04	0.02	0.02	0.01
UDP-Gal	0.03	0.02	0.02	0.02
PPi	0.01	0.00	0.01	0.00
Glc-1-P	0.44	0.30	0.22	0.19

that Glc-1-P separated clearly from UDP-Glc, and the rate of movement of each compound increased regularly with increasing salt concentration. Most important, though, was the fact that the 2.0 M formic acid solvent containing 0.4-0.5 M LiCl separated clearly the four substrates Glc-1-P, UDP-Glc, PPi, and UTP. This separation is shown by the graph in Fig. 1.

The formic acid solvent was then tried in combination with other salts instead of LiCl. NH_4Cl, NaCl, and KCl were all found to yield about the same results as LiCl. Only the separation of the substrates using a formic acid-NH_4Cl solvent is shown in Fig. 2. For some purposes NH_4Cl may have an advantage since this salt is easily volatilized. NH_4COOH and NaBorate were also tested in combination with formic acid, but did not separate the four substrates. However, the NaBorate salt did show some promise of separating UDP-Glc from UDP-Gal, but this was not pursued further. Boric acid with LiCl was also tried and gave very unsatisfactory results with general streaking of the spots.

In view of the resolving power of formic acid-LiCl for the compounds of principle interest, several preliminary experiments were conducted to see the effect of this solvent system on the separation of other nucleotides. Adenosine, guanosine, inosine, cytidine, uridine, and thymidine compounds were all tested using a 2.0 M formic acid-0.5 M LiCl solvent system. The results are shown in Table VIII. A mixture of the nucleoside diphosphate glucose derivatives were also separated with a 2.0 M formic acid-0.3 M LiCl

Figure 1. Separation of the four substrates Glc-1-P, UDP-Glc, PPi, and UTP, using a formic acid-LiCl solvent system.

Between 0.05 and 0.1 μmole of compound was spotted and chromatography was performed from 3-4 hr at room temperature.

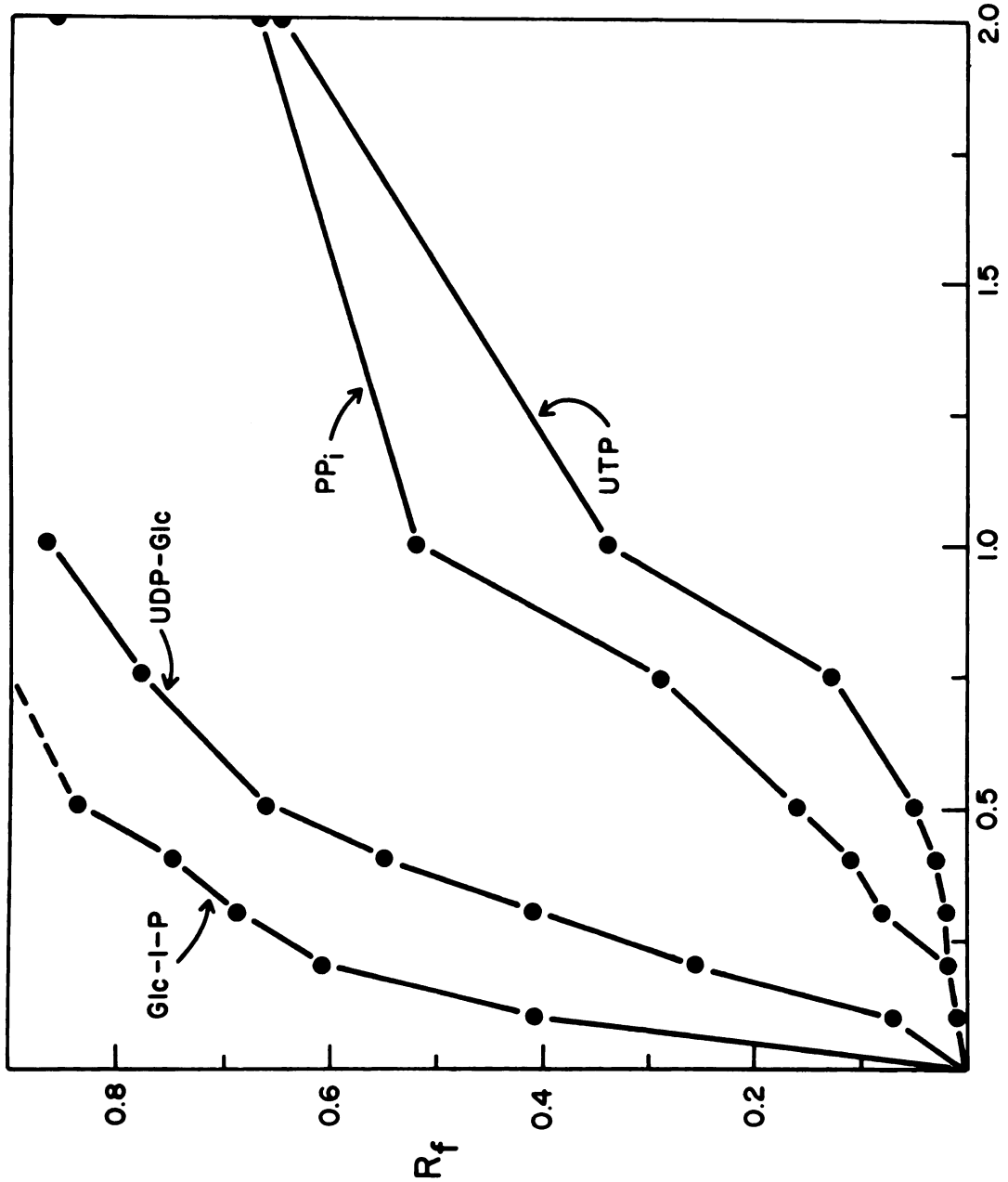

Glc-1-P
UDP-Glc
PPi
UTP
0.8
0.6
0.4
0.2
Rf
0.5
1.0
1.5
2.0
MOLES LiCl IN 2.0 M FORMIC ACID

Figure 2. Separation of the four substrates Glc-1-P, UDP-Glc, PPi, UTP, using a formic acid-NH_4Cl solvent system.

Between 0.05 and 0.1 μmole of compound was spotted and chromatography was performed from 3-4 hr room temperature.

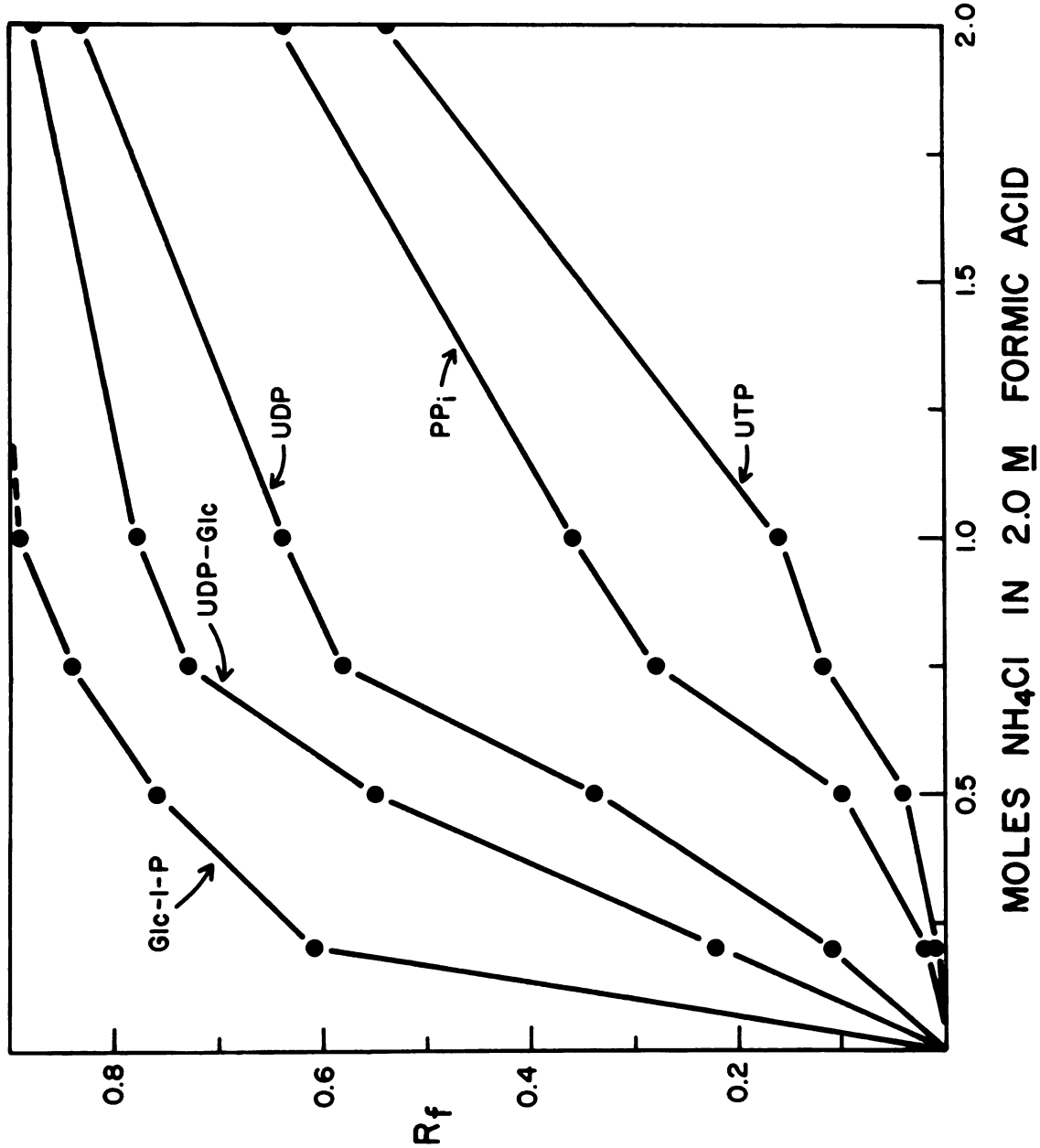

Glc-1-P
UDP-Glc
UDP
PPi
UTP
0.8
0.6
0.4
0.2
Rf
0.5
1.0
1.5
2.0
MOLES NH4Cl IN 2.0 M FORMIC ACID

Table VIII. R_f values for separation of nucleoside phosphates and sugars with 2.0 M formic acid-0.5 M LiCl.

Compound	R_f	Compound	R_f	Compound	R_f	Compound	R_f	Compound	R_f
Adenosine	----	Guanosine	----	Inosine	0.76	Cytidine	0.89	Thymidine	----
AMP	0.86	GMP	0.76	IMP	0.76	CMP	0.84	TMP	0.83
ADP	0.68	GDP	0.59	IDP	0.40	CDP	0.71	TDP	0.46
ATP	0.13	GTP	0.10	ITP	0.05	CTP	0.16	TTP	0.07
ADP-Glc	----	GDP-Glc	0.74	IDP-Glc	0.61	CDP-Glc	0.84		
ADP-Man	0.81	GDP-Man	0.74						
d-AMP	0.86								

solvent system. These results are shown in Table IX. Separations of the mono-, di-, and tri-phosphates are shown in Table X and their combination in Table XI.

Determination of Equilibrium Constant by Chromatography

A preliminary experiment was run to test if the substrates and reaction products of the incubation mixtures could, in fact, be separated on PEI paper with the 2.0 M formic acid-0.5 M LiCl solvent. Incubation mixtures were run as described in the methods section. 0.200 ml of each reaction mixture was streaked on the paper. For visualization purposes 0.005 ml of 10.0 μM solutions of Glc-1-P, UTP, UDP-Glc, and PPi were also applied coincidentally. The chromatogram was developed with a 2.0 M formic acid-0.5 M LiCl solvent for 3 or 4 hr at room temperature. The spots were detected as described in the methods section. The components of the incubation mixtures separated and cochromatographed with the four authentic compounds.

The purity of the radioactive substrates: U.L.-^{14}C-Glc-1-P, UTP-β,γ-^{32}P, UDP-Glc-^{14}C-U.L., and ^{32}PPi was next tested. The radioactive compounds were cochromatographed with authentic unlabelled standards using a 2.0 M formic acid-0.4 M LiCl solvent for 3 or 4 hr at room temperature. The unlabelled compounds were detected as before, and any radioactive compounds by cutting 2.0 cm strips of the chromatogram and counting them in the scintillometer. The radioactive compounds were coincident with the authentic

Table IX. R_f values for separation of nucleoside diphosphate sugars with 2.0 M formic acid-0.3 M LiCl.

Compound	R_f
CDP-Glc	0.70
ADP-Glc	----*
GDP-Glc	0.54
TDP-Glc	0.46
UDP-Glc	0.40
IDP-Glc	0.37

*Could not detect.

Table X. Separation of nucleoside mono-, di-, and tri-phosphates with 2.0 M formic acid and lithium chloride.

	Concentration of LiCl		
	----	0.5 M	1.0 M
Nucleoside	monophosphate	diphosphate	triphosphate
Cytidine	0.88	0.67	0.50
Adenosine	0.85	0.65	0.47
Guanosine	0.58	0.55	0.38
Thymidine	0.40	0.51	0.38
Inosine	0.35	0.39	0.31
Uridine	0.33	0.45	0.33

Table XI. R_f values for separation of nucleoside mono-, di-, and tri-phosphates with 2.0 formic acid-0.3 M LiCl.

Compound	R_f
CMP	0.86
CDP	0.53
CTP	0.06
AMP	0.85
ADP	0.48
ATP	0.04
GMP	0.66
GDP	0.32
GTP	0.02
TMP	0.77
TDP	0.28
TTP	0.02
UMP	0.74
UDP	0.23
UTP	0.02
IMP	0.68
IDP	0.19
ITP	0.02

compounds on all chromatograms. The U.L.-^{14}C-Glc-1-P was found to be pure, UDP-Glc-^{14}C-U.L. contained a trace of UDP, UTP-β,γ-^{32}P was pure except for a small amount of Pi. The ^{32}PPi appeared as if residual polyphosphates may have been present. This seemed not to affect the UTP estimations.

After validating the chromatographic system and radioactive chemicals, the first equilibrium experiment was performed using only the ^{14}C radioactive compounds. Two mixtures were prepared: the first containing 1.0 μmole of Glc-1-P and UTP with added U.L.-^{14}C-Glc-1-P; the second containing 1.0 μmole of UDP-Glc and PPi with added UDP-Glc-^{14}C-U.L. Each was incubated with 1.0 μmole of magnesium acetate and 0.17 units of crystallized enzyme in 1.0 ml of 0.1 M Tris-acetate buffer (pH 7.8) for 1 hr at 30°.

The reaction mixtures were then streaked on PEI paper and developed with a 2.0 M formic acid-0.3 M LiCl solvent for 3 or 4 hr at room temperature. The chromatogram was cut into 2.0 cm strips and each strip was counted. The radioactivity coincident with the Glc-1-P and UDP-Glc peaks was totaled and using a ratio of the counts with the preassayed amount of unlabelled substrates initially added to the mixtures, the amount of products formed was calculated. The results are shown in Table XII.

Experiments using both ^{14}C- and ^{32}P-labelled substrates were next performed. Again reaction mixtures were prepared, those starting with 1.0 μmole of Glc-1-P and UTP with added U.L.-^{14}C-Glc-1-P and UTP-β,γ-^{32}P and those with 1.0 μmole of

Table XII. Equilibrium study using ^{14}C-labelled substrates.

		Glc-1-P		UTP		UDP-Glc		PPi		
Mixture	Direction	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Keq
1	I	0.897	0.644	0.840	0.588	0.000	0.252	0.000	0.252	0.17
2	II	0.000	0.656	0.000	0.656	0.975	0.319	0.995	0.339	0.25

1.0 μmole quantities of the desired cold substrates, depending upon direction of synthesis, along with their respective radioactive substrates were incubated with 1.0 μmole of magnesium acetate and 0.17 units of crystallized enzyme in 1.0 ml of 0.1 M Tris-acetate buffer (pH 7.8) for 1 hr at 30°. The mixtures were then streaked on PEI paper and developed with 2.0 M formic acid-0.4 M LiCl for 3 or 4 hr at room temperature. The chromatograms were cut into 2.0 cm strips and each strip was counted. The initial amounts of cold substrates were pre-assayed. The direction and Keq definitions are the same as defined in Table III. All concentrations are micromolar.

UDP-Glc and PPi with added UDP-Glc-^{14}C-U.L. and ^{32}PPi. All mixtures were incubated with 1.0 μmole of magnesium acetate and the appropriate amount of enzyme in 1.0 ml of 0.1 M Tris-acetate buffer (pH 7.8) for 1 hr at 30°. The mixtures were each streaked on PEI paper and developed with a 2.0 M formic acid-0.4 M LiCl solvent for 3 or 4 hr at room temperature. The chromatograms were cut into 2.0 cm strips and each section counted. The counts under each peak were totaled with the results as shown in Table XIII. The Keq constant was calculated directly from the ratio of counts. Figures 3 and 4, representing experiments 5 and 6, respectively, show graphically how the peaks of each substrate separated on the chromatograms.

Table XIII. Equilibrium studies using ^{14}C- and ^{32}P-labelled substrates.

Experiment	Direction	Enzyme Units	Counts per Minute				Keq
			Glc-1-P	UTP	UDP-Glc	PPi	
1	I	0.21	3754	1473	1449	622	0.17
2	I	0.15	3917	1436	1190	536	0.12
	II	0.15	5070	8265	1780	3905	0.17
3	I	0.18	3334	1126	1800	458	0.22
	II	0.18	5975	6264	2100	2884	0.16
4	I	0.18	3743	1104	1343	638	0.21
	II	0.18	4112	5317	1421	2762	0.18
5	I	0.18	3809	973	1578	475	0.20
	II	0.18	4311	5617	1818	3017	0.23
6	I	0.14	3655	980	1494	387	0.16
	II	0.14	4125	5068	1857	2407	0.21

1.0 μmole quantities of the desired cold substrates, depending upon direction of synthesis, along with their respective radioactive substrates were incubated with 1.0 μmole of magnesium acetate and the appropriate amount of enzyme in 1.0 ml of 0.1 M Tris-acetate buffer (pH 7.8) for 1 hr at 30°. The mixtures were then streaked on PEI paper and developed with 2.0 M formic acid-0.4 M LiCl for 3-4 hr at room temperature. The chromatograms were then cut into 2.0 cm strips and counted. The direction and Keq definitions are the same as defined in Table III.

Figure 3. Equilibrium study using ^{14}C- and ^{32}P-labelled substrates.

Details of the experiment are described in the text. Graph A represents equilibrium starting with UDP-Glc^{14}C + ^{32}PPi ⟶ . Graph B represents equilibrium starting with ^{14}C-Glc-1-P + UTP-β,γ-^{32}P ⟶ .

CPM x 10^2

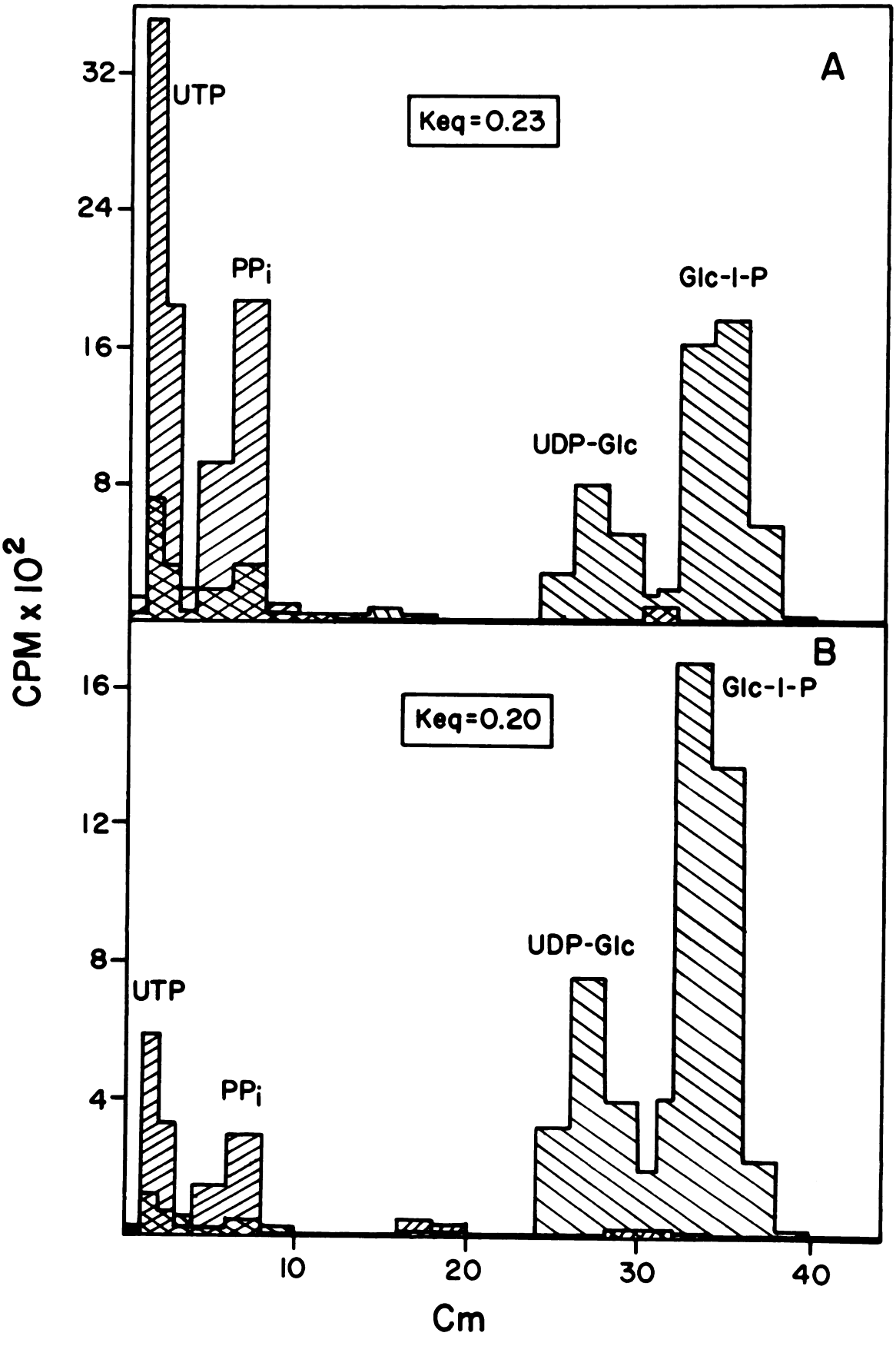
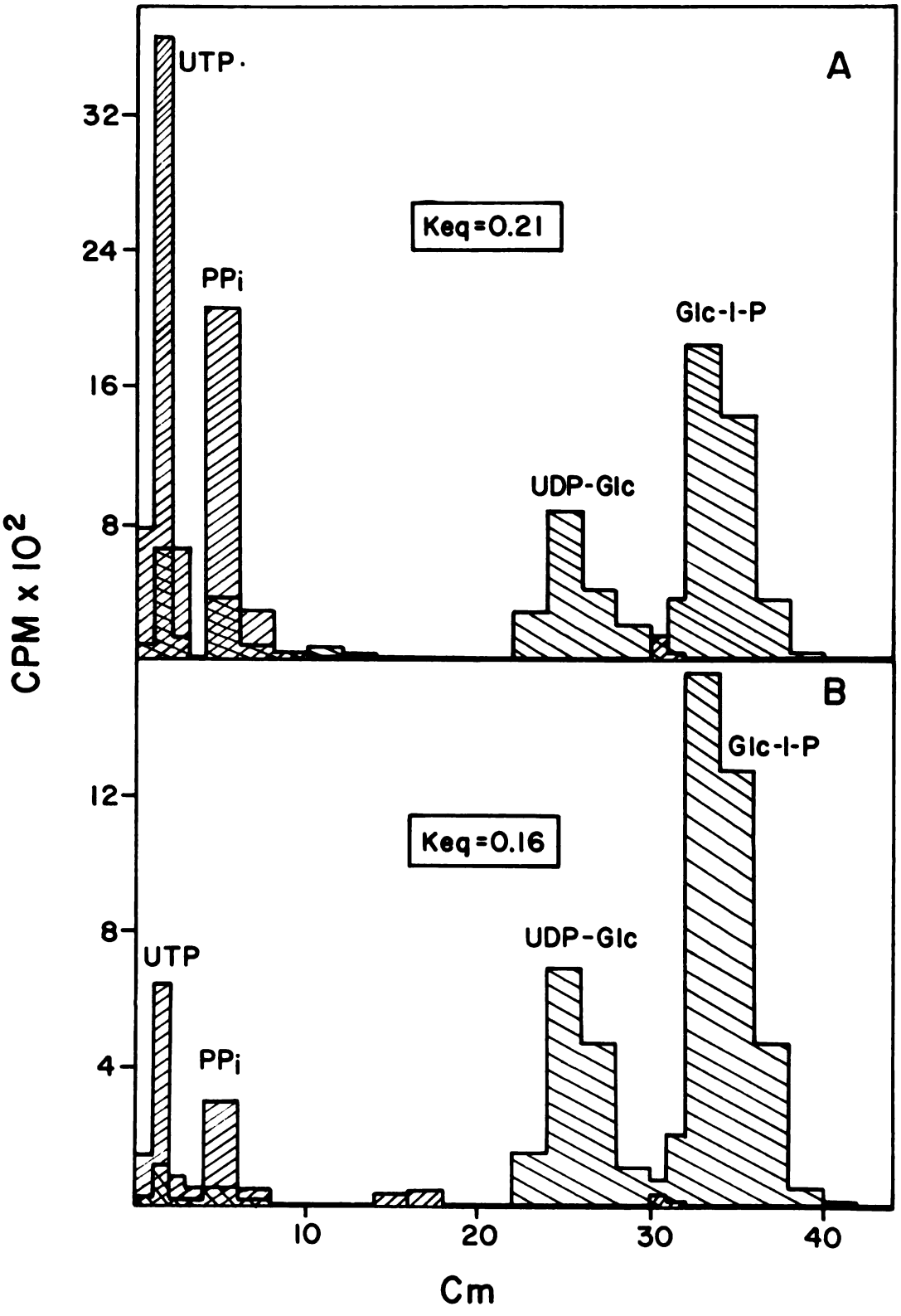

A
Keq=0.23
UTP
PPi
Glc-1-P
UDP-Glc
B
Keq=0.20
Glc-1-P
UDP-Glc
UTP
PPi
CPM x 10²
32
24
16
8
16
12
8
4
10
20
30
40
Cm

Figure 4. Equilibrium study using ^{14}C- and ^{32}P-labelled substrates.

Details of experiment are described in the text. Graph A represents equilibrium starting with UDP-Glc^{14}C + $^{32}PPi \longrightarrow$. Graph B represents equilibrium starting with ^{14}C-Glc-1-P + UTP-β,γ-$^{32}P \longrightarrow$.


A
UTP
PPi
Keq=0.21
Glc-1-P
UDP-Glc
B
Glc-1-P
Keq=0.16
UDP-Glc
UTP
PPi
CPM x 10²
32
24
16
8
12
8
4
10
20
30
40
Cm

DISCUSSION

The time course of the UDP-glucose pyrophosphorylase reaction studied by spectrophotometric means showed that equilibrium, in either direction, could be achieved within 1 hr at 30°, provided that 1.0 μmole quantities of each substrate and also magnesium were used. The value of Keq, which was defined as

$$Keq = \frac{[UDP\text{-}Glc][PPi]}{[Glc\text{-}1\text{-}P][UTP]} \qquad (2)$$

ranged from 0.23 to 0.25 in the spectrophotometric analysis. The presence of ammonium sulfate in the mixture showed no significant effect, yielding an equilibrium value of 0.14 to 0.27. Variations in the magnesium ion concentration appeared to have an effect on the constant. A favorable amount of magnesium was considered to be 1.0 mM. A level of 0.01 mM indicated that the turnover rate of the enzyme was inadequate for a 1 hr incubation. A 5.0 mM level of magnesium caused a precipitation of the PPi, effectively removing this product from the reaction mixture. Further consideration of the metal concentration should be interesting.

Assuming that ammonium sulfate did not greatly alter the Keq and the 1.0 mM magnesium was a favorable level to use, a series of 1 hr incubation mixtures were equilibrated and found to give an average constant of 0.20. The equilibrium,

therefore, favors the degradation of UDP-Glc, leaving approximately 30% of UDP-Glc and PPi, and 70% of Glc-1-P and UTP. This value agrees with that observed in E. Coli K_{12} (14), falls within the 0.10 to 0.21 range reported by Turner and Turner (11), and is slightly lower than that reported by Albrecht (15). However, it is appreciably lower than the value of 1.0 originally obtained by Munch-Petersen (4). The fact that she did not have a crystalline enzyme may account for this difference. The presence of other enzymes and substrates in an unpurified preparation would be expected to alter the constant.

A chromatographic method that was developed using a 2.0 M formic acid-0.4 M LiCl solvent on PEI paper was found to clearly separate the four components of the reaction mixture. This was the first time that Glc-1-P could be separated from UDP-Glc, and UTP from PPi using one solvent system in a minimum of time. Applying this method to equilibrium studies gave very satisfactory results. Using only ^{14}C-labelled substrates, the equilibrium constant was between 0.17 and 0.25. Using both ^{14}C- and ^{32}P-labelled substrates, Keq ranged from 0.12 to 0.23 and was averaged to be 0.18. This agrees nicely with the average value of 0.20 obtained by the spectrophotometric method. It is concluded that this new chromatographic method provides an excellent opportunity for further equilibrium studies of enzymatic reactions and can be performed quite simply in a short amount of time.

The application of the formic acid-LiCl solvent system

on the separation of other nucleotides yielded some interesting results. Under the acidic conditions the rate of migration decreased in the order: monophosphate > nucleotide sugars > diphosphate > triphosphates. Since polyethyleneimine is a resin that exchanges anions, the difference in negative charges of the compound affected their movement. The pattern is identical to that obtained by Randerath who used PEI-cellulose thin-layer plates instead of paper (24). Under neutral conditions using only LiCl as the solvent, Verachtert reported that the nucleotide sugars migrated faster than the monophosphates (25). Randerath also reported this reversal on the thin-layer plates (24). Again under acidic conditions, the general rate of migration as affected by the base component was cytidine > adenosine > guanosine > thymidine > uridine > inosine. There was some variation of this pattern when spots migrated near the solvent front or moved only a short distance from the origin. The level of salt also appeared to have an effect on their pattern of separation. It is important to note the separation of adenosine and inosine compounds on the PEI paper. Until now, this was impossible using only a LiCl solvent (25).

In summary, the separation of almost any two nucleotide substrates can be achieved using PEI paper and either an acidic or a neutral solvent. The rates of migration depend upon the salt concentration and the pH of the solvent, the net charge and the size and spatial configuration of the molecule.

SUMMARY

The enzyme, uridine diphosphate glucose pyrophosphorylase, catalyzing the biosynthesis of uridine diphosphate glucose from uridine triphosphate and glucose-1-phosphate, is available for the first time in crystalline form. The equilibrium constant for this enzymatic reaction was determined by spectrophotometric and chromatographic means.

A new spectrophotometric method for the determination of inorganic pyrophosphate was used. The spectrophotometric determinations of all the components of the reaction were also performed as a basis for the equilibrium calculations.

A new chromatographic method for the separation of all four components of the reaction was developed using polyethyleneimine-impregnated paper and a 2.0 M formic acid-0.4 M LiCl solvent. Employing ^{14}C- and ^{32}P-labelled substrates, the concentration of reactants at equilibrium was determined chromatographically.

The separation of other nucleotides and sugars was also achieved using polyethyleneimine-impregnated paper and a formic acid-LiCl solvent system.

The equilibrium constant determined by both the spectrophotometric and the chromatographic procedures was in good agreement at about 0.20. Accordingly, when the products were allowed to accumulate, the reaction proceeded to about

30% uridine diphosphate glucose formation from equivalent amounts of the substrates uridine triphosphate and glucose-1-phosphate.

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