PURIFICATION, PROPERTIES, AND KINETIC ANALYSIS OF UDP-GLUCOSE PYROPHOSPHORYLASE FROM BOVINE MAMMARY TISSUE

By

ROBERT ROSCOE AKSAMIT

Bachelor of Science

University of Wyoming

Laramie, Wyoming

1965

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
DOCTOR OF PHILOSOPHY
May, 1971

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Thesis Approved:

K. E. Ekner
Thesis Adviser

H. Olin Spivey

George V. Odell

Ernest M. Hodnett

Calvin A. Beames, Jr.

D. Durham
Dean of the Graduate College

ACKNOWLEDGEMENTS

The author gratefully acknowledges the guidance and advice of his adviser, Dr. Kurt E. Ebner, during the course of these investigations and the preparation of this dissertation. He sincerely thanks Drs. G. V. Odell, E. M. Hodnett, and C. L. Beames for serving as members of the advisory committee, and Dr. H. O. Spivey for serving as a member of the advisory committee and for his help during the sedimentation velocity experiments. The author is grateful to Dr. J. F. Morrison, NSF Visiting Professor from the Australian National University, Canberra, Australia, for the many timely discussions which promoted the author's confidence during the kinetic studies.

The author wishes to express his appreciation to Tom Nelson of the Animal Science Department for his help in the atomic absorption spectrometry, to Max Whaley for his help in the pH stability studies and preparation of enzyme for the fixed-time assay, and to Janelle Johnson and Sharon Cunningham for the fine technical assistance in running the fixed-time assays.

A very special thanks goes to the author's wife, Linda, for her patience and help during these studies and the preparation of the thesis.

The financial assistance and facilities provided by the Oklahoma State University Biochemistry Department and the Oklahoma Agriculture Experiment Station are gratefully acknowledged.

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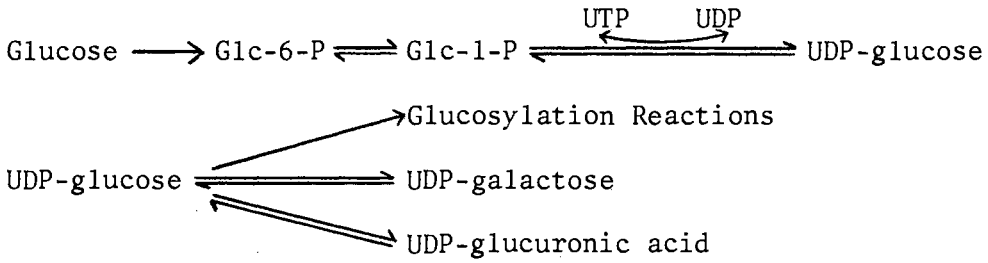
CHAPTER I

INTRODUCTION

The first nucleotide sugar isolated was UDP-glucose by Caputto et al. (1) in 1950. The general metabolism of this compound has since been elucidated and is presented below.



In certain tissues glucosylation reactions are probably the principal pathways of UDP-glucose metabolism. These include the synthesis of glycogen in mammals (2); the attachment of glucose to proteins, lipids, and DNA; and the glucosylation of aromatic amines and phenols for excretion in the urine (3). Transformation to UDP-galactose similarly provides the galactose required in saccharides, proteins, and lipids. The formation of lactose by this pathway has been the chief interest in this laboratory. The last pathway provides glucuronic acid for mucopolysaccharide synthesis and upon decarboxylation provides a source of xylose. The existence of UDP-glucose dehydrogenase (UDP-glucose: NAD oxidoreductase, EC 1.1.1.22) in rat mammary tissue has not been demonstrated (4).

In catabolic reactions UDP-glucose also has an essential role. It

is required catalytically in the Leloir pathway for galactose metabolism in man and microorganisms (5,6) as shown below.

$$\text{Galactose} + \text{ATP} \rightleftharpoons \text{Gal-1-P} + \text{ADP}$$

$$\text{GAL-1-P} + \text{UDP-glucose} \rightleftharpoons \text{UDP-galactose} + \text{Glc-1-P}$$

$$\text{UDP-galactose} \quad \text{UDP-glucose}$$

$$\text{Sum:} \quad \text{Galactose} + \text{ATP} \rightleftharpoons \text{Glc-1-P} + \text{ADP}$$

Tsuboi et al. (7) have proposed that the pyrophosphorolysis of UDP-glucose as the terminal reaction of galactose entry into the glycolytic pathway appears to be a primary, if not the sole, function of UDP-glucose pyrophosphorylase (UTP: α-D-glucose-1-P uridylyltransferase, EC 2.7.7.9) in erythrocytes.

In addition to UDP-glucose, approximately 100 other sugar nucleotides have been identified from biological sources. The nucleotide moiety may be UDP, ADP, GDP, dTDP, or CDP in combination with numerous sugar moieties such as glucose, mannose, galactose, rhamnose, xylose, arabinose, glucuronic acid, galacturonic acid, glucosamine, galactosamine, and several dideoxyhexoses. Extensive lists of sugar nucleotides occurring in biological systems and the reactions responsible for their synthesis have been published (3,8,9).

Many of the sugar nucleotides are the products of pyrophosphorylase reactions. If a sugar nucleotide is not formed directly by a pyrophosphorylase reaction, then it is usually formed by transformations of pre-existing sugar nucleotides (3). The first pyrophosphorylase, UDP-glucose pyrophosphorylase, was discovered and isolated by Munch-Petersen and his co-workers in yeast (10,11,12). Its existence had previously been implied by Trucco (13). The reaction is shown below.

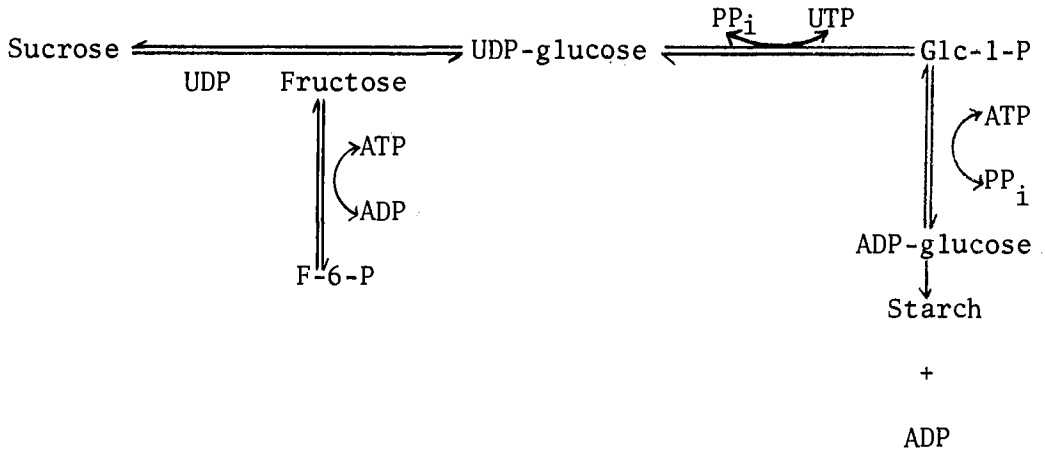
$$\text{UTP} + \text{Glc-1-P} \overset{Mg^{2+}}{\rightleftharpoons} PP_i + \text{UDP-glucose}$$

Most pyrophosphorylases appear to be specific for the nucleotide moiety and slightly less specific for the sugar moiety. UDP-glucose pyrophosphorylase activity has been separated from TDP-glucose pyrophosphorylase (TTP: α-D-glucose-1-P thymidylyltransferase, EC2.7.7) activity in E. coli B (14) and S. typhimurium (15). Upon Sephadex G-200 chromatography of an extract of S. typhimurium ADP-glucose, GDP-glucose, UDP-glucose, CDP-glucose, and TDP-glucose pyrophosphorylase activities were all separated from each other (16). GDP-hexose pyrophosphorylase activity has been separated from UDP-glucose pyrophosphorylase activity in bovine liver by Sephadex G-200 and DEAE-cellulose chromatography (17). However, UDP-galactose pyrophosphorylase activity is apparently a result of the nonspecificity of UDP-glucose pyrophosphorylase in liver (18). Similarly, pyrophosphorylase activity toward GDP-glucose and UDP-glucose have been separated in plant extracts by Sephadex G-200 chromatography (19).

The chemical mechanism of the UDP-glucose pyrophosphorylase reaction can be visualized as an attack by the phosphate of either Glc-1-P or PP_i on the pyrophosphate linkage of $MgUTP^{2-}$ or UDP-glucose, respectively (20). Similar reactions involving the attack of a nucleoside triphosphate may use phosphoric acid derivatives, fatty acids, amino acids, sulfuric acids, and carbonic acids as nucleophiles. A table of such reactions is presented by Kornberg (20).

Current evidence points to the nucleotide moiety of a sugar nucleotide as directing the use of the sugar moiety (3,9). For example, glycosyl nucleotides in plants give rise to different products;

ADP-glucose to starch (21), GDP-glucose to cellulose (22), UDP-glucose to callose (23), and sucrose (21,24). The interdependence of UDP-glucose and ADP-glucose sugar nucleotides is shown by the mechanism of starch formation as proposed by Turner (25,26).



The influence of several glycolytic intermediates on ADP-glucose pyrophosphorylase in plants and microorganisms has been summarized by Furlong and Preiss (27). 3-Phosphoglycerate acts as a potent activator, fructose-6-P activates to a lesser extent, and inorganic phosphate inhibits the reaction. Also it has been shown that the same nucleotide moiety may direct different acceptors in different organisms. Thus GDP-glucose is the glucosyl donor for cellulose in plants (22), but UDP-glucose is the donor in Acetobacter xylium (28). Likewise ADP-glucose acts as the glucosyl donor for starch, the storage polysaccharide in plants (21,25,26); but UDP-glucose performs the same function in mammals during the synthesis of glycogen (2).

Since UDP-glucose is utilized by three different pathways, it is possible that some of the end products may regulate the activity of the enzyme. The approach was to purify UDP-glucose pyrophosphorylase and to determine some of its physical and kinetic properties in order to

determine if multiple forms of the enzyme existed or if it possessed any unusual kinetic properties.

CHAPTER II

LITERATURE REVIEW

Introduction

The discussions in this chapter are directed with few exceptions to the various UDP-glucose pyrophosphorylases that have been studied. The review emphasizes the physical properties of the enzymes. The properties of UDP-galactose pyrophosphorylase activity in human liver (29) are included in this review because the enzyme is probably UDP-glucose pyrophosphorylase. Its stability in the presence of dithiolthreitol is similar to the human erythrocyte (7) and human liver (30) enzymes. In addition, the activity of the enzyme is very low compared to Gal-1-P uridyl transferase. Ting and Hansen (18) have shown that the UDP-galactose pyrophosphorylase activity in bovine liver is really due to UDP-galactose acting as a substrate for UDP-glucose pyrophosphorylase. The ratio of activity with UDP-glucose as a substrate to the activity with UDP-galactose as a substrate is constant throughout the purification of the bovine and human liver enzymes (18,30). Since UDP-glucose pyrophosphorylase is present in such large quantities in the liver (31), activity toward UDP-galactose is easily detected.

UDP-glucose pyrophosphorylase has been found in all tissues and organisms studied. However, in green algae a cellular gradient of enzymatic activity has been shown (32). The activity of the enzyme is highest in the apical region of the cell, whereas toward the basal end

it decreases sharply. It has been demonstrated that the distribution of the enzyme is the result of a preferential synthesis of the enzyme in the apical region of the stock.

Role of UDP-glucose Pyrophosphorylase During Synthesis

Developmental studies have shown that UDP-glucose pyrophosphorylase increases during the synthesis of a saccharide and decreases when synthesis decreases. Examples are presented below.

During development from a microscopic organism to a macroscopic spore forming organism, UDP-glucose pyrophosphorylase activity in the slime mold D. discoideum rises to a maximum and then falls until it reaches 50% of the maximum activity, at which point the activity remains constant. A detailed discussion of the various stages has been presented by Sussman and co-workers (33,34). The protein synthesis inhibitors, cycloheximide and actinomycin D, inhibit the increase of UDP-glucose pyrophosphorylase activity (34). The relationship of morphogenesis to UDP-glucose pyrophosphorylase activity has been substantiated by morphogenetically aberrant mutants which also have abnormal UDP-glucose pyrophosphorylase activity.

In the uninucleate green algae Acetabularia mediterranea, a close temporal relationship exists between cap formation and the activity of UDP-glucose pyrophosphorylase (35). The activity of the enzyme increases sharply when cap formation begins.

The change in UDP-glucose pyrophosphorylase activity from pregnancy to late lactation has been studied by a number of workers in rat mammary gland (4,36,37,38,39,40). UDP-glucose pyrophosphorylase activity increases gradually during pregnancy and then rapidly increases after

parturition (40). The enzymatic activity rapidly falls after weaning.

Genetics

Galactose nonfermenting mutants of E. coli K12, designated as Group E by J. and E.M. Lederberg, were found to have diminished levels of UDP-glucose pyrophosphorylase (41,42,43). As a result, these mutants have a diminished amount of hexoses in their cell walls. The location of UDP-glucose pyrophosphorylase on the E. coli chromosome has been mapped (44,45).

Mutants of E. coli K12 at the capR locus are mucoid, overproduce capsular polysaccharides, and are derepressed for synthesis of UDP-glucose pyrophosphorylase approximately 13 fold (45). Several other enzymes involved in capsular polysaccharide synthesis are also derepressed. The capsular polysaccharide contains D-glucose, D-galactose, D-glucuronic acid, and L-fucose. UDP-glucose pyrophosphorylase is also derepressed in a haploid capR9 mucoid mutant. Heterozygous mucoid partial diploids with the capR9 allele on the episome and the wild type ($capR^+$) allele on the chromosome (F'$capR9^+$/capR9) are derepressed for UDP-glucose pyrophosphorylase and GDP-mannose pyrophosphorylase. The reciprocal nonmucoid heterozygotes (F'$capR^+$/capR9) are repressed for these enzymes. These results provide evidence that episomal capR allele is dominant with respect to synthesis of these two enzymes (45). Since structural genes for UDP-galactose-4-epimerase and UDP-glucose pyrophosphorylase map at different loci than either capR of man (46), the capR regulator gene controls expression of several widely separated structural genes.

In S. typhimurium, Naikaido et al. (47) have mapped UDP-glucose

pyrophosphorylase activity in the rfb cluster between metG and his. The location on the overall chromosome has been discussed by Sanderson (48).

Assays

The following enzymatic assays have been described in the literature. Many of them have been used both as a fixed time assay and as a continuous spectrophotometric assay. Assays using paper chromatography for estimation of products have been omitted since the availability of instruments and reagents has made these assays of limited importance. Assays measuring pyrophosphorolysis are generally two to three times the velocity measured in the direction of synthesis.

Assay I - Estimation of Glc-1-P by Absorbance

The most commonly used assay was devised by Munch-Petersen et al. (11,12). Pyrophosphorolysis is measured by coupling glucose-6-P dehydrogenase and phosphoglucomutase to the Glc-1-P that is formed. Under conditions of excess indicator enzymes, one µmole of Glc-1-P quantitatively produces one µmole of NADPH which is measured by its absorbance at 340 nm.

Assay II - Estimation of Glc-1-P by Fluorescence

Tsuboi, Fukunago, and Petricciani (7) have applied fluorometry to the assay described by Munch-Petersen (11,12). NADPH which is formed as described above is measured by its natural fluorescence (49). This assay can detect UDP-glucose pyrophosphorylase activity at a concentration two or three fold less than the previous one. The increased

sensitivity allows the determination of product formed when only a small percentage of the reaction has occurred or when micromolar concentrations of substrate are used. This assay has been used as a fixed-time assay by inactivating UDP-glucose pyrophosphorylase by heat (7). A modification of this assay is presented in Chapter V.

Assay III - Estimation of UDP-glucose Using Snake Venom

In the direction of sugar nucleotide synthesis, UDP-glucose has been hydrolyzed with snake venom pyrophosphatase and the Glc-1-P determined by coupling with phosphoglucomutase and glucose-6-P dehyrogenase (50).

Assay IV - Estimation of Radioactive UDP-glucose

In the direction of UDP-glucose synthesis, Ashworth and Sussman have used a radioactive assay in slime mold (34). UTP, Glc-1-P-^{14}C, Mg^{2+}, and buffer are incubated with the enzyme for a fixed time. The reaction is stopped by heating, cooled, and incubated with alkaline phosphatase to remove unreacted Glc-1-P-^{14}C. The remaining phosphorylated compounds were precipitated with mercuric acetate in alcohol and collected on a Millipore filter. The filters were dried and counted on a gas flow planchet counter.

Assay V - Estimation of Radioactive Nucleotides

The enzyme from S. typhimurium has been assayed by incubation of Glc-1-^{32}P with nucleoside triphosphate, $MgCl_2$, buffer and enzyme at 37°C for 30 minutes (16). The reaction was stopped by precipitation with trichloroacetic acid and the nuclectides were absorbed onto

charcoal and separated for counting.

Assay VI - Estimation of UTP by Absorbance Utilizing the Hexokinase Reactions

In the direction of pyrophosphorolysis, Munch-Petersen (12) applied a system of enzymatic reactions described by Berg and Joklik (51) to measure the formation of UTP. Nucleoside diphophokinase utilizes UTP to covert ADP to ATP, which phosphorylates glucose to form glucose-6-P, the substrate for glucose-6-P dehydrogenase. Again the reaction is followed by the absorbance increase at 340 nm of NADPH. Any glucosyl nucleotide whose nucleotide moiety will react with nucleoside diphosphokinase can be used in this assay.

Assay VII - Estimation of UTP Using 3-Phosphoglycerate Kinase and Glyceraldehyde-3-Phosphate Dehydrogenase

Verachtert et al. (52) have described an assay to measure the production of ATP, GTP, ITP, or UTP. The nucleoside triphosphate is used to phosphorylate 3-phosphoglycerate catalysed by 3-phosphoglycerate kinase. The product is then converted to glyceraldehyde-3-P and NAD^+. The reaction is driven to completion by the addition of hydrazine. The assay measures the decrease in absorbance of NADH at 340 nm. This assay was found to be fast enough to use in the spectrophotometric assay of UDP-glucose pyrophosphorylase from bovine liver. The measurement of pyrophosphate; reducing sugar in the presence of phosphomonoesterase; nucleoside triphosphates in the presence of ADP, nucleoside diphosphokinase, hexokinase, glucose, and glucose-6-P dehydrogenase; and chromatography of nucleotide diphosphate sugars was not sufficiently rapid or specific to assay the liver enzyme. The assay will not

measure CTP or TTP.

Assay VIII - Estimation of UDP-glucose by Absorbance

Oliver (53) has measured UDP-glucose formation by a two step procedure. The enzyme was inactivated by heating followed by the addition of NAD^+ and UDP-glucose dehydrogenase. The NADH produced has been measured by absorbance at 340 nm (53) or by its natural fluorescence (7). Similarly, Shatton et al. (4) and Steelman and Ebner (54) have described a spectrophotometric assay in which UDP-glucose dehydrogenase is used as the indicator enzyme.

Assay IX - Estimation of Pyrophosphate

Basu and Bachhawat (55) have assayed UDP-glucose pyrophosphorylase in brain tissue by measuring the disappearance of inorganic pyrophosphate in the presence of UDP-glucose. The substrates, UTP and Glc-1-P, are incubated at 37°C with $MgCl_2$ in an appropriate buffer and the inactivated by heating at 100°C for one minute. After incubation of the heat denatured mixture with inorganic pyrophosphatase for 10 minutes at 37°C, inorganic phosphate is measured by the method of Fiske and Subbarow (56). Malpress (36) has measured inorganic pyrophosphate by precipitation with Mn^{2+}.

Assay X - Separation of Radioactive Products by DEAE-cellulose Paper

A radioactive assay adaptable to measuring either synthesis or pyrophosphorolysis has been described by Abraham and Howell (29). The ^{14}C-sugar phosphate is reacted under appropriate conditions with UTP to form ^{14}C-UDP-sugar. At the end of the incubation, the reaction mixture

is treated with alkaline phosphatase to hydrolyze the residual ^{14}C-UDP-sugar phosphate, leaving ^{14}C-D-sugar and ^{14}C-UDP-sugar as the only radioactive compounds in solution. These two substances are then readily separated by chromatography on DEAE-cellulose paper (57,58). The radioactivity in the sugar nucleotide is then determined quantitatively in a scintillation counter. This method could probably be improved by separating the components of the reaction mixture on DEAE-cellulose paper developed by an appropriate buffer containing Mg^{2+} (59). This could possibly eliminate the need for alkaline phosphatase treatment.

Purification

UDP-glucose pyrophosphorylase has been purified from E. coli (60), yeast (Saccharomyces fragilis) (12), mung beans (61), bovine (31,54,62), rat (53,63), rabbit (64), guinea pig (53), dog (7), and human (7,30,55). The organs used in mammalian systems are liver (7,30,31,53), muscle (64), heart (7), erythrocytes (7), brain (53,55), thyroid (62), and the mammary gland (54,63). Table I summarizes the results of these purifications. The best preparations have been obtained from liver where the enzyme comprises as much as 0.2 to 0.3% of the extractable protein (30,31).

Similarities among the purifications can be found. All the procedures utilize ammonium sulfate fractionation and most contain a DEAE-cellulose step. Calcium phosphate gels are frequently used when the enzyme is readily available since this treatment usually gives a poor yield of enzyme. The enzyme is generally stored best as a concentrated alkaline ammonium sulfate suspension.

TABLE I

SUMMARY OF PURIFICATIONS OF UDP-GLUCOSE PYROPHOSPHORYLASE OBTAINED FROM VARIOUS SOURCES

Source	Fold Purification	Per Cent Yield	Specific Activity (I.U.)	Assay	Protein	Comments	Reference
E. coli K12	256	18	11	I	Lowry (65)	Free of interfering enzymes	60
Saccharomyces fragilis	257	15	7.7	I	Lowry	"	12
Mung Beans	800	25	815	I	Lowry Biuret (66) U.V. (67)	Sensitive to -SH reagents	61
Bovine Liver	300	11.5	240	I	Lowry	Crystallized to constant specific activity, 1 to 4 bands on disc gel electrophoresis	31, 68
Human Liver	527	66	200	I	Lowry	Crystals sensitive to -SH reagents; on disc gel one band with fresh preparation	30
Rabbit Skeletal Muscle	1300	35	82.5	I	Biuret U.V.		64
Human Erythrocyte	1450	22	127	II	Lowry	Multiple bands by acrylamide electrophoresis; contaminated by GDP-PP_i	7
Human Brain	32	36	155	IX	U.V.		35
Bovine Thyroid	192	32.6	1.92	I	Lowry		62
Bovine Mammary Gland	50	41	5.2	VIII	Lowry	Electrophoresis on Sephaphore III (Gelman) 1 sharp band, 1 faint	54
Rat Mammary	225	29.4	53.9 162	VIII I	Lowry	Disc gel electrophoresis at 8.9 or 4.8 gave 1 major, 1 minor, and sometimes 3rd band	63

It must be pointed out that only a semiquantitative comparison can be made between many of the listings in Table I. The assays vary and the rate is dependent upon the direction of assay as well as the conditions of the assay such as pH, ionic strength, unintended inhibitors or activators, side reactions, temperature, and possibly the concentration of the enzyme. The reaction in the direction of pyrophosphorolysis has been reported to be 1.6, 2, 3, and 5 times as fast as the reaction in the direction of synthesis (33,54,60,63). The enzymes from different classes of organisms (microorganisms, plants, animals) probably possess different amino acid sequences which can affect the various kinetic constants and therefore the specific activity. Since there is no accurate way of measuring total protein in a complex mixture, the specific activity will also depend upon the method of protein estimation.

Two of the procedures presented in Table I have been used for the purification of UDP-glucose pyrophosphorylase from other sources. The method of Villar-Palasi and Larner (64) has been applied to dog heart (7) and rat liver (53). The rat liver preparation gave one band on starch electrophoresis and showed a minor component upon sedimentation velocity in the Model E analytical centrifuge. A slight modification of the method used to purify bovine liver (31,68) has been used to purify the enzyme from rat liver (69). Ammonium sulfate fractionation and chromatography on DEAE-cellulose has been used to purify the enzyme from guinea pig brain (53).

A crude extract from wild type S. typhimurium has been applied to a DEAE-cellulose column (47). The UDP-glucose pyrophosphorylase activity was separated into four peaks, all of which contained activity towards TDP-glucose. Since the first peak had a much lower apparent K_m

for TDP-glucose than for UDP-glucose, it was considered to be TDP-glucose pyrophosphorylase. The remaining three peaks had low apparent K_m values for UDP-glucose relative to TDP-glucose and were assumed to be UDP-glucose pyrophosphorylases.

Stability

As mentioned previously, UDP-glucose pyrophosphorylase is most stable in concentrated alkaline solutions at 0 to 4°C. In some cases the enzyme has been successfully frozen, particularly in crude extracts (21,29,34,55,63,70). In the presence of an equal volume of glycerol the E. coli K12 enzyme could be stored at -20°C for several months with only a gradual loss of activity (60). Bovine thyroid enzyme is more stable in neutral or slightly acid solutions (62), but the enzyme from E. coli B is denatured by acetate buffers at pH 4.5 to 6.0 (14). The enzyme from slime mold is unaffected by pH in the range 5.5 to 9.0 (34). Incubation of the rabbit muscle enzyme at 30°C for 30 minutes gives a pH stability maximum of 9.8 (64). In some cases the enzyme has been stored with substrates or metal (55,63). The enzyme from bovine mammary gland was not stabilized by 10 mM mercaptoethanol, 10 mM cysteine, 10 mM ascorbic acid, 5 mM EDTA, 10 mM $MgCl_2$, 1% serum albumin or 50% ethylene glycol (54). Munch-Petersen was not able to stabilize the yeast enzyme with 3 mg/ml bovine serum albumin (12).

Mung bean, human erythrocyte, and human liver enzymes all require a sulfhydryl reagent such as dithiolthreitol for stability (7,29,30,61). Activity lost upon vacuum dialysis of erythrocyte enzyme from column chromatographic separations can be restored by the addition of dithiolthreitol (7). Dithiolthreitol was found to increase UDP-galactose

pyrophosphorylase activity in human liver as much as 100% (29). A lesser increase occurred with glutathione. However, Basu and Bachhawat (55) found that cysteine or glutathione had no effect on the activity of the brain enzyme.

Rodriguez et al. (62) found that the heat stability of the thyroid enzyme was a function of ammonium sulfate concentration. The enzyme was heated at 55°C for 4 minutes at various concentrations of ammonium sulfate. Under these conditions optimum activity occurred between 25 to 30% ammonium sulfate with 90% or more of the activity remaining. From 0 to 10% and at 40% ammonium sulfate, 20% of the activity remained. The E. coli B enzyme (14) was denatured by briefly heating at 50°C. Heating the enzyme from pea seeds for 10 minutes at 60°C resulted in a 75% loss in activity (70). The enzyme from the slime mold D. discoideum is stable for at least one hour at 35°C (34).

The effects of buffers on the stability of UDP-glucose pyrophosphorylase from slime mold has been investigated (33). In one hour at 37°C a crude homogenate at a concentration of 1 mg/ml lost 80% activity in Tris, pH 7.5 or pH 8.5, while only 10% was lost in Tricine, pH 7.5. Further investigation showed that the apparent instability depended upon two factors, the protein concentration of the extract and the developmental stage of the organism. When the slime mold was grown for 3 hours, the crude extract showed a stability optimum at 3 mg/ml when heated for one hour at 37°C in 0.1 M Tris, pH 7.5. On the other hand when the slime mold had grown for 19 hours and then was subjected to the same treatment, no stability optimum was observed up to 6.5 mg/ml of protein. Instead, the enzymatic activity continued to increase with protein concentration.

Under assay conditions in Tris or Tricine, the enzyme is stable for at least 30 minutes at 37°C. Tests of single components of the mixture showed that inorganic pyrophosphate is the probable stabilizing agent. At 10 mM pyrophosphate the enzyme was almost completely stable over a 60 minute period. UTP has been reported to protect the enzyme (71) and is as effective as pyrophosphate at equimolar concentrations. Wright and Dahlberg (72) have found a slight stabilization of a crude extract of D. discoideum by UDP-glucose.

Molecular Properties

Levine et al. (73) have found that the basic protein unit of the bovine liver UDP-glucose pyrophosphorylase is a monomer composed of eight identical or nearly identical subunits. The evidence was obtained by disc gel electrophoresis, sucrose density gradient centrifugation, ultracentrifugal analysis, and electron microscopy.

The basic eight subunit structure (monomer) associates to a dimer, trimer, and tetramer. A sample of enzyme centrifuged on a 5 to 20% sucrose gradient showed four separated peaks of protein each of which have the same specific activity. The separate peaks were collected, dialyzed in 10,000 to 20,000 volumes of 0.01 M Tricine, pH 8.5, for three days, and concentrated. The original sample showed the same four bands upon disc gel electrophoresis. Each of the single associated species had begun to re-equilibrate to the other polymeric forms. Ultracentrifugal analysis showed up to three peaks with Schliern optics. The sedimentation coefficients, $s^{0.4\%}_{20,w}$, were 13.22 S for the principal component and 19.66 S and 25.08 S for the two minor components. Extrapolation to infinite dilution yielded a $s^{0}_{20,w}$ of 14.10 S to 14.45 S and

a $D^0_{20,w}$ of 2.72 to 2.87 x 10^{-7} cm^2 per sec for the major component. Combination of these values, assuming a spherical molecule with a partial specific volume of 0.725, yielded a molecular weight of about 480,000. This compares to a molecular weight of 350,000 $\pm$ 50,000 estimated from sucrose density gradient centriguation (31) and of 424,000 calculated from a previous sedimentation velocity constant of 14.8 (31). The frictional ratio was calculated to be 1.5.

In 6 M guanidine hydrochloride the enzyme dissociates into eight subunits with an $s^0_{20,w}$ of 2.15 S and a $D^0_{20,w}$ of 2.90 x 10^{-7} cm^2 per sec. A combination of these values gives a molecular weight of 69,100. The frictional ratio was 2.8. The addition of mercaptoethanol to the dissociated monomer in guanidine hydrochloride or to the aggregated enzyme did not change the results. Thus, sulfhydryls do not play a major role in the quaternary structure of bovine liver UDP-glucose pyrophosphorylase.

Human liver UDP-glucose pyrophosphorylase also appears to undergo polymerization upon aging (30). A slower migrating band appeared on disc gel electrophoresis after storage. However, upon sucrose density gradient centrifugation or sedimentation velocity centrifugation, a slower component was observed. Thus the component which appears to be of higher molecular weight on disc gel electrophoresis may only be more highly charged. Knop and Hansen (30) suggest that the slower moving component on disc gel electrophoresis may be a dimer, and perhaps the slower sedimentating component is one of two interconvertible forms of the same protein. The sedimentation coefficient, $s^{0.4\%}_{20,w}$, was found to be 12.8 S for the major component.

Sephadex G-200 chromatography has been used to estimate the

molecular weight of the human erythrocyte (7) and S. typhimurium (16) enzymes. Human erythrocyte enzyme has a molecular weight of 440,00 ± 25,000 (7) and S. typhimurium has a molecular weight of about 100,000 (16). Rat mammary gland enzyme has been reported to pass unhindered through Sephadex G-200 (63). Using sucrose density gradient centrifugation, a molecular weight of about 450,000 is found for the rat mammary gland enzyme (73). Thus, there is apparently a large difference in the size of mammalian and enterobacterial enzymes.

Enzyme from slime mold cells harvested after 19 hours shows two peaks of activity upon sucrose density gradient centriguation (33). The major peak is stable in Tricine at 37°C for 15 minutes, but is partially labile in Tris. The second minor peak is slower moving and represents 5 to 10% of the total activity. It is equally labile in Tris or Tricine at 37°C, losing nearly all its activity in 40 minutes. The two peaks were not freely interconvertible. An extract harvested after 4 hours of incubation showed the same two peaks plus a third faster moving peak. The data is consistent with two forms of enzyme differing in their stability in Tris and Tricine. With this assumption the kinetics of loss in activity for the unstable form in Tris can be shown to be first order with a half-life of 3 1/2 minutes (33).

Crude homogenates of UDP-glucose pyrophosphorylases from various sources have been shown to increase 2 to 50 fold in reactivity without further treatment as a function of time (74). The optimum activity usually occurs after 10 hours. It is dependent upon temperature, concentration of extract, initial level of enzymatic activity, pH, and urea concentration. The "activation" process in rat explants was independent of 5 µg/ml prolactin, 5 µg/ml hydrocortisone, 5 µg/ml insulin,

0.2 mM puromycin, 0.5 mM phenylmethylsulfonyl fluoride (a proteolytic inhibitor), and 1% sodium cholate. The apparent K_m for UTP and the molecular weight are not altered during the increase in activity. Passing the homogenate through a G-25 column did not interfere with its ability to increase in activity. The "activation" was also observed in an extract of acetone powder and in a 30 to 50% ammonium sulfate cut. It is suggested that the enzyme undergoes a structural change to a more active form of the enzyme.

The Catalytic Reaction

pH Activity Optimum

Nearly all of the UDP-glucose pyrophosphorylases studied have a broad pH profile between approximately pH 7 to 9. Two cases which report a sharp profile (55,61) were obtained with relatively long incubation periods of 20 minutes at 25°C and 30 minutes at 37°C. The pH optimum in these two cases occurred at pH 8. It may be that the sharpness of the pH profile is dependent upon the length of incubation during a fixed time assay (55,64). The longer the time of incubation the sharper the pH profile appears. Thus a stability function is superimposed upon the pH profile. The pH profile of UDP-galactose pyrophosphorylase activity in human liver is sharp with an optimum at pH 8 (29).

Turnover Number

The turnover number reported by Albrecht et al. (31) for the crystalline bovine liver UDP-glucose pyrophosphorylase is 83,000 moles of substrate converted per minute per mole of enzyme at 25°C. However, this value is based on the earlier molecular weight of 350,000. Using

the most recent data reported by Levine et al. (73), namely, a molecular weight of 480,000 and a specific activity of 238 units per mg protein, a value of 114,000 moles of substrate per minute per mole of enzyme at 25°C is obtained.

Metals

A divalent metal is an absolute requirement of UDP-glucose pyrophosphorylase in all cases studied, with Mg^{2+} the most effective. Tsuboi et al. (7) in human erythrocytes have presented some of the best evidence for UTP and inorganic pyrophosphate participating in the reaction as their metal complexes and for Glc-1-P and UDP-glucose participating in the noncomplexed form. The activation by Mg^{2+} was found to be largely independent of UDP-glucose or Glc-1-P while the maximum occurred when the concentration of Mg^{2+} ion was approximately equal to the concentration of inorganic pyrophosphate or UTP. Inhibition at high levels of Mg^{2+} ion concentration could be due to increased ionic strength or inactive complexes such as formation of Mg·UDP-glucose and Mg·Glc-1-P.

In bovine mammary gland enzyme, Mg^{2+} can be replaced by 5 mM Mn^{2+} and Co^{2+} with 95% and 47% of the activity remaining, respectively (54). In the same tissue Ca^{2+}, Ba^{2+}, and Zn^{2+} have measurable activity but interfer with the assay. Ni^{2+} gave 60% inhibition, but in crude pea seed extracts (70) Ni^{2+} was stimulatory as was Co^{2+}. In bovine liver Mn^{2+} and Co^{2+} gave 25% of the activity of Mg^{2+}; Ca^{2+} gave only slight activity (31). No activity was found with Fe^{2+}, Cu^{2+}, Ni^{2+}, and Zn^{2+}, but all of the divalent cations tested were inhibitory with Mg^{2+} (30, 31). Sodium ions were without effect.

In human liver, Mg^{2+} was optimal at 3 mM but Mn^{2+} was optimal at 1 mM (30). Co^{2+} at 1 mM was 40% as effective as Mg^{2+}. In human erythrocytes the order of effectiveness was $Mg^{2+} > Mn^{2+} > Co^{2+} > Ca^{2+}$ when measured in the direction of UDP-glucose synthesis (7). In the direction of pyrophosphorolysis, Ca^{2+} was ineffective. Mn^{2+}, Zn^{2+}, Ni^{2+}, Ca^{2+}, and Cu^{2+} are ineffective in replacing Mg^{2+} in human brain (55). In slime mold, Mn^{2+} substituted for Mg^{2+} with approximately 50% of the rate obtained with Mg^{2+} (34).

Substrate Specificity

Under most physiological conditions UDP-glucose pyrophosphorylase is specific for both nucleotide and sugar phosphate. However, Albrecht et al. (31) have termed the bovine liver nonspecific because UDP-galactose will substitute with a rate as high as 3% of the rate obtained with UDP-glucose. In addition to UDP-galactose, TDP-glucose and UDP-xylose will give 1 to 3% of the rate obtained with UDP-glucose in bovine liver (31). In human erythrocytes, UDP-glucose pyrophosphorylase will give 1.3% of the activity with TDP-glucose (7). It is interesting to note that the pyranose form of xylose is equivalent to the pyranose form of glucose minus C-6. It may be that the pyranose form of UDP-xylose composes 1 to 3% of UDP-xylose in solution, the remainder being the preferred furanose form.

Sugar nucleotides which show less than 1% of the activity obtained with UDP-glucose, but yet are detectable include CDP-glucose, GDP-glucose, IDP-glucose, UDP-mannose (30,31); ADP-glucose, UDP-glucuronic acid, and UDP-glucosamine (31).

Sugar nucleotides with no detectable activity include GDP-glucose

in rabbit muscle (64); ADP-glucose in human liver (30) and E. coli (60); UDP-N-acetylglucosamine, GDP-mannose, and NAD^+ in yeast (12); and 1 mM UDP-galactose in E. coli (60).

In the direction of synthesis no activity was detected with the following sugars: β-D-glucose-1-P (54,61,64), α-L-arabinose-1-P (64), β-L-arabinose-1-P (61), α-D-galactose-1-P (64), β-D-galactose-1-P (64), α-D-xylose-1-P (61,64), β-D-xylose-1-P (64), α-D-ribose-1-P (61,64), glucuronic acid-1-P (61), galacturonic acid-1-P (61), and mannose-1-P (54). In human erythrocytes galactose-1-P, mannose-1-P, and xylose-1-P gave less than 1% of the activity obtained with glucose-1-P (7). GTP gave 6% of the rate obtained with UTP in human erythrocytes (7). This was considered to be due to contamination by GDP-glucose pyrophosphorylase.

The amount of activity shown by the various substrate analogs above will depend upon the levels of the analogs relative to substrate concentration, upon the sensitivity of the assay, and upon the amount and purity of the enzyme used. With these considerations, the data from the bovine liver enzyme (31) is probably the most reliable.

With one exception, Table II tabulates the reported apparent K_m's of UDP-glucose pyrophosphorylase from various sources studied. The notable exceptions are the true K_m values obtained from initial velocity studies on human erythrocyte enzyme. K_m values for UTP, Glc-1-P, and inorganic pyrophosphate generally fall between 10^{-4} to 10^{-3} M. The K_m value for UDP-glucose is in the 10^{-5} M range.

The apparent K_m values are dependent upon the levels of fixed substrate and must be extrapolated to infinite substrate concentration to obtain the true K_m value. Mg^{2+} ion and buffer can also affect the K_m

TABLE II

APPARENT K_M VALUES FOR UDP-GLUCOSE PYROPHOSPHORYLASE FROM VARIOUS SOURCES

Source	Substrate	Apparent K_m	Fixed Substrate	$MgCl_2$	Buffer	Reference
		mM	mM	mM		
E. Coli K12	UDP-glucose	0.13	1	3	25 mM Tris, pH 7.5, 25°	60
	PP_i	0.13	2	3		
	Glc-1-P	0.048	2	3		
	UTP	0.029	1	3		
E. Coli Strain B	UDP-glucose	0.25	4	10	100 mM Tris-HCl, pH 7.8, 25°	14
	PP_i	3.1	0.2	10		
Sacch. fragilis	UDP-glucose	0.07	1	4	40 mM Tris, pH 7.2, room temperature	12
Yeast	UDP-glucose	0.09	4	10	100 mM Tris-HCl, pH 7.9, 25°	14
D. discoideum	UTP	0.4	5	10	70 mM Tris, pH 7.4, 35°	34
	Glc-1-P	0.03	10	10		
Mung Beans	UDP-glucose	0.11	1	2	85 mM Tris, pH 7.5, room temperature, 6.5 mM mercaptoethanol	61
	PP_i	0.23	0.2	2		
Mung Beans	UTP	0.16	3	1.5xUTP	50 mM Tris, pH 8.5, 30°	7
	UDP-glucose	0.11	3	1.5xUTP		
Rat Liver	Glc-1-P	0.18-0.28	0.58	2.3	38.5 mM Tris, pH 7.4, 30°	53
Rat Liver	UDP-glucose	0.09	-	-	-	81
Rat Liver	Glc-1-P	0.19	0.4	2	30 mM Tris, pH 7.4, 25°	69
Rat Mammary Gland	Glc-1-P	0.39	3.33	6.67	167 mM Glycine, pH 8.8, 25°	63

TABLE II (Continued)

Source	Substrate	Apparent K_m	Fixed Substrate	$MgCl_2$	Buffer	Reference
Rat Mammary Gland	Glc-1-P	2.6	1.25	2.5	100 mM Glycine, pH 8.7, 25°	4
	UTP	0.22	7.5	2.5	2.5 pH 8.7, 25°	
Rat Mammary Gland	UTP	0.23, 0.19	5	2	25 mM Tris, pH 8.0, 23-25°	74
Rabbit Skeletal Muscle	UDP-glucose	0.045	1	2	25 mM Tris, pH 7.45, 30°	64
	PP_i	1	0.4	2	120 mM Tris, pH 8.0, 37°	
Guinea Pig Brain	Glc-1-P	0.045-0.086	0.58	2.3	38.5 mM Tris, pH 7.4, 30°	53
Dog Heart	UTP	0.36	3	1.5xUTP	50 mM Tris, pH 8.5, 30°	7
	UDP-glucose	0.028	3	1.5xUTP		
Bovine Liver	Glc-1-P	0.055	-	-	-	31
	UDP-glucose	0.06	2	2	90 mM Tris-acetate, pH 7.8, 25°	
	PP_i	0.084	0.4	2		
	UTP	0.2	-	-		
	TDP-glucose	0.35	2	2		
Bovine Mammary Gland	UTP	0.29	5	1	25 mM Tris, pH 8.0, 23-25°	54
	UTP	0.14	5	5		
	Glc-1-P	0.21	1	1		
	Glc-1-P	0.11	1	5		
	UDP-glucose	0.085	1	5		
	PP_i	1.0	0.2	2		
Human Brain	UDP-glucose	0.075	4	8		55
	PP_i	0.2	2	8		

TABLE II (Continued)

Source	Substrate	Apparent K_m	Fixed Substrate	$MgCl_2$	Buffer	Reference
Human Liver	UDP-glucose	0.05	-	-	90 mM Tris-acetate, pH 7.8,	30
	UTP	0.048	-	-	2mM mercaptoethanol, 25°	
	Glc-1-P	0.095	-	-		
	PP_i	0.21-0.26	-	-		
Human Liver	Gal-1-P	1.8	3.95	11.6	46.5 mM Tris, pH 8.1, 37°	29
	UTP	0.8	0.015	11.6		
Human Erythrocyte	UTP	0.33	3	1.5xUTP	50 mM Tris, pH 8.5, 30°	7
		0.33	0.1-3.0	1.5xUTP		
	Glc-1-P	0.17	0.2-1.0	1.5xUTP		
	UDP-glucose	0.023	3	3		
		0.04	0.3-3.0	equal to PP_i		
	PP_i	0.48	0.05-1.0	equal to PP_i		

value. Considering the stability constants for $MgUTP^{2-}$ (7×10^4) (75, 76) and for magnesium pyrophosphate (2.5×10^5) (77), UTP and pyrophosphate will essentially exist as their metal complexes whenever Mg^{2+} ion is equimolar or greater. A small but insignificant concentration of noncomplexed UTP and PP_i also exists. Since optimum rates occur when the ratio of Mg^{2+} to UTP or pyrophosphate is one to one, it is assumed that the metal complexes are the true substrates. In early studies the metal substrate concentration was not varied to obtain K_m values, but rather, Mg^{2+} would be fixed and UTP or pyrophosphate varied. As would be expected, this gave nonlinear Lineweaver-Burk (78) plots (55,64). Thus many investigators have not reported values for UTP or more frequently for inorganic pyrophosphate. No studies have answered whether or not the metal may interact directly with the enzyme. Glc-1-P and UDP-glucose can bind Mg^{2+}, but at quantities which are insignificant compared to UTP or pyrophosphate. The stability constant for Glc-1-P is 20 (79,80). The stability constant for UDP-glucose is not known, but might be expected to be similar to that of ADP or UDP, namely 4×10^3 (75,76).

UTP and pyrophosphate combine with Mg^{2+} ion as bidentate ligands and thus are highly pH dependent (75). At pH 8.0 the pyrophosphate linkages are in a single ionic form and therefore only one ionic species need be considered. Below pH 8.0 multiple ionic forms occur.

Equilibrium

The reversibility of the UDP-glucose pyrophosphorylase reaction was first shown by Munch-Petersen *et al.* (11) using ^{32}P labelled UTP or Glc-1-P. Equilibrium constants shown in Table III range from

TABLE III

APPARENT EQUILIBRIUM CONSTANTS FOR UDP-GLUCOSE PYROPHOSPHORYLASE FROM VARIOUS SOURCES

Source	°C	$K_{eq} = \frac{(UTP)(Glc\text{-}1\text{-}P)}{(UDP\text{-}glucose)(PP_i)}$	Initial Concentration UTP	PP_i	Mg^{2+}	$\frac{Mg^{2+}}{UTP+PP_i}$		Reference
			mM	mM	mM			
E. coli K12	25	5		0.275	3	10.9	50 mM Tris, pH 7.5	60
Sacch. fragilis	22-25	approximately one		0.25	5	20	50 mM Tris, pH 7.4	12
Pea Seeds	30	7.2		9.2	5	0.54	200 mM Tris, pH 7.9	70
		3.8		9.2	10.0	1.09	"	
		8.4			2.5	0.27	"	
		3.5		9.2	2.5	0.27	200 mM Tris, pH 7.0	
Bovine Liver	30	2.9-3.6	0.56		2	3.6	100 mM Tris acetate, pH 7.8	31
			2.82		2	0.7		
			0.35		2	5.7		
Human Liver	30	6.7	0.79		1	1.3	90 mM Tris-acetate, pH 7.8, 2 mM mercaptoethanol	30
Human Erythrocytes	30	3.8	20	or 20	20	1	50 mM Tris, pH 8.5	7

approximately 1 to 8.4 and favor pyrophosphorolysis. All equilibrium constants were calculated using the total concentrations of UTP and pyrophosphate. Equilibrium constants which were measured at a ratio of total metal to UTP plus PP_i greater than one probably represent true equilibrium constants. When the ratio is less than one, only apparent constants are calculated. The apparent equilibrium constants can be seen to be a function of pH and Mg^{2+} ion concentration (70). In pea seeds lowering the pH from 7.9 to 7.0 lowers the equilibrium constant from 8.4 to 3.5 (70). Changing the ratio of total metal to toal UTP plus inorganic pyrophosphate from 0.54 to 1.09 lowers the equilibrium constant from 7.2 to 3.8 (70).

Inhibition

A list of competitive inhibitors of UDP-glucose pyrophosphorylase is presented in Table IV with their apparent inhibition constants. Galactosamine-1-P is an effective inhibitor of purified rat and bovine liver enzymes (69,82). Evidence for *in vivo* inhibition has been obtained by measuring the levels of appropriate metabolites in the presence and absence of galactosamine-1-P (69).

An interesting inhibition of UDP-glucose pyrophosphorylase by 5'-AMP has been reported by Kornfeld (81). 3',5'-cyclic AMP or ATP had no significant effect. AMP decreased the apparent K_m of UTP by 2 to 3 fold while lowering V_{max}. As the concentration of Glc-1-P increased in the presence of 5'-AMP a progressive and marked substrate inhibition occurred. It was found that 1.6×10^{-3} M AMP inhibited 70% when the concentration of Glc-1-P was 1.2×10^{-3} and only 13% when Glc-1-P concentration was 1.2×10^{-4} M. In the nomenclature of Cleland (83) when

TABLE IV

COMPETITIVE INHIBITORS OF UDP-GLUCOSE PYROPHOSPHORYLASE FROM VARIOUS SOURCES

Source	Inhibitor	K_i	Varied Substrate	Fixed Substrate	$MgCl_2$	Reference
		mM		mM	mM	
E. coli B	TDP-glucose 1-2 mM	2	UDP-glucose 0.05-1 mM	4	10	14
Yeast	TDP-glucose 2 mM	10	UDP-glucose 0.05-1 mM	4	10	14
E. coli B	TDP-rhamnose	0.6	UDP-glucose 0.05-1 mM	4	10	14
Rat Liver	Gal-1-P 41 mM	8.7-9.2	Glc-1-P	0.6	2.3	53
Guinea Pig Brain	Gal-1-P 35 mM	15-16	Glc-1-P	0.6	2.3	53
Bovine Mammary Gland	Gal-1-P	8	Glc-1-P	1	1	54
Rat Liver	GalN-1-P 14 mM	5	Glc-1-P 0.15-1.5 mM	0.4	2	69
Rat Liver	GalN-1-P 14 mM	3.2	Glc-1-P 0.15-1.5 mM	1.4	2	69
Bovine Liver	UDP 2 mM	0.15	UDP-glucose 0.008-0.064 mM	2	2	31
Human Liver	UDP	0.1	UDP-glucose	-	-	30
Bovine Liver	P_i 3.2 mM	3.7	PP_i 0.005-0.03 mM	0.4	2	31

both V_{max} and K_m vary, the inhibition may be described as noncompetitive.

Inorganic phosphate has been found by Albrecht et al. (31) to be competitive with pyrophosphate at 3.2×10^{-3} M. Oliver (53) found no significant effect of inorganic phosphate at 8×10^{-4} M, but at a concentration of 2×10^{-2} M showed noncompetitive inhibition in rat liver. Unfortunately the variable substrate was not reported. Since all substrates contain phosphate, it would not be surprising if phosphate formed a number of different dead-end complexes which would give rise to noncompetitive inhibition. No effect by 10 mM phosphate was found in pea seeds (70).

Only the human brain enzyme has been reported to be sensitive to p-chloromercuribenzoate (55). A 10^{-4} M solution inhibited 50%. It would be expected that human liver (30), human erythrocyte (7) and mung beans (61) which require a sulfhydryl reagent for maximum stability would also be sensitive to this compound.

No inhibition was found by 10 mM iodoacetate, 1 mM p-chloromercuribenzoate, or 0.1 mM Hg^{2+} ions in pea seeds (70); 2 mM p-chloromercuribenzoate in E. coli K12 (60); cysteine in yeast (12), human brain (55), or E. coli K12 (60); and glutathione in human brain (55).

Galactose (0.2 M) in rat liver (53) or 10 mM rhamnose in rat liver (53) and E. coli B (14) did not inhibit. Sodium fluoride (12,60,70), arsenate (70), penicillin (12), UMP (12,60) and UDP (12) have all been reported to have no significant effect on UDP-glucose pyrophosphorylase. However, UDP is a good competitive inhibitor of both human and bovine liver enzymes (30,31).

Several anions have been shown to inhibit the UDP-glucose

pyrophosphorylase from bovine mammary gland including 5 mM sulfate, 1 mM arsenate and 1 mM citrate (54). Citrate may be acting as a complexing agent. EDTA shows no effect at low concentrations (12), but at higher concentrations where Mg^{2+} complexing becomes significant, inhibition occurs. The enzyme from bovine mammary gland is inhibited 90% by 10 mM EDTA in the presence of 10 mM Mg^{2+} (54).

Product inhibition has been reported for mung beans (7), bovine mammary gland (54), rat liver (81), dog heart (7), and human erythrocyte (7). Inorganic pyrophosphate at 0, 1 and 3 mM gave rates of 124, 97, and 64 I.U. per ml of bovine mammary gland enzyme, respectively (54). In rat liver, UDP-glucose was a competitive inhibitor of UTP with a K_i of 4×10^{-5} M (81). The product inhibition pattern of human erythrocyte enzyme is presented in Table V with the associated kinetic constants in Table VI (7). All the secondary plots of slope and intercept were linear for all the inhibitions. In dog heart and mung beans UDP-glucose and UTP were competitive with UTP and UDP-glucose, respectively. Again the secondary plots were linear in both cases. The kinetic constants are shown in Table VI (7).

Initial Velocity Studies

Initial velocity studies have been performed on the human erythrocyte enzyme (7). An intersecting pattern was obtained with plots of slope and intercept linear. The intersection point was below the abscica indicating that $K_{ia} < K_a$. The kinetic constants are shown in Table VII (7). No statistical analysis was reported. The kinetic constants were checked by substitution into the following Haldane expression for a steady-state ordered mechanism.

TABLE V

PRODUCT INHIBITION PATTERN FOR UDP-GLUCOSE PYROPHOSPHORYLASE FROM HUMAN ERYTHROCYTES

Product Inhibitor	Variable Substrate			
	Glc-1-P	UTP	UDP-glucose	PP_i
UDP-glucose	NC	C	-	-
Inorganic Pyrophosphate	NC	NC	-	-
UTP	-	-	C	NC

TABLE VI

INHIBITION AND MICHAELIS CONSTANTS OF MAMMALIAN AND PLANT UDP-GLUCOSE PYROPHOSPHORYLASE FOR UDP-GLUCOSE AND UTP

Enzyme	UDP-glucose		UTP	
	K_i x10^5	K	K_i x10^4	K
Human Erythrocyte	1.5	2.3	1.0	3.3
Dog Heart	2.3	2.8	0.7	3.6
Mung Beans	16.0	11.0	0.8	1.6
Rat Liver	4.0	9.0		

$$K_{eq} = \frac{V_f K_{PP_i} K_{iUDP\text{-}glucose}}{V_r K_{iUTP} K_{Glc\text{-}1\text{-}P}} = 0.24$$

The experimental value (Table III) is 0.26.

TABLE VII

KINETIC CONSTANTS FOR HUMAN ERYTHROCYTE UDP-GLUCOSE PYROPHOSPHORYLASE FROM INITIAL VELOCITY STUDIES

Kinetic Constant	Value
K_{UTP}	0.33 mM
K_{iUTP}	0.11
$K_{Glc\text{-}1\text{-}P}$	0.17
K_{PP_i}	0.48
$K_{UDP\text{-}glucose}$	0.04
$K_{iUDP\text{-}glucose}$	0.028
V_f/V_r	0.33

Isotopic Exchanges

The first exchange reaction was reported by Munch-Petersen et al. (11) in yeast. It was shown that ^{32}P labelled inorganic pyrophosphate was incorporated into the two terminal phosphates of UTP. Trucco (13, 84) found that ^{14}C-Glc-1-P exchanged with UDP-glucose in a crude extract of yeast and ascribed it to the combined effect of Gal-1-P uridyltransferase and UDP-galactose epimerase. Munch-Petersen (12) showed the

following exchange reactions in yeast required enzyme only.

$$\text{Glc-1-}^{32}\text{P} \longleftrightarrow \text{UDP-glucose} \quad \text{(I)}$$

$$^{32}\text{P}^{32}\text{P} \longleftrightarrow \text{UTP} \quad \text{(II)}$$

The following reactions did not occur with only enzyme present.

$$\text{UMP-}^{14}\text{C} \longleftrightarrow \text{UDP-glucose} \quad \text{(III)}$$

$$\text{UMP-}^{14}\text{C} \longleftrightarrow \text{UTP} \quad \text{(IV)}$$

$$\text{Glc-1-}^{32}\text{P} \longleftrightarrow \text{UDP-glucose} \quad \text{(V)}$$

These exchange reactions ruled out the possibility of a uridyl-enzyme intermediate.

In contrast to the data of Munch-Petersen (12), Neufeld et al. (85) found exchange II to require enzyme and Glc-1-P. The results of Neufeld et al. (85) are consistent with the proposed mechanism for UDP-glucose pyrophosphorylase. Studies using human brain UDP-glucose pyrophosphorylase also have shown exchange II to require enzyme, Glc-1-P, and magnesium ion. Equimolar Gal-1-P did not inhibit the exchange in brain. It may be possible that Glc-1-P was a contaminant in the experiment of Munch-Petersen (12). In agreement with Munch-Petersen (12), Neufeld et al. (85) showed that exchange V required enzyme and inorganic pyrophosphate.

Binding Studies

Incubation of UTP ($2\text{-}^{14}\text{C}$; $\beta,\gamma\text{-}^{32}\text{P}$) or UDP-glucose with crystalline UDP-glucose pyrophosphorylase form calf liver results in complexes which are stable to chromatography on Sephadex G-25 (86). One mole of

substrate was bound per mole of enzyme. Mg^{2+} or Mn^{2+} ions were not required for binding. Complexes could not be demonstrated when the enzyme was incubated with $^{32}PP_i$, ^{14}C-Glc-1-P, UMP-2-^{14}C, or UDP-2-^{14}C. However, enzyme-UDP-glucose-^{14}C and enzyme-UTP-β,γ^{32}P complexes were formed when incubated with $^{32}P^{32}P_i$ and Glc-1-P-^{14}C, respectively.

Knop and Hansen (30) report that the human liver enzyme shows sigmoidal kinetics when PP_i is varied with a Hill coefficient of 2.5. If the enzyme is incubated for 5 minutes with 2 mM PP_i before addition to the reaction mixture the Hill coefficient is reduced to 1.5.

Mechanism

The initial velocity studies show that the reaction is sequential (7). The product inhibition pattern shown in Table V is consistent with a steady-state ordered mechanism. The competitive inhibitors listed in Table IV may be considered as substrate analogs and are therefore consistent with the steady-state ordered mechanism shown in Figure 1, since they are competitive with the first substrate to add. The binding data of Gillett *et al.* (86) further supports this mechanism. The strong binding of UTP by the enzyme in the absence of Mg^{2+} may indicate strong inhibition by UTP. The possibility of a ping pong mechanism was ruled out by the initial velocity studies (7) and the isotope exchange studies (12).

Uses

UDP-glucose pyrophosphorylase can be useful as an analytical reagent (87). Coupled with phosphoglucomutase and glucose-6-P dehydrogenase, UDP-glucose pyrophosphorylase can be used to quantitatively

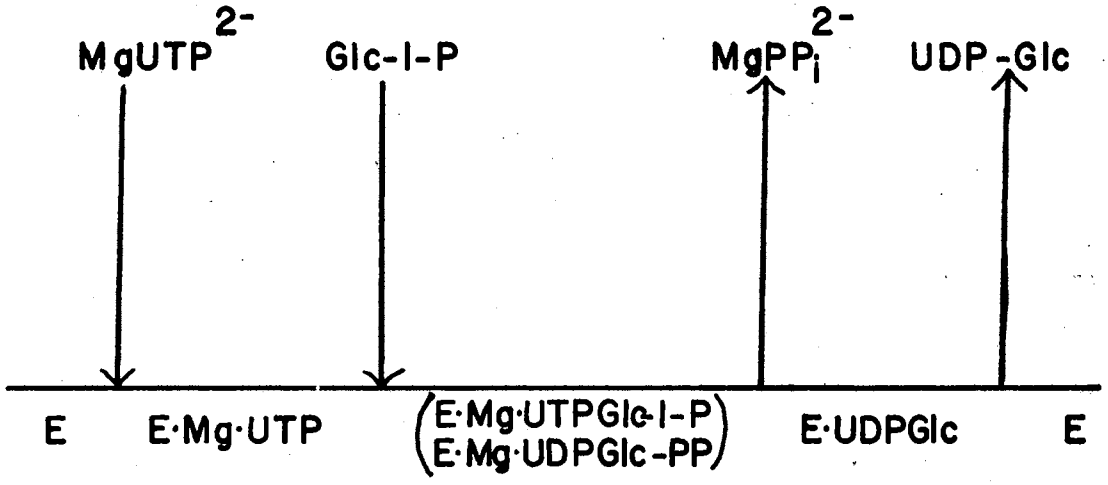


Figure 1. Mechanism of UDP-Glucose Pyrophosphorylase

estimate inorganic pyrophosphate in biological systems. The same principle has enabled Johnson et al. (87) to assay for RNA polymerase spectrophotometrically.

Proposed Control Mechanisms

A highly selective product inhibition by UDP-glucose has been found in human erythrocytes, dog heart, mung beans, and rat liver enzymes which suggests that UDP-glucose pyrophosphorylase may be participating in both the regulation and synthesis of UDP-glucose (7,81). As seen in Table VI, the inhibition constant for UDP-glucose is an order of magnitude lower for the mammalian enzymes than for the mung bean enzyme. This may reflect the differences in their particular metabolic needs (7). No significant differences in the kinetic constants between the mammalian and mung bean enzymes occurred when UTP was used as the product inhibitor (7).

Kornfeld (81) has suggested that the inhibition by AMP could prevent the recycling of Glc-1-P back to glycogen via UDP-glucose. This inhibition would be consistent with the effects of AMP on other enzymes of the glycolytic scheme (81).

In D. discoideum Newell and Sussman (33) have found as many as three different molecular weight forms of UDP-glucose pyrophosphorylase which presumably are not interconvertible. A mechanism of regulation is proposed in which four different forms of UDP-glucose pyrophosphorylase exist, each responsible for the production of its corresponding saccharide. The four saccharides which are known to depend upon UDP-glucose pyrophosphorylase for their synthesis are cellulose (88), glycogen (89), trehalose (90,91), and mucopolysaccharide (92).

Albrecht et al. (31) have suggested that the competitive inhibitors, inorganic phosphate and UDP, may have a physiological function. When glucose and ATP are in excess and inorganic phosphate is low, the pyrophosphorylase could function in the provision of UDP-glucose for glycogen storage; but when inorganic phosphate is high and the cell is in need of energy, the formation of UDP-glucose is limited by inorganic phosphate. Similarly, for glycogen formation to proceed, the UDP must be removed at a sufficient rate to prevent its accumulation and inhibition of the formation of the glucosyl donor, UDP-glucose.

It has been suggested that the UDP-galactose pyrophosphorylase activity may play a role in the galactosemic patient (30). Isselbacher (93) has indicated that a pyrophosphorylase is responsible for the increased ability of some galactosemics to metabolize galactose by synthesizing UDP-galactose from Gal-1-P and UTP. On the other hand, Gitzelmann (94) has proposed that a pyrophosphorylase may be responsible for the elevated galactose-1-P levels found in the blood of some galactosemics on supposedly galactose-free diets.

CHAPTER III

PURIFICATION OF UDP-GLUCOSE PYROPHOSPHORYLASE

Experimental Procedure

Materials and Reagents

Tris, UTP, Glc-1-P, NAD^+, bovine serum albumin, lactic dehydrogenase (Type III from beef heart), thyroglobulin, and protamine sulfate were purchased from Sigma Chemical Co., St. Louis, Missouri. Apoferrin was obtained from Mann Research Laboratories; magnesium chloride from Fisher Chemical Co.; Whatman DE-32, DE-23, and CM-32 from H. Reeve Angel & Co. Ltd; Bio-Gel P from Bio-Rad Laboratories; Sephadex G from Pharmacia; and catalase (sterilized solution in 10 ml vials from beef liver, 30,000 units per ml) from Worthington. All other chemicals were of reagent grade.

UDP-glucose dehydrogenase was purified from bovine liver through Step V by the procedure of Strominger et al. (95). Hydroxylapatite was prepared following the procedure of Siegelman et al. (96). Urease was a gift from Dr. D. P. Blattler, Department of Chemistry, Oklahoma State University. Lactating bovine udders were obtained immediately after slaughter from the Wilson Packing Plant, Oklahoma City, Oklahoma.

Spectrophotometric Assay for UDP-Glucose Pyrophosphorylase

UDP-glucose pyrophosphorylase was assayed in the direction of

UDP-glucose synthesis by determining UDP-glucose with UDP-glucose dehydrogenase as previously described (54). Assays were done in a 1.0 ml final volume and contained 0.2 M Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 5 mM Glc-1-P, 1 mM NAD^+, 4 to 6 units UDP-glucose dehydrogenase, enzyme, and 1 mM UTP. The reaction was initiated by the addition of 0.1 ml of 10 mM UTP. Assays with a change in absorbance at 340 nm of 0.04 per minute or less were proportional to enzyme concentration. One unit is defined as that amount of enzyme which forms one μmole of UDP-glucose per minute and equals 12.4 A_{340}/min. Assays were performed at 23° on a Cary-14 or occasionally on a Beckman DB recording spectrophotometer.

During purification, ammonium sulfate was added to the assay mixture. Table VIII shows that under the normal conditions used for assaying UDP-glucose pyrophosphorylase ammonium sulfate strongly inhibits the assay. To assure no ammonium sulfate was present initially, the enzyme from Step VII was desalted by passing it through a Bio-Gel P-10 column equilibrated with 20 mM triethanolamine, pH 8.5. The data are consistent with the 50% inhibition reported by Ebner and Steelman (54) in the presence of 5 mM potassium sulfate. The effect of sulfate on UDP-glucose dehydrogenase was tested by omitting UTP, Glc-1-P, and UDP-glucose pyrophosphorylase from the assay mixture and adding 0.4 mM UDP-glucose as a substrate. With 5.5% ammonium sulfate in the assay cuvette, the reaction was only 45% complete in three minutes, whereas the reaction in the absence of ammonium sulfate had reached completion in this time. It was concluded that most of the inhibition observed was due to inhibition of UDP-glucose dehydrogenase rather than inhibition of UDP-glucose pyrophosphorylase.

TABLE VIII

AMMONIUM SULFATE INHIBITION OF THE SPECTROPHOTOMETRIC ASSAY

Per Cent Ammonium Sulfate	I.U./ml	Per Cent Activity
0	0.0128	100
5.0	0.0024	19
5.5	0.0016	12

Ion Exchange and Hydroxylapatite Chromatography

Cellulose ion exchange resins were precycled and regenerated by procedures recommended in the Whatman technical bulletin (97). For the final equilibration in the starting buffer, the cellulose resin was suspended in the starting buffer and the pH readjusted with an electrode immersed in the slurry.

Cellulose or hydroxylapatite columns were packed by adding a thick slurry to a column filled half way with starting buffer. If more than one addition was required the buffer on top was drawn off with a pipet and additional slurry was added followed by gentle stirring. The pressure head was maintained to give the desired flow rate. After packing, three volumes of starting buffer were passed through the column and the pH and conductivity were checked to assure equilibration.

A Bio-Gel P-10 or Sephadex G-25 (40 or more cm high with an appropriate diameter such that the sample volume was 10% or less of the gel bed volume) was packed and equilibrated in a similar manner. The sample

was applied to the desalting column. After the sample had passed approximately three-fourths of the way down the column, the effluent was run directly onto the cellulose or hydroxylapatite column. The desalting column was stopped as soon as the conductivity meter, which was connected between the effluent of the desalting column and the cellulose or hydroxylapatite column by P-200 tubing, indicated the beginning of the salt peak. The cellulose or hydroxylapatite columns were then washed with one or two volumes of starting buffer and eluted by a linear gradient or batch technique. Hydroxylapatite columns were eluted with the aid of a LKB peristaltic pump.

Gel Filtration Chromatography

The appropriate gels were swollen and equilibrated by the procedures described in the technical bulletins (98,99). With Sephadex G-200, Bio-Gel P-300, and Bio-Gel A-5m gels special care was taken to use only a minimal pressure head during all stages of column preparation and operation. A 10 cm head was never exceeded. Good flow rates and separations were obtained. Columns less than 3 cm in diameter were silanized by passing a solution of 1% dichlorodimethyl silane in benzene throughout their length. The columns were then washed with ethanol to react with any excess silanizing reagent.

Sedimentation Velocity Centrifugation

Sedimentation velocity experiments were performed on a Beckman Model E analytical ultracentrifuge. Sedimentation coefficients were calculated using a microcomparitor to measure the distance the peak moved down the cell.

Preparative Sucrose Density Gradient Centrifugation

Sucrose density gradient centrifugation (5 to 20%) was performed by the method of Martin and Ames (100). Buffer was added to the sucrose solutions at the appropriate concentrations. Centrifugation was performed in a Beckman L2-65 preparative ultracentrifuge at the desired speed and temperature. Fractions were collected immediately after centrifugation by piercing the tube through the bottom with a 20 gauge hypodermic needle and forcing the gradient out slowly through the needle with controlled air pressure. Fractions of 0.66 to 0.68 ml were usually collected.

Electrophoresis

Disc gel electrophoresis was performed as described in the Canalco manual (101) with the standard 7% separating gel. No sample gel was used.

Polyacrylamide horizontal electrophoresis was used with little success. A gel layer of desired dimensions and polyacrylamide concentration was poured between two glass plates with a spacer between them. After polymerization, one of the plates was removed and wells were cut in the slab. The polyacrylamide layer on the glass plate was placed in a Buchler Universal Electrophoresis Cell and paper wicks placed on the ends of the gel. The sample was placed in the well and subjected to electrophoresis for an appropriate time. The system could separate the polymers of urease into separate bands. However, solutions containing UDP-glucose pyrophosphorylase always streaked with only hints of bands. Gels of 3% and 5% acrylamide in Tris-borate, pH 9.0, and phosphate, pH 7.0, buffers were used. Dialyzing the sample extensively against buffer

did not help.

Concentration Methods

The best procedure for the concentration of protein from chromatographic pools was ammonium sulfate precipitation. Ultrafiltration with a Dia-Flow X-50 or UM-10 membrane was too slow. Putting the material into a dialysis bag and packing either Biodryex or Sephadex G-25 around the bag usually gave significant concentration but could not be used routinely because of the large volumes. This method also allowed an A_{280} material to enter the solution in the dialysis bag. Addition of a dry Bio-Gel P-10 gel material to a solution, allowing it to swell, and then centrifuging in specially adapted tubes (99,102). gave 80% recovery and a large increase in A_{280} material. Only a two fold concentration was obtained by a single treatment with gel.

Other Methods

Protein was routinely estimated by the absorbance at 280 nm assuming that an A_{280} of 1.0 is equal to one milligram of protein in a 1 cm light path. Assays for lactic dehydrogenase and catalase were performed as described in the Worthington technical manual (103). A small crystal of 1,1,1-trichloro-2-methyl-2-propanol was routinely added to buffers and enzyme solutions that were to be stored for more than two days to prevent bacterial contamination. No effect on enzyme activity was detected.

Results

Acetone powder from bovine mammary glands was used as starting material for the purification. The acetone powder was prepared by the procedure described by Tsai (104). Unlike the epimerase enzyme, the powder could be stored for up to two years at -20°C without significant loss of UDP-glucose pyrophosphorylase activity. The yield was 20 grams of dry powder for each 100 grams wet tissue processed.

A detailed procedure for the purification of UDP-glucose pyrophosphorylase will be described. The procedure is a modification of the one described by Ebner and Steelman (54). Unless otherwise stated all steps were performed at 0° to 4°C. Centrifugation during the purification was performed at 10,000 x g for 30 minutes.

Step I: Extraction With 0.1 M Tris-HCl, pH 8.0

For extraction of the enzyme, 750 ml of 0.1 M Tris-HCl, pH 8.0, was stirred into 50 grams of acetone powder. The mixture was stirred for 1 to 1 1/2 hours and centrifuged. The supernatant solution was passed through glass wool and the precipitate was discarded.

Step II: Treatment With Protamine Sulfate

To every 12 ml of supernatant solution from Step I, 1 ml of 0.2% protamine sulfate was slowly added with a pipet. The protamine sulfate solution was prepared by dissolving 2 grams protamine sulfate in 3.2 ml of 0.1 M potassium hydroxide and diluting to 100 ml. The pH of the protamine solution was not adjusted to 6.8 as described by Ebner and Steelman (54) since a greater loss of enzymatic activity occurs if the adjustment is made at this step. The solution was stirred an additional

15 minutes after the last addition of protamine sulfate and then centrifuged. The precipitate was discarded.

Step III: Precipitation With 50% Ammonium Sulfate

To the supernatant solution from Step II, 313 grams of ammonium sulfate was slowly added per liter of solution. After stirring for 15 minutes, the solution was centrifuged. The precipitate was dissolved in 250 ml of 0.2 M potassium phosphate, pH 8.0.

Step IV: Heat Treatment

The dissolved precipitate of Step III was heated to 50 to 51°C in a glass beaker submerged in a water bath. Exceeding 52° for even a short time will destroy activity. During heating, the solution was stirred constantly by an overhead stirrer. After 30 minutes, the beaker was removed and plunged into ice-water. An ammonium sulfate fraction (0 to 40%) of bovine liver UDP-glucose pyrophosphorylase subjected to this heat treatment also showed little loss in activity.

Step V: Precipitation of Denatured Protein by Ammonium Sulfate

When the heat-treated solution had cooled to 0 to 5°, 100 grams of ammonium sulfate per liter of solution was slowly added. After stirring for 15 minutes, the solution was centrifuged and the precipitate discarded.

Step VI: Precipitation With Ammonium Sulfate

To the supernatant solution from Step V, 114 grams of ammonium sulfate was added per liter of solution. After stirring for 15 minutes,

the solution was centrifuged and the precipitate dissolved in a minimum volume of 0.2 M potassium phosphate, pH 8.0.

Step VII: DEAE-Cellulose Chromatography

A column (typically 6.4 x 12 cm) was prepared which contained 2 ml bed volume of DEAE-cellulose for each 10 mg of protein. The column was equilibrated in 20 mM potassium phosphate buffer, pH 8.0, and the sample was applied as described in the Experimental Procedure. The column was washed successively with two volumes of 20 mM potassium phosphate, pH 8.0, two volumes of 50 mM potassium phosphate, pH 8.0, and finally with 500 mM potassium phosphate, pH 8.0. Fractions of 20 ml were collected. The material was eluted from the column by 500 mM potassium phosphate and contained 80 to 100% of the activity. This material was pooled and precipitated by the addition of 313 grams ammonium sulfate per liter of solution. After stirring for 15 minutes, the solution was centrifuged and the precipitate dissolved in a minimum volume of 0.2 M potassium phosphate, pH 8.0.

Elution of the column with a linear gradient from 20 mM to 500 mM potassium phosphate, pH 8.0, was more effective in separating the protein components. However, in order to obtain the best resolution the total volume of the gradient was usually 4 to 6 liters. The time required to add ammonium sulfate to such large volumes of solution to precipitate the protein was considered too long for the increased resolution. A combined pH and ionic strength gradient from 10 mM potassium pyrophosphate, pH 8.0, to 100 mM potassium pyrophosphate, pH 9.9 (1 liter each), can also be used.

A summary of the purification obtained to this point is presented

in Table IX. A 50 fold purification and a 65% yield of activity was obtained. This procedure was adopted as routine and further purification was attempted from this point.

TABLE IX

SUMMARY OF THE PURIFICATION OF UDP-GLUCOSE PYROPHOSPHORYLASE FROM 50 GRAMS OF BOVINE MAMMARY ACETONE POWDER

	Step	Volume (ml)	Activity (I.U.)	Protein (mg)	Specific Activity	Purification Fold
I.	Extraction	640	681	22,100	0.03	1
II.	Protamine Sulfate	680	429	13,000	0.03	1
III.	Ammonium Sulfate	250	668	4,280	0.16	5.3
IV.	Heat	250	688	5,180	0.13	4.3
V.	Ammonium Sulfate	242	472	2,400	0.20	6.7
VI.	Ammonium Sulfate	17.8	433	1,175	0.37	12.3
VII.	DEAE-cellulose	14.5	440	280	1.57	52.3
VIII.	DEAE-cellulose pH 9.7 Sephadex G-200	-	-	-	16.0	533

Hydroxylapatite Chromatography

The results from six different hydroxylapatite columns are presented in Table X. All of the columns were approximately the same size (2 x 12 cm). A batch elution was carried out using increasing concentrations of potassium phosphate buffer. Three milliliter fractions were collected. Columns 4 to 6 from Table X are presented in Figures 2 and 3, respectively, as examples of the elution profiles.

At pH 8.0 to 8.5 UDP-glucose pyrophosphorylase activity came off in two peaks using the same concentration buffer. Eluting a column batchwise usually results in several peaks of activity, but only one activity peak at a given concentration of the buffer. The two peaks observed in Figure 2 may be due to different conformational forms, different polymeric species, isozymes, or two different nonspecific pyrophosphorylases. Technical problems such as a nonhomogeneous gel or severe channelling cannot be ruled out. The purification history of the sample did not significantly alter the elution pattern. Care must be taken in assessing the specific activity of the peaks eluted with high concentrations of phosphate. Phosphate is an inhibitor of UDP-glucose pyrophosphorylase (31) and may have inhibited the assay. Therefore, the true specific activity in the peaks at higher phosphate concentrations may be higher. Disc gel electrophoresis of the active pools from the various hydroxylapatite columns always showed one slow moving component in the separating gel. One or more additional bands were also present.

Although a two to five fold purification may be achieved by hydroxylapatite column chromatography, the recovery of such purified material is quite small, and a large proportion of the enzyme is

TABLE X

SUMMARY OF HYDROXYLAPATITE CHROMATOGRAPHY OF UDP-GLUCOSE PYROPHOSPHORYLASE

Column Number	pH	Sample	Elution Buffer	Number of Peaks	Specific Activity of Peak Tube
1	8.0	Step VII without heat step, 45.6 mg, 59.3 units, S.A.[1] 1.3	20 mM	1	1.7
			50	2	2.26, 1.83
			100	1	1.8
			200	1	0.64
2	8.0	Step VII without heat step, 45.6 mg, 59.3 units, S.A. 1.3	1 mM	none	-
			2	1	1.4
			5	1	1.9
			100	1	< 8.6
			200	1	1.9
			1000	1	-
3	8.5	Step VII then Sephadex G-200	1 mM	1	none
			2	1	13.4
			50	2	15.8,20.0
			60	2	16.2,15.8
			100	1	14.0
			200	2	11.0, 8.0
			500	1	5.0
			1000	1	none
4	8.5	Step VII, 38.6 mg, 1000 units, S.A. 25.9	2 mM	3	47.0,35.0 1.0
			20	3	24.0,28.5 19.0
			40	1	1.0
			80	none	-
			160	1	6.1
			1000	1	5.0
5	9.0	Step VII, 75 mg, 714 units, S.A. 9.0	1 mM	2	34.1,31.4
			5	3	11.0,21.0,21.8
			10	2	23.0,26.7
			20	2	27.7,19.0
			40	1	$\leq$ 15.8
			80	2	9.5,10.9
			160	1	2.8
			320	1	4.8
			640	1	4.1
			1000	1	none

TABLE X (Continued)

Column Number	pH	Sample	Elution Buffer	Number of Peaks	Specific Activity of Peak Tube
6	9.6	Step VII, 125 mg, 1.19 units, S.A. 9.0	H_2O	1	15.0
			1 mM	1	13.4
			5	1	14.8
			10	1	18.2
			20	1	47.5
			40	1	9.5
			80	1	15.7
			160	1	9.7
			320	1	4.6
			640	5	6.7,0,0,0,0
			1000	1	none

[1]S.A. = specific activity.

actually less pure. In addition, the elution profile of a column varies with the individual preparation of gel. Therefore, this procedure was abandoned as a general and reliable method for routine purification.

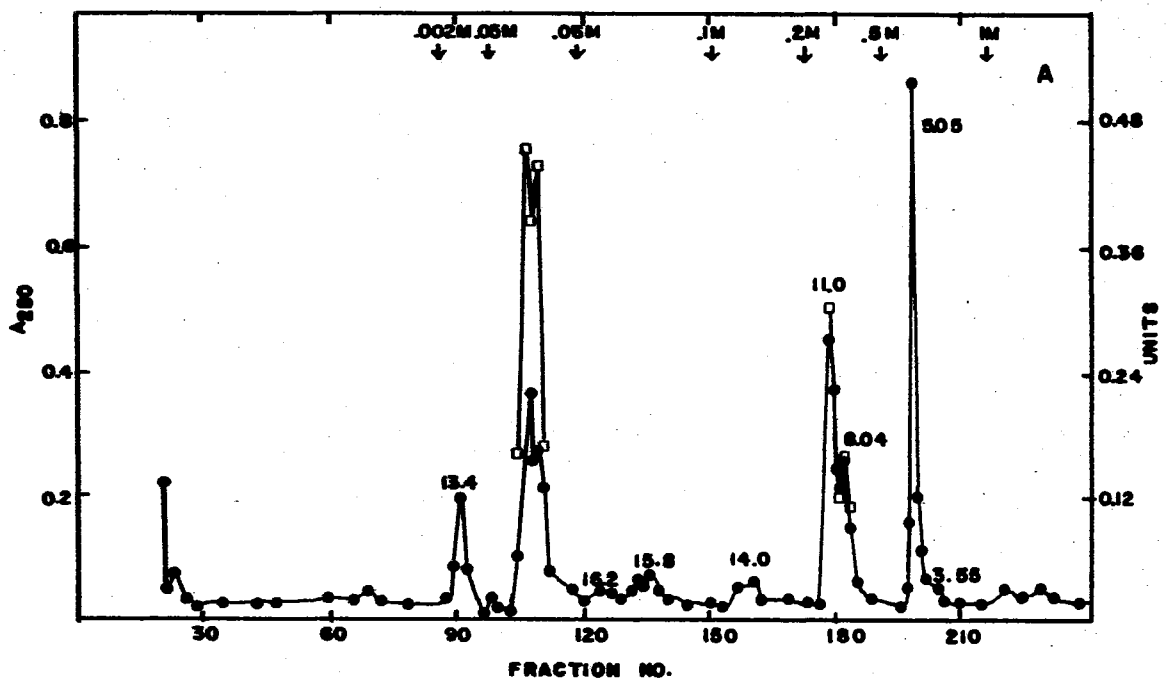


Figure 2. Elution Profile of Hydroxylapatite Column Chromatography at pH 8.5

The column was 2 x 12 cm and 3 ml fractions were collected. A batch elution was used with the concentrations of potassium phosphate buffer at pH 8.5 shown by the arrows at the top of the figure. The number beside each peak is the specific activity in units per ml. Specific activities of fractions 107 and 109 were 15.8 and 20.0, respectively. The sample of UDP-glucose pyrophosphorylase was purified through Step VII and chromatographed on Sephadex G-200. ●, A_{280}; □, activity in units per ml.

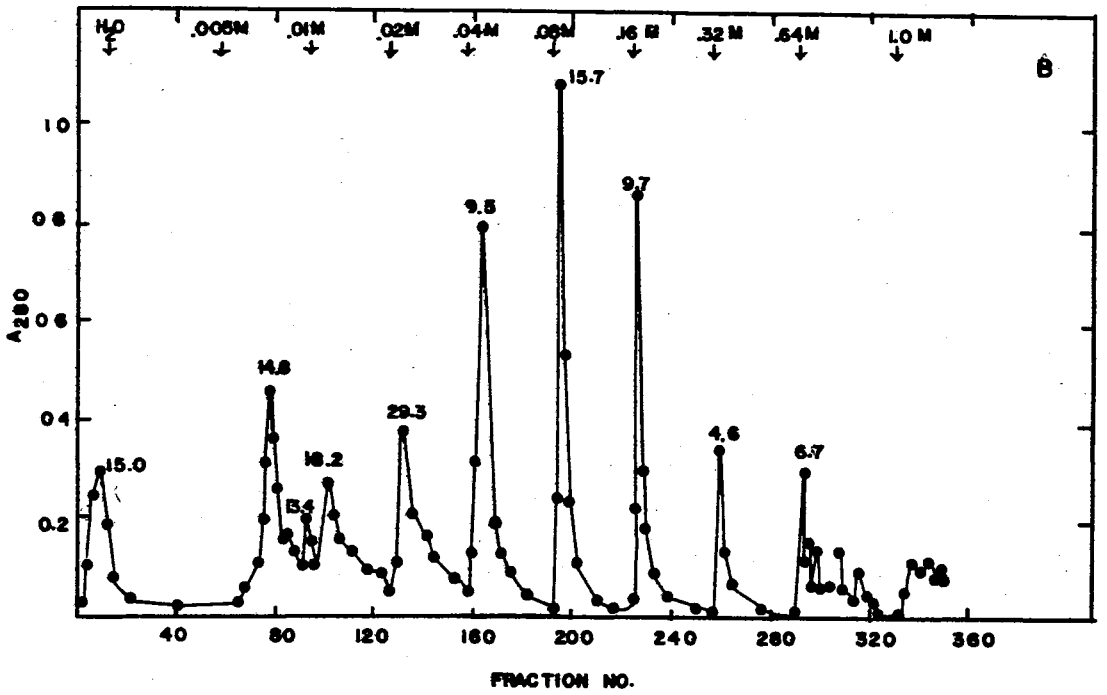


Figure 3. Elution Profile of Hydroxylapatite Column Chromatography at pH 9.6

The column was 2 x 12 cm and 3 ml fractions were collected. A batch elution was used with the concentrations of potassium phosphate buffer at pH 9.6 shown by the arrows at the top of the figure. The number beside each peak is the specific activity in units per ml. The sample of UDP-glucose pyrophosphorylase was purified through Step VII.

DEAE-Cellulose Chromatography at pH 8.8 to 10.0

Several DEAE-cellulose columns eluted under different conditions are presented in Table XI. The pH of the columns range from 8.8 to 10.0. No distinct advantage was found in using one pH over another. However, in the pH range 9.7 to 10.0, sharper peaks with greater recovery usually occurred. The sharpness and therefore resolution of the peaks on the column were dependent upon the gel treatment. It was found that the generation of fines by vigorous stirring, treatment with base over 0.5 N for any length of time, or keeping the gel in the pH range of 8.8 to 10.0 for periods of several weeks resulted in severe loss in resolution.

Two elution profiles are shown in Figures 4 and 5. Three distinct activity peaks occur which are similar to the shoulders observed in Figure 2 upon hydroxylapatite chromatography. A second column at pH 10.0 gave an identical profile to that in Figure 5. However, subsequent elution profiles did not show a third activity peak distinctly separated from the other activity peaks. A sample from each tube in the elution profile shown in Figure 5 was analyzed by disc gel electrophoresis. A schematic representation of the results is shown in Figure 6. The main band found in the first peak is carried throughout the column and is in the same position as the major band found on hydroxylapatite chromatography. The second peak shows an additional band. The material in the second peak, fractions 99 to 104, was precipitated by ammonium sulfate and subjected again to disc gel electrophoresis. The disc gel pattern remained the same. In addition, another gel was sliced, extracted with buffer at room temperature ($22^{o}C$) and assayed. The results are shown in Figure 7. No band could be detected in the area of

TABLE XI

SUMMARY OF DEAE-CELLULOSE CHROMATOGRAPHY OF UDP-GLUCOSE PYROPHOSPHORYLASE

pH	Column and Fraction Size	Sample	Equilibration Buffer	Elution Buffer	Peaks	Comments
9.0	Column: 2.15 x 15 cm Fraction: 6 ml	Step VII 9.25 I.U. 12.1 mg	20 mM Tris-HCl	200 ml of 20 mM Tris-HCl Linear Gradient: 1500 ml of 20 mM Tris-HCl 1500 ml of 1 M Tris-HCl 500 ml of 800 mM KH_2PO_4, pH 8.0	One active peak over all tubes	
10.0	Column: 2.15 x 15 cm Fraction: 6 ml	Step VII 9.25 I.U. 12.1 mg	20 mM Glycine	700 ml of 20 mM Tris-HCl Linear Gradient: 500 ml of 20 mM Glycine 500 ml of 1.5 M Glycine 500 ml of 800 mM KH_2PO_4, pH 8.0	Three major peaks; 2nd active	Recovered 262 I.U.; 28.3 fold increase
10.0	Column: 3.09 x 16 cm Fraction: 10 ml	Step VII 142 I.U.	150 mM Glycine	450 ml of 150 mM Glycine Linear Gradient: 800 ml of 150 mM Glycine 800 ml of 700 mM Glycine	Nearly all material Small peak; not active	
10.0	Column: 3.09 x 17 cm Fraction: 10 ml	Step VII 130 I.U.	20 mM Glycine	650 ml of 20 mM Glycine Linear Gradient: 1000 ml of 20 mM Glycine 1000 ml of 1 M Glycine	Three major peaks; 2nd active; one minor peak; active	Recovered 2720 I.U.; 20.9 fold increase
10.0	Column: 3.09 x 30 cm Fraction: 3.2 ml	Step VII 45 I.U. 60 mg	20 mM Glycine	130 ml of 20 mM Glycine Linear Gradient: 1000 ml of 20 mM Glycine 1000 ml of 1.5 M Glycine	None Two major peaks; 1st active	

TABLE XI (Continued)

pH	Column and Fraction Size	Sample	Equilibration Buffer	Elution Buffer	Peaks	Comments
9.7	Column: 3.09 x 27.8 cm Fraction: 3.2 ml	Step VII 45 I.U. 60 mg	20 mM Glycine	600 ml of 20 mM Glycine Linear Gradient: 1000 ml of 20 mM Glycine 1000 ml of 1.5 M Glycine 500 ml of 0.5 M KH_2PO_4, pH 8.0	Eight major peaks; 3rd and 4th active	Two major and two minor bands on disc gel electrophoresis
9.4	Column: 3.09 x 30 cm Fraction: 3.2 ml	Step VII 45 I.U. 60 mg	20 mM Tris-HCl	150 ml of 20 mM Tris-HCl Linear Gradient: 1 l of 20 mM Tris-HCl 1 l of 1.5 M Tris-HCl Linear Gradient: 1 l of 50 mM Borate 1 l of 50 mM Borate 250 ml of 0.5 M KH_2PO_4, pH 8.0	One major peak active one minor peak not active	Three major and one minor bands on disc gel electrophoresis
9.1	Column: 2.16 x 32.5 cm Fraction: 3 ml	Step VII 45 I.U. 60 mg	20 mM BO_3	150 ml of 20 mM Borate Linear Gradient: 1 l of 20 mM Borate 1 l of 316 mM Borate 500 ml of 0.5 M KH_2PO_4, pH 8.0	Small peak active A_{280} smear Two peaks not active	Very little A_{280} and large smear of activity; one major, three minor bands on disc gel electrophoresis
8.8	Column: 3.09 x 28.9 cm Fraction: 6 ml	Step VII 36 I.U. 48 mg	5 mM PP_i	1 l of 5 mM KPP_i Linear Gradient: 1.2 l of 5 mM KPP_i 1.2 l of 400 mM KPP_i	Three active peaks	

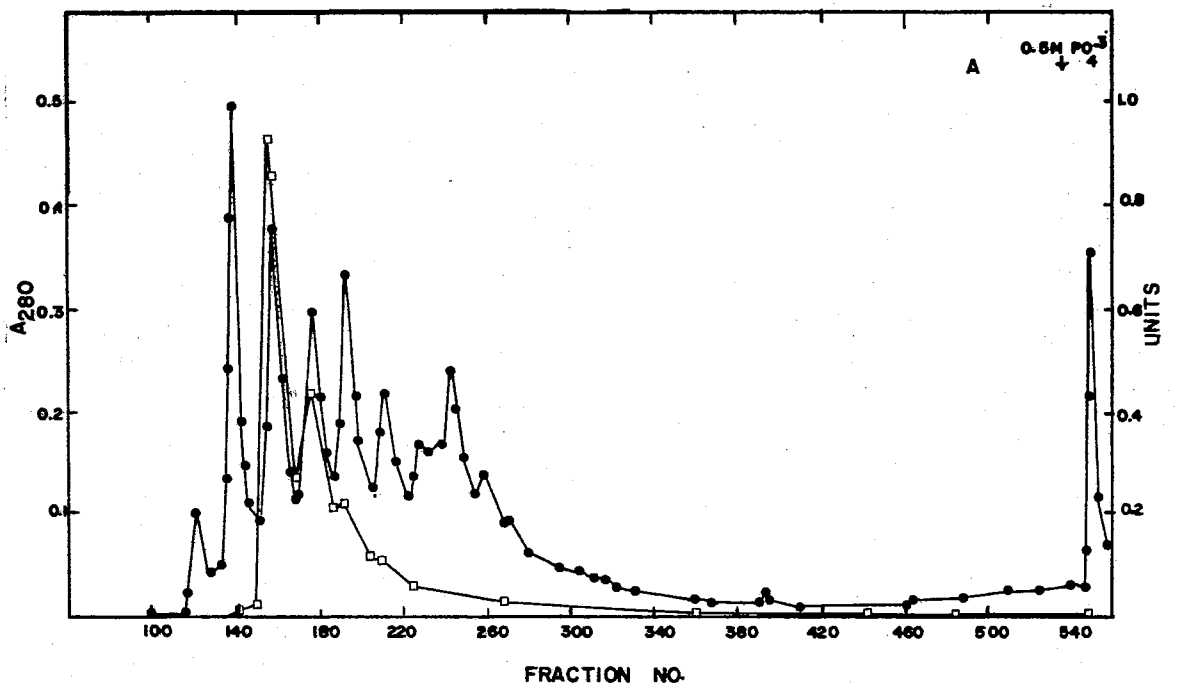


Figure 4. Elution Profile of DEAE-Cellulose Column Chromatography at pH 9.7

A column (3.09 x 30 cm) was equilibrated with 20 mM glycine, pH 9.7, washed with 600 ml of the same solution, eluted with a linear gradient formed by 1000 ml each of 20 mM glycine and 1.5 M glycine, pH 9.7, and finally washed with 500 mM potassium phosphate, pH 8.0, at the point indicated by the arrow. A 5 ml sample of UDP-glucose pyrophosphorylase prepared through Step VII containing 45 units and 60 mg of protein was applied. ●, A_{280}; □, activity in units per ml.

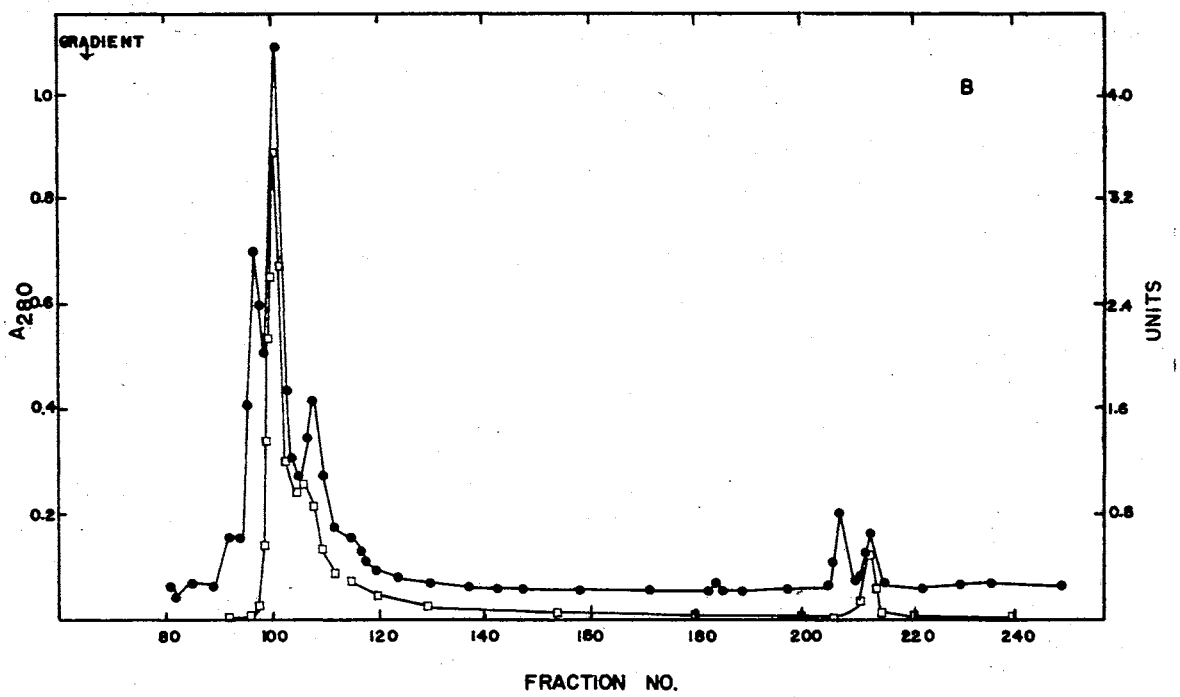


Figure 5. Elution Profile of DEAE-Cellulose Column Chromatography at pH 10.0

A column (3.09 x 17 cm) was equilibrated with 20 mM glycine, pH 10.0, washed with 600 ml of the same solution, and eluted with a linear gradient of 1000 ml each of 20 mM glycine and 1 M glycine, pH 10.0. The start of the gradient is indicated by the arrow. A 5 ml sample of UDP-glucose pyrophosphorylase prepared through Step VII containing 130 units. ●, A_{280}; □, activity in units per ml.

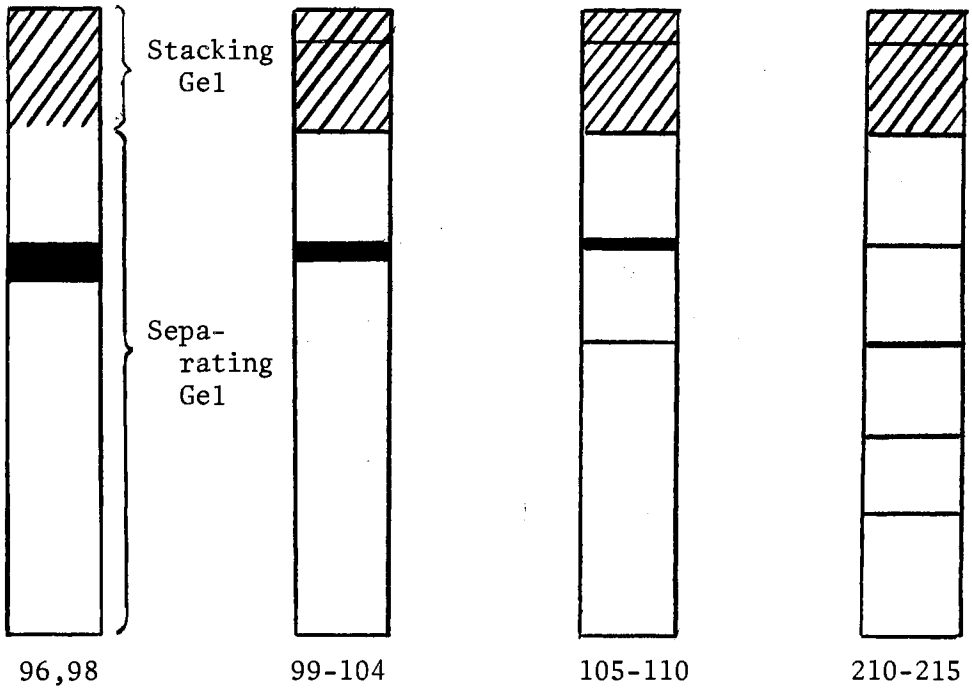


Figure 6. Disc Gel Electrophoresis of Various Fractions From a DEAE-Cellulose Column at pH 10.0

Various fractions from the DEAE-cellulose column shown in Figure 5 were subjected to electrophoresis in 7% polyacrylamide gels at pH 9.5 and stained with amido black. The fraction numbers are shown below the appropriate schematic representation. Fraction 96 and 98 are from the first inactive protein peak; fraction 99 through 104, from the second protein peak which has enzymatic activity; fractions 105 through 110, from the third protein peak; and fractions 210 through 215, from the trailing edge of the third protein peak.

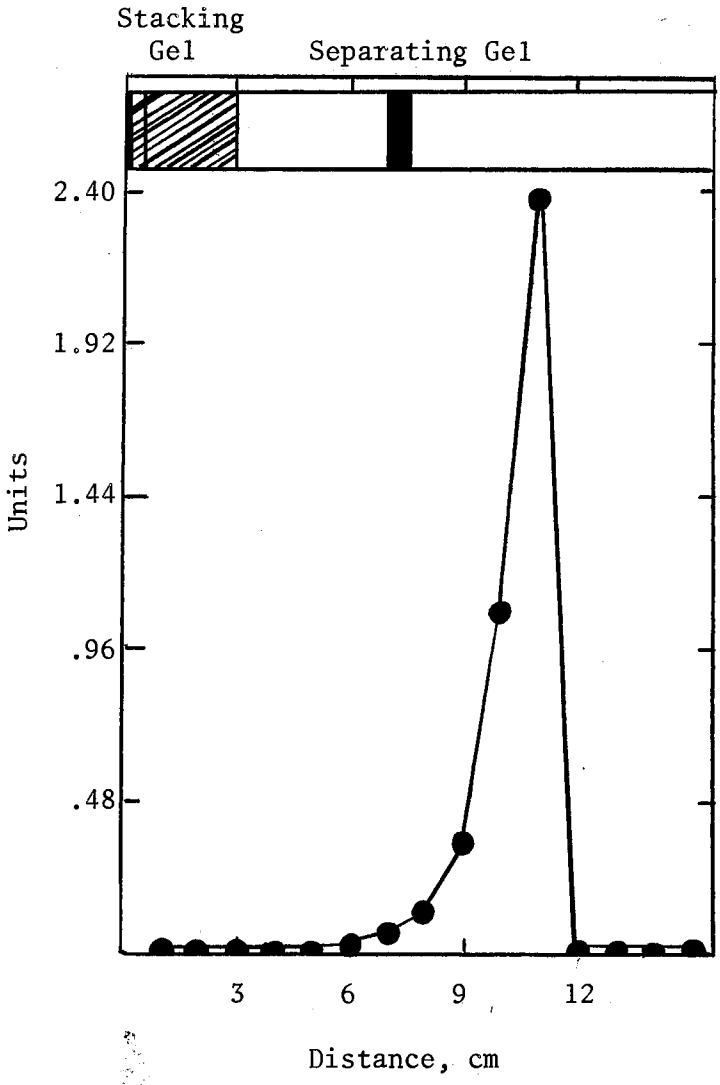


Figure 7. UDP-Glucose Pyrophosphorylase Activity Pattern on a Disc Gel

A sample from the pooled fractions (99 to 104) of the DEAE-cellulose column at pH 10.0 (Figure 5) was subjected to electrophoresis in a 7% polyacrylamide gel at pH 9.5. The gel was sliced horizontally into 0.3 cm sections. Each section was triturated with 0.2 ml of 0.2 M Tris, pH 8.0, and an aliquot assayed. At the top of the figure is a schematic representation of a second gel run simultaneously but stained with amido black.

activity when dyed with amido black. This is not surprising since calculations based on the specific activity of the liver enzyme show that only 0.0003 mg of protein were applied to the gel. The position of the activity band was further verified by surrounding the disc gel with 1% agar containing 0.2 M Tris-HCl, pH 8.0, 4 mM $MgCl_2$, 10 mM Glc-1-P, 2mM NAD^+, 10 units UDP-glucose dehydrogenase, 2 mM UTP, 0.05 mg/ml phenazine methosulfate, and 0.5 mg/ml nitroblue tetrazolium. The gel was placed in the dark. Within an hour a blue band appeared at the same position as the sliced gel.

The absorbance spectra of the two small A_{280} peaks appearing last in the elution profile of Figure 5 indicated the first peak was not protein but that the second one was. The first peak began to absorb at 300 nm and continued to increase in intensity as the wavelength decreased. No peak could be observed in the spectra upon several fold dilution nor could a shoulder be found at any wavelength.

No increase in resolution occurred upon doubling the height of the column, as shown by comparing the first four columns of Table XI with the remaining ones. In fact the longer columns showed more diffusion. Disc gel analysis of pooled active fractions of the remaining columns always showed two or three major bands and one to three minor ones.

A very important feature of these DEAE-cellulose columns is the increase in total units. The peak tube of activity will usually contain more units than were applied to the column. The total units recovered are 10 to 30 times those applied. Two explanations may account for all or part of the increase. First, ammonium sulfate in the concentrated sample, which was assayed prior to application, may inhibit UDP-glucose dehydrogenase in the assay to such an extent that the dehydrogenase

becomes rate limiting. Second, the increase in total units may be real and similar to the activation observed by Fitzgerald et al. (74) in crude homogenates. In the purification of UDP-glucose pyrophosphorylase a second DEAE-cellulose column at high pH was found very useful because it was reproducible, yielded a high specific activity material, and required no lengthy gel preparation.

Molecular Weight Separations

Sedimentation velocity experiments on the material pooled from a DEAE-cellulose column, pH 10.0, showed two peaks with sedimentation constants, $s^{0.4\%}_{20,w}$, of 7.15 S and 20.15 S, representing approximate molecular weights of 100,000 to 150,000 and 500,000 to 600,000, respectively. Thus a molecular weight separation of these two components should be feasible.

Sucrose density gradient centrifugation using a SW 25.2 rotor gave adequate separation of the activity and protein (Figure 8). Material from three different DEAE-cellulose columns were used. The only differences observed between the tubes is in quantity of activity and protein. This method was not pursued because of the limited quantity of material that can be applied to a single tube and the necessity of assaying each tube for activity. The method could be useful for small volumes of material.

As an alternative method, gel filtration was examined in detail. Thyroglobulin or apoferritin was used as high molecular weight markers and bovine serum albumin as a low molecular weight marker in order to test the separating ability of gel filtration columns.

The chromatography of apoferritin and bovine serum albumin on a

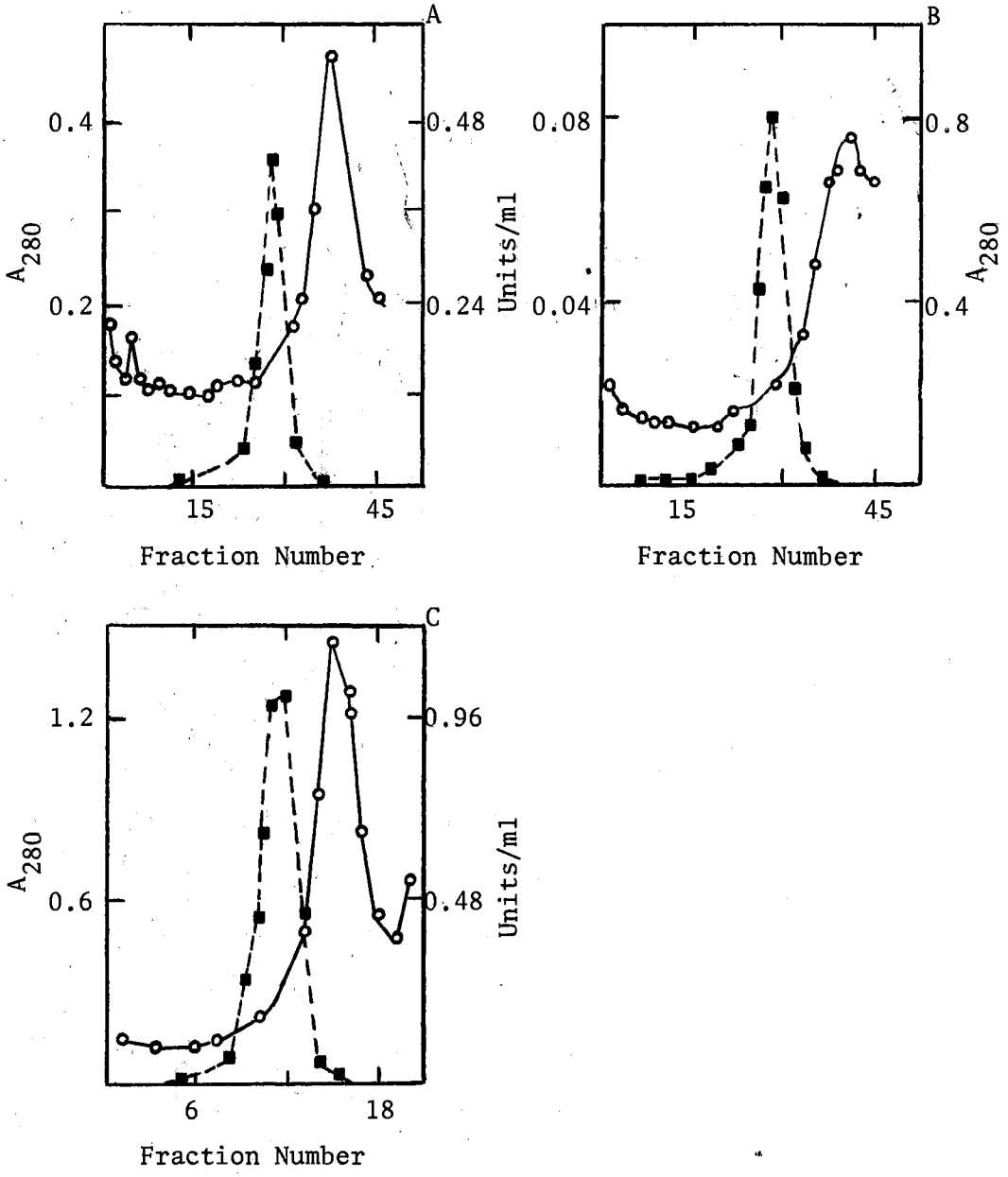


Figure 8. Preparative Sucrose Density Gradient Centrifugation of UDP-Glucose Pyrophosphorylase

5-20% sucrose gradients containing 10 mM triethanolamine, pH 8.0, were centrifuged for 24 hours at 25,000 RPM at 2°C. One ml samples were applied. Before centrifugation the samples were precipitated by ammonium sulfate, dissolved in a minimal volume of 0.2 M potassium phosphate, pH 8.0, and dialyzed for 24 hours against 10 mM potassium phosphate, pH 8.0. O-O, A_{280}; ■--■, UDP-glucose pyrophosphorylase activity. Tube A was an active fraction from a DEAE-cellulose column at pH 9.1; tubes B and C were active fractions from DEAE-cellulose columns at pH 10.0.

100 cm Bio-Gel P-300 column is shown in Figure 9A. Bovine serum albumin is clearly separated from apoferritin, and in addition thyroglobulin and apoferritin are slightly separated. Figure 9C shows the elution profile of a sample from a DEAE-cellulose column at pH 9.4. Two runs on the same sample gave identical results, including the low point in the activity profile at the expected peak. This suggests the possibility of two forms, but interpretations based on a single point cannot be reliably made with only two experiments. It can be seen that little of the A_{280} material is active enzyme. Evaluation of the sucrose density gradient centrifugation was made by applying a concentrated, pooled sample. Little A_{280} material is detectable (Figure 9D). Again multiple activity peaks could be present. Molecular weight marking of the column (Figure 9B) did not yield a linear relationship between the logarithm of the molecular weight and elution volume (105). Nonetheless, the molecular weight of the smallest component can be deduced as 200,000 $\pm$ 50,000 since it falls between catalase (M.W. 244,000) and lactic dehydrogenase (M.W. 130,000 to 140,000). By the same reasoning the larger contaminant would have a molecular weight approximately equal to apoferritin (M.W. 460,000). UDP-glucose pyrophosphorylase elutes between apoferritin and catalase, which is not consistent with a molecular weight of 480,000 reported by Levine *et al.* (73) for the bovine liver enzyme. A possible explanation is binding of UDP-glucose pyrophosphorylase to polyacrylamide, suggested by the results of the Bio-Gel A-5m column and the lack of success using horizontal polyacrylamide electrophoresis.

In an attempt to obtain a linear relationship between the logarithm of the molecular weight and the elution volume, a 100 cm Sephadex G-200

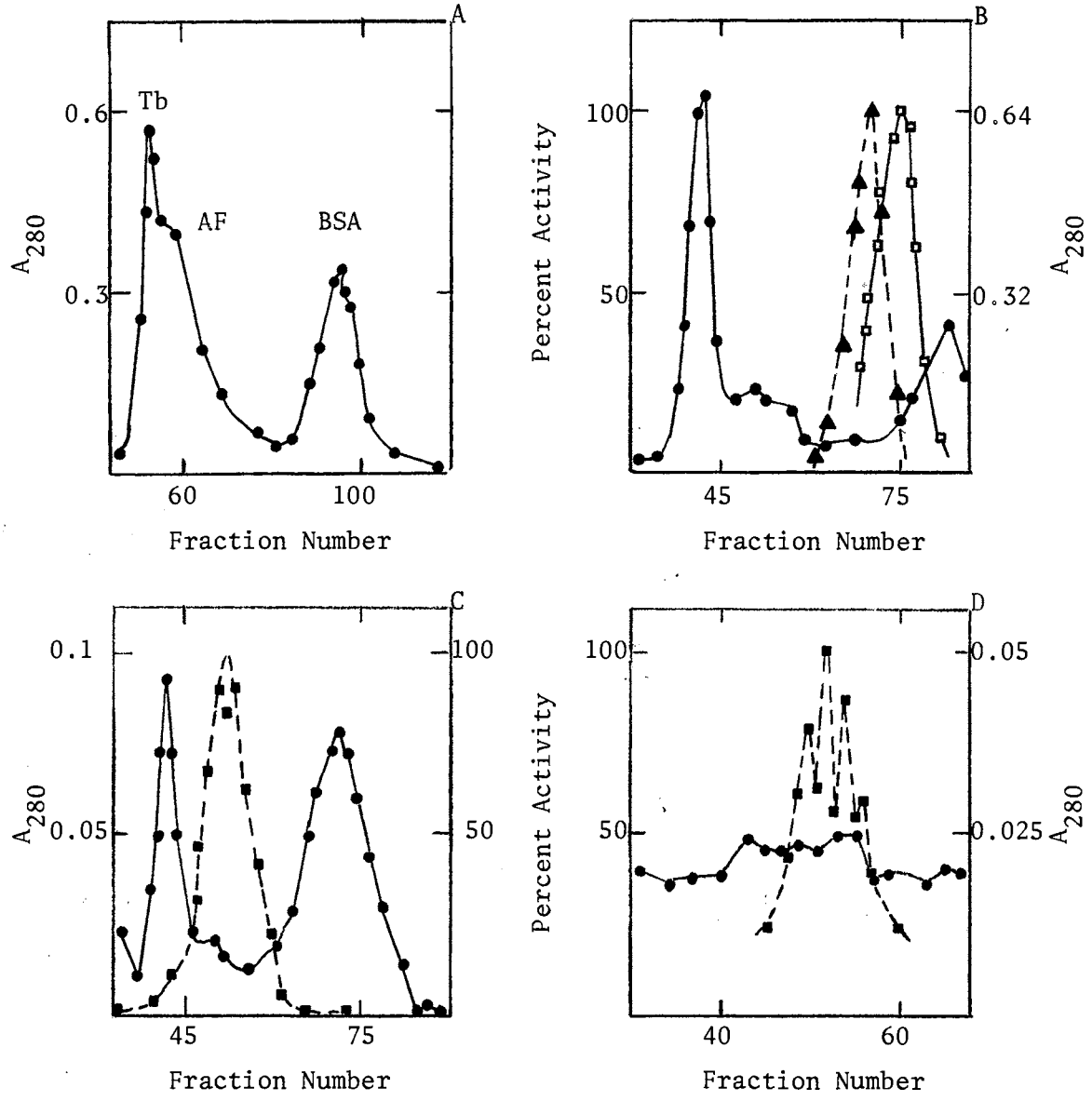


Figure 9. Bio-Gel P-300 Chromatography of UDP-glucose Pyrophosphorylase

Columns (1 x 95 cm) were equilibrated at 4°C with 20 mM triethanolamine and 250 mM KCl, pH 8.5. 0.5 ml fractions were collected. ●—●, A_{280}; ▲--▲, catalase (CAT); □—□, lactate dehydrogenase (LDH); ■--■, UDP-glucose pyrophosphorylase. [A] 3.6 mg thyroglobulin (Tb), 2.5 mg apoferritin (AF), and 5.4 mg bovine serum albumin (BSA) applied in 0.5 ml; [B] 3.1 mg BSA, 10 µl LDH (100% activity = 1.31 I.U./ml), 10 µl CAT (100% activity = 0.80 I.U./ml), and 2.5 mg AF applied in 0.5 ml; [C] 0.5 ml of UDP-glucose pyrophosphorylase from DEAE-cellulose, pH 10.0 (100% activity = 10.8 I.U./ml); [D] 0.5 ml of UDP-glucose pyrophosphorylase from sucrose density gradient centrifugation (Figure 8A) (100% activity = 3.6 I.U./ml).

column, equilibrated in 20 mM triethanolamine and 250 mM KCl, ph 8.5 was prepared. The markers gave a linear relationship identical to the one obtained previously on a 44 cm Sephadex G-200 column (Figures 10A and 10C). The 50 cm Sephadex G-200 column had been used to chromatograph material purified through Step VII but before the heat step had been incorporated into the purification procedure. The elution profile indicated that UDP-glucose pyrophosphorylase activity was associated with a high molecular weight shoulder, migrating slightly behind the void volume in the nonlinear region. After the heat treatment was added to the purification procedure, larger quantities of material appeared in the void volume, destroying the separation. Material obtained from a DEAE-cellulose, pH 9.7, column was chromatographed (Figures 10B and 10D). A large amount of higher molecular weight material was not observed but the slow eluting component was still present with an approximate molecular weight of 210,000. The UDP-glucose pyrophosphorylase activity elutes just after apoferritin, which is in the nonlinear region. However, apoferritin eluted from the column sooner than was expected. This may also have been the case on the Bio-Gel P-300 column. The specific activity of the material from gel filtration was about 18 measured in the direction of UDP-glucose synthesis or 55 in the direction of pyrophosphorolysis. This compares to a specific activity of 240 for crystalline bovine liver UDP-glucose pyrophosphorylase measured in the direction of pyrophosphorolysis. Thus the preparation is at least 25% pure, assuming the specific activity of the mammary gland enzyme is the same as that from bovine liver.

Recycling of UDP-glucose pyrophosphorylase on a Sephadex G-200 or Bio-Gel P-300 column gave poor results. The peaks became so diffuse

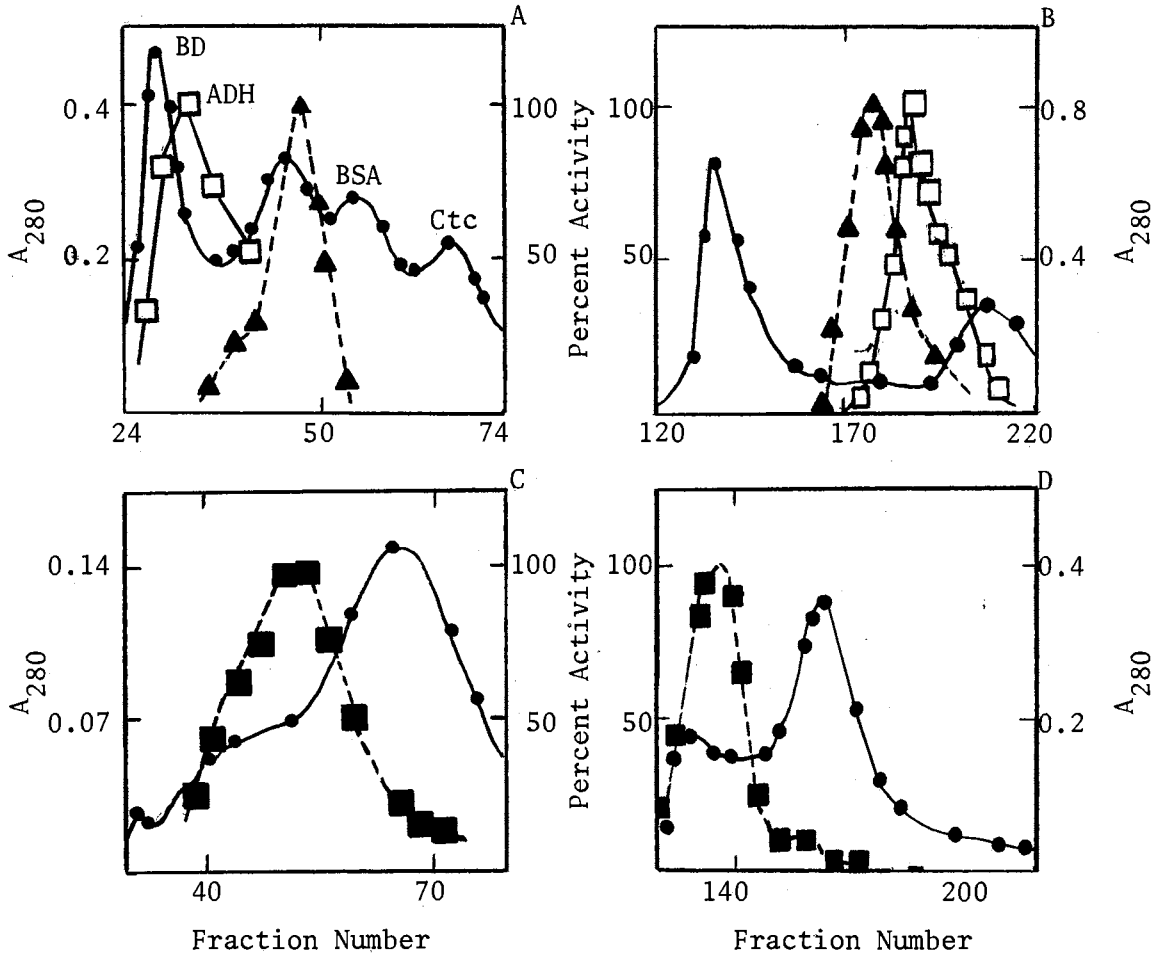


Figure 10. Sephadex G-200 Chromatography of UDP-Glucose Pyrophosphorylase

All columns were equilibrated at 4°C with 20 mM triethanolamine, pH 8.5 containing 250 mM KCl. ●—●, A_{280}; ▲--▲, catalase, (CAT); □—□, lactate dehydrogenase (LDH); ■--■, UDP-glucose pyrophosphorylase. [A] 2.5 x 44 cm column, 1 ml sample containing 4 mg blue dextran (BD), 20 µl CAT (100% activity = 0.275 I.U./ml), 10 mg bovine serum albumin (BSA), 4 mg cytochrome c (Ctc), and UDP-glucose pyrophosphorylase purified through Step VII without heat step (100% activity = 0.075 I.U./ml). 2.9 ml fractions collected. [B] 3 x 100 cm column, 3.0 ml sample containing 4.4 mg BD, 13.2 mg AF, 75 µl CAT (100% activity = 0.48 I.U./ml), 30 µl LDH (100% activity = 0.27 I.U./ml), and 29.4 mg BSA. 2.6 ml fractions collected. [C] 2.5 x 44 cm column, 1.5 ml of UDP-glucose pyrophosphorylase without heat step (100% activity = 0.088 I.U./ml). 1.8 ml fractions collected. [D] 3 x 100 cm column, 2.3 ml sample of UDP-glucose pyrophosphorylase purified through DEAE-cellulose column, pH 9.7 (100% activity = 1.4 I.U./ml), 25 ml fractions.

after recycling once that further recycling lowered the absorbance at 280 nm to below detection limits.

Bio-Gel A-5 chromatography of an enzymatic solution prepared through Step VII resulted in a smear of both A_{280} and activity throughout the elution profile. The column had been equilibrated with 20 mM triethanolamine, pH 8.5, containing 250 mM KCl. It was assumed that no adsorption would occur under these conditions. In retrospect, this column might be transformed into a purification step. A protein sample of low ionic strength could be applied to a column equilibrated in a low ionic strength buffer and washed exhaustively. If adsorption is occurring, elution with a high ionic strength buffer such as molar phosphate may elute the activity in a sharp peak.

Other Methods

Cation exchange celluloses were not tried because it was assumed the enzyme was unstable below pH 8.0, and because attempts using CM-cellulose at pH 8.7 (106) had not proved successful. However, in view of the results of the pH stability studies presented in the next chapter and a small scale batch experiment with CM-cellulose and bovine liver extract, CM-cellulose chromatography at pH values below seven may prove very useful.

It was also observed during the pH stability study, presented in the next chapter, that a coagulated precipitate of denatured protein formed when the protein solution was stored at pH 6.0 for one month or longer. A 5 µl sample of the clear solution was carefully removed so as not to disturb the precipitate and assayed. The solution was then stirred until the precipitate was uniformly suspended and again assayed.

The activity of the two assays was the same. To test for bacterial contamination, a sample of the solution was stained with crystal violet and observed under the microscope. Only amorphous protein was found. Although it required three to four weeks for the precipitate to form at 0^{o} to 4^{o}C, this process might be accelerated at a higher temperature. A liver extract of an acetone powder in water when adjusted to pH 5.5 precipitated protein without appreciably affecting the UDP-glucose pyrophosphorylase activity. These two observations could be used as a starting point to develop a pH step after extraction or elsewhere during the purification.

The precipitate obtained in Step VII was extracted sequentially at 0^{o}C with 50 ml of 50, 45, 40, 35, 30, and 25% saturated ammonium sulfate at pH 8.5. After extraction with each of the above solutions, the suspension was centrifuged for 30 minutes at 10,000 x g. The final precipitate was dissolved in 0.2 M potassium phosphate, pH 8.5. The results are presented in Table XII. No increase in specific activity in any of the solutions occurred using this procedure.

TABLE XII

AMMONIUM SULFATE FRACTIONATION OF UDP-GLUCOSE PYROPHOSPHORYLASE

	Per Cent Ammonium Sulfate						
	50	45	40	35	30	25	0
Units/ml	0.0	0.0	0.0012	0.014	0.074	0.034	2.48
Protein/ml	0.56	0.33	0.51	0.76	1.14	0.78	7.70
Specific Activity	0.0	0.0	0.002	0.018	0.065	0.043	0.032

In an attempt to crystallize the enzyme, material prepared by chromatography on a second DEAE-cellulose column, pH 9.7, was extracted at 0 to 4°C with decreasing concentrations of ammonium sulfate according to Jakoby (107). The 55, 45, 41, 38, 34, 30, 26, and 22% saturated ammonium sulfate solutions contained 25 mM potassium phosphate, pH 8.0. The protein was initially precipitated by adding saturated ammonium sulfate, pH 8.0, to a concentration of 55%. After centrifugation, the supernatant solutions were allowed to warm to room temperature. In one experiment the solutions were allowed to stand until turbidity appeared. In a second experiment, using 50 mM triethanolamine at pH 8.5, the solutions were held at room temperature for two hours and were then placed in the cold room. In both cases only amorphous material and dust particles were observed under a microscope.

Summary

Several changes in the isolation of UDP-glucose pyrophosphorylase from bovine mammary tissue have been made in the method described by Steelman and Ebner (54). The first major change was the elimination of a second ammonium sulfate fractionation and the addition of the heat step. The heat step did not result in any significant purification until chromatography on DEAE-cellulose. At this step approximately three times as much material was washed through the column with the starting buffer. The alkaline ammonium sulfate step was not used to collect the material off DEAE-cellulose chromatography at pH 8.0 because of the unreliability of the pH meter in this lab and the time required using the pH meter. An alkaline ammonium sulfate fractionation was made, although not at constant pH, when the material was collected from

a second DEAE-cellulose column at pH 9.7 to 10.0. The average buffer molarity was 0.5 M glycine.

Before any future purifications are attempted, it is recommended that a crude tissue homogenate and an extract of an acetone powder should be prepared using Tricine buffer and compared with the previous methods. Newell and Sussman (33) have found one form of UDP-glucose pyrophosphorylase that is unstable in Tris but stable in Tricine.

If only a small purified sample is required, it is suggested that UDP-glucose pyrophosphorylase be purified through Step VII and followed by a second DEAE-cellulose column and either gel filtration or preparative sucrose gradient centrifugation. If the preparation of a large amount of material is required, a pH step and chromatography on CM-cellulose should be considered.

The bovine liver UDP-glucose pyrophosphorylase enzyme should be nearly the same if not identical to the bovine mammary gland enzyme. Therefore, using a specific activity value of 240 for the crystalline liver enzyme (31) the amount of UDP-glucose pyrophosphorylase in the extract of 50 grams of acetone powder can be estimated as follows: From Table IX the total activity of the extract is 681 I.U. Since the reaction is two to three times faster in the direction assayed by Albrecht et al. (31), the total activity in the reverse direction is 2043 I.U. Therefore,

$$2043(1/240) = 8.5 \text{ mg.}$$

Since most purifications give 10% or less yield, approximately 850 μg of protein can be expected. This represents 0.04% of the total initial protein as determined in the acetone powder extract by A_{280}. This can

be compared to the liver homogenate concentration of 0.2 to 0.3% (31). Higher levels of activity might be found in the mammary gland at an appropriate stage of lactation. In addition the material obtained from the slaughter house is probably old or not functioning normally, and therefore would be expected to have a lower UDP-glucose pyrophosphorylase content. The increased difficulties in purifying the enzyme from bovine mammary tissue is apparent. The most practical purification procedure would include a trapping system such as affinity chromatography.

CHAPTER IV

PROPERTIES OF UDP-GLUCOSE PYROPHOSPHORYLASE

Experimental Procedure

Materials and Reagents

Piperazine and glycylglycine were purchased from Sigma Chemical Co.; boric acid, and citric acid from Baker Chemical Co.; potassium phosphate, ethylenediaminetetraacetic acid, and sucrose from Fisher Chemical Co. All other chemicals were of reagent quality.

Methods

All of the assays performed in this chapter were done with the standard assay presented in the Experimental Procedure of Chapter III. The temperature was approximately 22°C. Enzyme, UTP, Glc-1-P, and UDP-glucose dehydrogenase were kept in an ice-bath until addition to the assay cuvette. The remaining assay reagents were at room temperature (about 22°C).

Analytical sucrose gradients were prepared according to Martin and Ames (100). 0.2 ml aliquots were layered onto the top of the gradients. Centrifugation was performed in a Beckman L2-65 preparative ultracentrifuge using a SW 65 rotor at 65,000 RPM and at the desired temperature. Approximately 0.13 ml fractions were collected as described in Chapter III.

Results

During the course of the purification of UDP-glucose pyrophosphorylase from bovine mammary tissue, several of the properties of the enzyme were determined. These properties were very similar to other UDP-glucose pyrophosphorylases.

Temperature Stability

A purification was carried through Step III and the stability of the enzyme toward heat was established. The pH of the enzymatic solution was adjusted by adding 1 N KOH. The purified enzyme was taken from an ice-bath at zero time; and after incubation at the selected temperature and time, it was immediately plunged into an ice-bath. The control was kept in the ice-bath and assayed with the incubated sample. The results are shown in Table XIII.

The enzyme is stable up to 50°C. At 52°C the activity of the enzyme falls rapidly and at 60°C no activity remains after 5 minutes of incubation. Since pH in the range of 7.5 to 9.0 apparently had no effect upon heat stability up to 40°C, pH 8.0 or 8.5 was selected as a convenient pH to continue the test of stability at higher temperatures. The heat stability upon incubation for 30 minutes at pH 8.0 is plotted as a function of temperature in Figure 11. The enzyme has been incubated for as long as two hours at 50 to 51° without significant loss of activity. A 0-40% ammonium sulfate fraction of a water extract of bovine liver UDP-glucose pyrophosphorylase was stable when incubated at pH 8.0 for 30 minutes at 50 to 51°.

During the heat stability experiments the bovine mammary UDP-glucose pyrophosphorylase was in the presence of 250 mM potassium

TABLE XIII

TEMPERATURE STABILITY OF UDP-GLUCOSE PYROPHOSPHORYLASE AS A FUNCTION OF TIME AND pH

Temperature	pH	Control	Time (minutes) 2	5	10	15	20	30
°C		I.U./ml	I.U./ml	I.U./ml	I.U./ml	I.U./ml	I.U./ml	I.U./ml
30	7.5	1.60	-	1.92	1.71	1.96	1.80	1.88
	8.0	1.69	-	1.90	1.96	1.53	1.66	1.68
	8.5	2.00	-	1.64	1.67	1.68	1.67	1.80
	9.0	1.60	-	1.80	1.74	1.74	1.40	1.75
40	7.5	1.60	-	1.90	1.60	-	1.86	2.00
	8.0	1.69	-	1.71	1.60	-	1.40	1.40
	8.5	2.00	-	1.76	1.74	-	1.60	1.38
	9.0	1.60	-	1.34	1.90	-	1.28	1.66
50	8.0	2.18	-	2.11	2.22	-	2.33	2.14
52	8.0	1.25	-	0.68	0.55	-	0.40	0.40
55	8.5	2.16	2.00	1.53	0.58	-	0.46	0.34
60	8.0	1.89	0.47	0	0	-	0	0

0.5 ml of an enzymatic solution prepared through Step III was heated at the indicated time and temperature and assayed as described in Experimental Procedure.

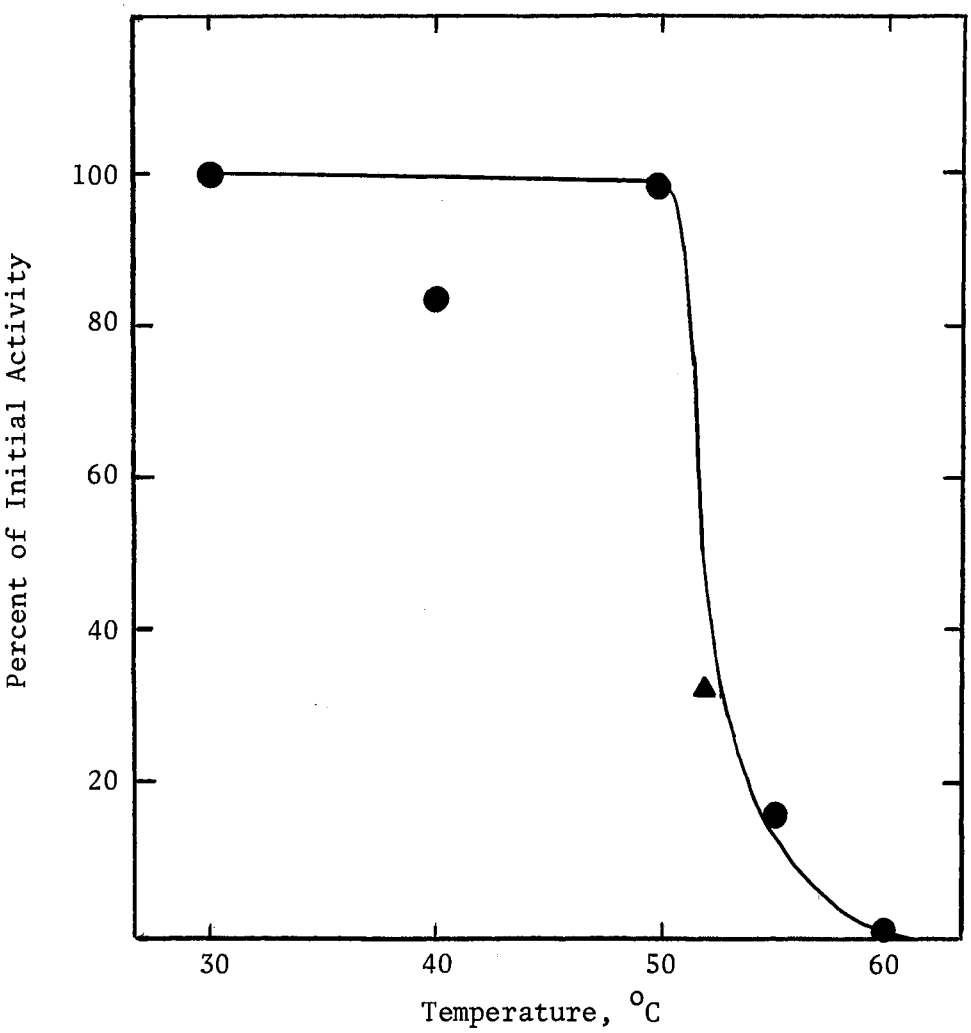


Figure 11. UDP-Glucose Pyrophosphorylase Activity Remaining After Incubation for Thirty Minutes at pH 8.0

0.5 ml of an enzymatic solution prepared through Step III was incubated for 30 minutes and then assayed as described in Experimental Procedure. ●, incubated at pH 8.0; ▲, incubated at pH 8.5. 100% activity equals 1.60 I.U./ml.

phosphate and approximately 12 to 20% ammonium sulfate. Albrecht et al. (31) have shown that phosphate is a competitive inhibitor of the bovine liver enzyme which may account for its stability to heat. In addition, Rodrigues (62) has shown that ammonium sulfate protects the bovine thyroid enzyme toward heat. It may be that sulfate ion is binding at the "phosphate" binding site of the enzyme.

A 0.5 ml aliquot of a preparation purified through Step VII and a 0.5 ml aliquot purified through Step VII followed by DEAE-cellulose column chromatography at pH 10.0, and stored as a concentrated ammonium sulfate solution were diluted 1:100 and stored at 22°C and at -10°C, respectively. The enzymatic solution stored at 22°C retained 87% of its activity after 2.5 days and 32% after 9 days. The enzymatic solution frozen at -10°C retained 93.9% and 0.9% of its activity after 25 and 9 days, respectively. The results are summarized in Table XIV.

TABLE XIV

STORAGE OF UDP-GLUCOSE PYROPHOSPHORYLASE AT 22°C AND AT -10°C

Time	Stored at 22°C		Stored at -10°C	
Days	Activity (I.U.)	Per cent Activity	Activity (I.U.)	Per cent Activity
0	16.0	100	23.4	100
2.5	13.7	85.5	22.0	93.9
9	0.54	3.2	0.21	0.9

pH Stability

A series of buffers were prepared from pH 3.0 to 7.5 to test the pH stability of the enzyme after Step VII of the purification procedure. The buffers were composed of 57.3 mM borate, 33.3 mM citrate, and 72.5 mM phosphate (108). An aliquot (0.02 ml) of the enzyme was diluted to 0.2 ml and stored in a cold-room maintinaed at 0 to 5°C. A control was diluted in a similar manner except that Tris buffer at pH 8.0 was used. Tris, pH 8.0, was chosen because it is the usual assay buffer. In all cases zero time is the time immediately after adding the enzyme, mixing, and placing the cuvette in the spectrophotometer. Less than 10 seconds were required for these operations. An additional 30 to 60 seconds lag was observed on the direct recording of absorbance change.

To test for buffer effects the control enzyme was assayed in the presence and absence of the pH 3.0 buffer. It was found that 0.005 ml of the buffer inhibited the reaction only slightly. At higher levels of buffer inhibition became appreciable and could not be relieved by adding Mg^{2+}. Therefore to avoid buffer effects, all assays were conducted with 0.005 ml of the incubation mixture. The results are shown in Figure 12. The pH 5.5 curve is not shown in the figure to reduce complexity since it closely followed the control. All values are expressed as percentages of the Tris control at zero time. The variation in repeated assays was tested by performing five separate assays over a one hour period. The maximum range of variation was $\pm$ 3%.

At zero time the activity increases as the pH increases. This cannot be due to the lower pH of the buffer since no significant effect upon the control was observed upon adding the same amount of the lowest pH buffer. The pH stability at pH 3.0 showed the most dramatic loss.

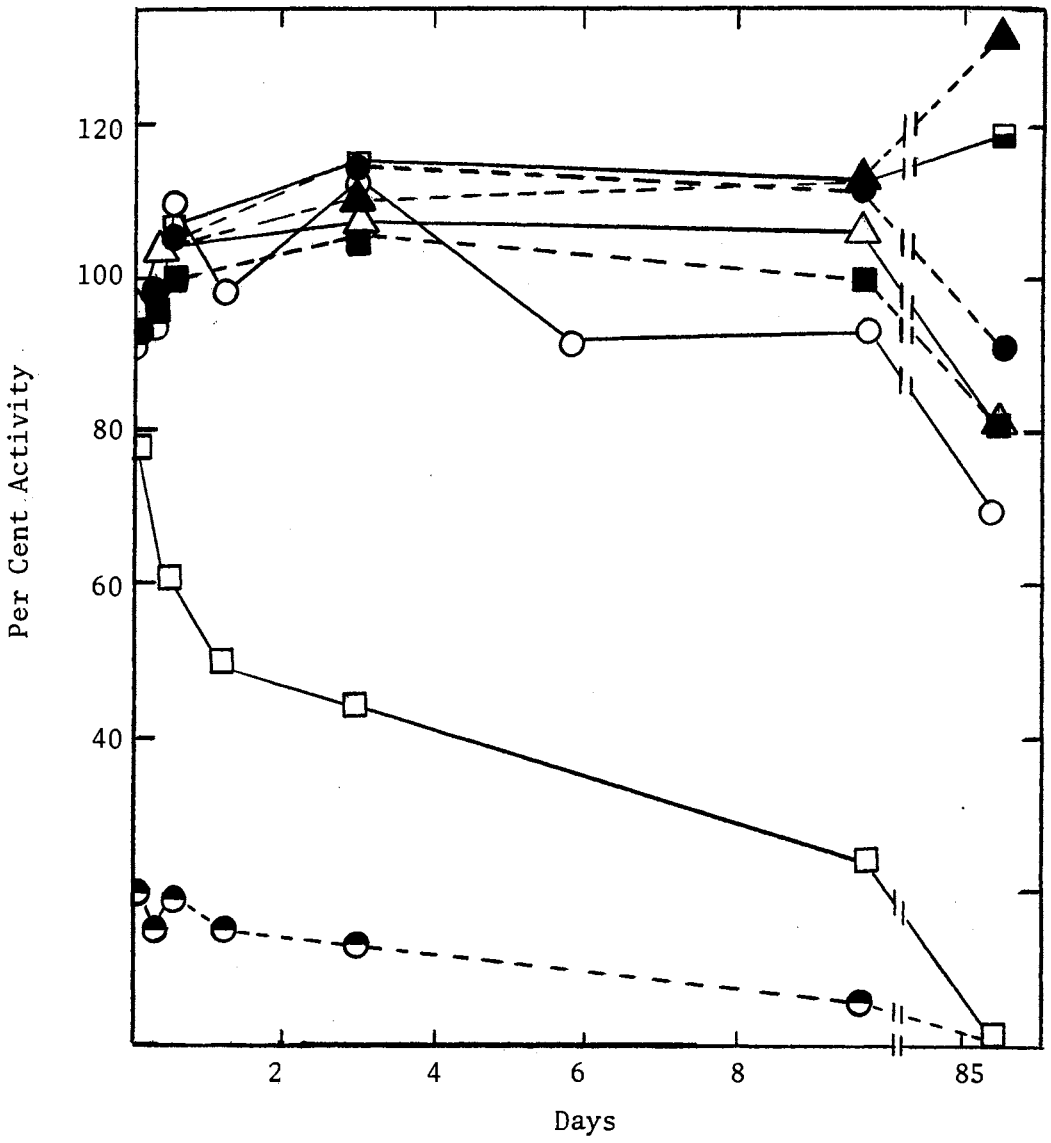


Figure 12. pH Stability of UDP-Glucose Pyrophosphorylase in Borate-Citrate-Phosphate Buffer, pH 3.0 to 7.5

Experimental details are presented in the text. 100% activity equals 0.51 I.U./ml at all pH values and for the control. ⊖—⊖, pH 3.0; □—□, pH 4.0; ○—○, pH 5.0; ■--■, control; △—△, pH 6.0; ●—●, pH 6.5; ◪—◪, pH 7.0; ▲--▲, pH 7.5.

In the time required to record an assay the enzyme lost 80% of its activity. However, it requires 86 days for the remainder of the activity to be lost. The enzyme stored at pH 4.0 lost 20% of its activity at zero time and still retained a detectable amount after 86 days. At pH 5.0 to 7.5 the enzyme retained 70 to 100% or more of the activity after 86 days. At pH's above 4.0 the enzyme appeared to rise in activity and to reach a maximum after approximately 70 hours. Since this suggested the "activation" described by Fitzgerald et al. (74) in crude homogenates of mammary gland, the pH stability was observed over a shorter time course and in a buffer system which did not contain an inhibitor. The buffer was 0.1 M piperazine and 0.1 M glycylglycine adjusted to pH values between 4.4 and 7.5 (109). These results are presented in Table XV and Figure 13. Since no large change in activity was seen at pH 4.4 or above at zero time, all the values have been expressed as percent of the activity at zero time for each pH. A Tris control was again chosen. Addition of pH 4.4 buffer to the control did not produce any inhibition. Assays were performed every six hours the first day, and thereafter performed at approximately the same time on the various days assayed.

It is again observed that at lower pH values the activity is lower than at the higher pH values. The apparent oscillatory behavior is accentuated. This may be the result of an "activation" phenomenon (74) or changes in temperature. Unfortunately, these experiments were not performed under strict temperature control. Assuming a Q_{10} of 2 for the reaction, a 50% change in rate would indicate a 5^{o} difference in temperature. If an "activation" phenomenon is assumed, it increases two fold as compared to the crude homogenate of rat mammary gland in

TABLE XV

pH STABILITY OF UDP-GLUCOSE PYROPHOSPHORYLASE IN PIPERAZINE-GLYCYLGLYCINE, pH 4.4 TO 7.5

Time (Days)	pH	Activity (Units/ml)	Per Cent of Activity at Zero Time
0	4.4	0.40	100.0
	5.0	0.39	100.0
	5.5	0.38	100.0
	6.0	0.41	100.0
	6.5	0.39	100.0
	7.0	0.54	100.0
	7.5	0.39	100.0
	C[1]	0.39	100.0
0.5	4.4	0.43	106.7
	5.0	0.45	115.9
	5.5	0.46	119.7
	6.0	0.47	113.0
	6.5	0.49	127.3
	7.0	0.61	112.6
	7.5	0.50	126.4
	C	0.52	135.3
2	4.4	0.48	118.6
	5.0	0.48	123.7
	5.5	0.48	125.5
	6.0	0.52	124.8
	6.5	0.53	137.2
	7.0	0.66	120.9
	7.5	0.53	134.6
	C	0.52	134.4
6	4.4	0.33	81.8
	5.0	0.33	85.3
	5.5	0.36	93.3
	6.0	0.38	92.2
	6.5	0.37	96.7
	7.0	0.51	93.2
	7.5	0.40	102.0
	C	0.39	100.4
7.5	4.4	0.42	104.3
	5.0	0.47	120.4
	5.5	0.48	126.4
	6.0	0.51	123.2
	6.5	0.50	128.5
	7.0	0.65	120.3
	7.5	0.52	131.7
	C	0.50	130.1

TABLE XV (Continued)

Time (Days)	pH	Activity (Units/ml)	Per Cent of Activity at Zero Time
13.5	4.4	0.49	121.3
	5.0	0.51	131.0
	5.5	0.56	146.4
	6.0	0.58	140.3
	6.5	0.59	151.2
	7.0	0.76	140.6
	7.5	0.60	151.6
	C	0.57	149.0
14.5	7.5	0.52	130.6
15.5	7.5	0.48	122.0
16.5	7.5	0.41	104.9
18.5	4.4	0.34	84.6
	7.5	0.41	104.1
19.5	7.5	0.50	127.6
20.5	7.5	0.40	102.8
21.5	7.5	0.47	119.5
22.5	7.5	0.58	146.7
25.5	7.5	0.42	106.1
26.5	7.5	0.51	129.7
27.5	7.5	0.55	139.0
28.5	7.5	0.48	121.1
29.5	7.5	0.43	108.1
30.5	7.5	0.40	101.2
31.5	7.5	0.40	102.8
36.5	7.5	0.33	84.9

[1]C is the control at pH 8.0 in 100 mM Tris-HCl buffer.

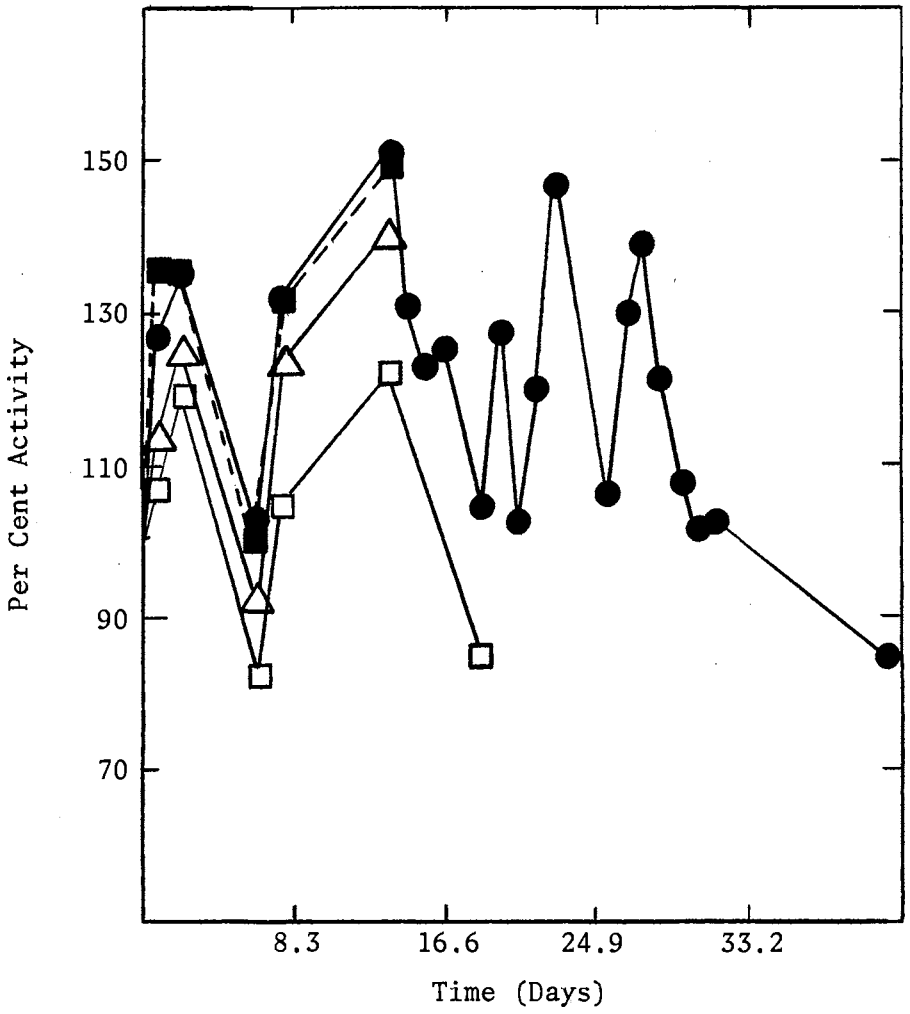


Figure 13. pH Stability of UDP-Glucose Pyrophosphorylase in Piperazine-Glycylglycine Buffer, pH 4.4, 6.0, and 7.5

Details are presented in the text. 100% activity equals 0.39 I.U./ml for the control, 0.40 I.U./ml at pH 4.4, 0.41 I.U./ml at pH 6.0, and 0.39 I.U./ml for pH 7.5. □—□, pH 4.4; △—△, pH 6.0; ■--■, control; ●—●, pH 7.5.

which a 3 to 4 fold increase was observed upon dilution (74). The maximum was reached in the rat mammary gland at about 10 hours and compares to the "first maximum" of 6 hours in these experiments. Without question, the enzyme is stable under these conditions with over 100% of the activity remaining at all pH values tested after 13.5 days of storage at 0 to 5°C. The pH 8.0 stability was carried out to 36.5 days with 85% activity remaining. The enzyme still retained 103% activity after 31.5 days.

Another experiment was performed in the same manner in piperazine-glycylglycine buffer from pH 8.0 to pH 10.8. The values are expressed as the percent of the activity at the particular pH at zero time. The results are presented in Table XVI and Figure 14. Under these conditions the differences between pH values are very small. For this reason only two stability curves are plotted in Figure 14. With the exception of the pH 10.8 curve which lies slightly below the control curve, all the remaining curves are indistinguishable from the data plotted in Figure 14. The oscillatory behavior is again seen, and the enzyme is stable at alkaline pH.

A stability experiment using material directly off a second DEAE-cellulose column at pH 10.0, which contained approximately 200 mM glycine, was assayed after eluting from the column and then at 2.5 and 5.5 days afterward. Complete stability was observed.

The stability of the enzyme under such a wide variety of conditions of pH is surprising in view of experimental observations that activity is frequently lost below pH 8 or below pH 5 during ammonium sulfate steps. Possibly at lower pH values the UDP-glucose pyrophosphorylase may be subject to attack by proteolytic enzymes which are lost upon

TABLE XVI

pH STABILITY OF UDP-GLUCOSE PYROPHOSPHORYLASE IN PIPERAZINE-GLYCYLGLYCINE, pH 8.0 TO 10.8

Time (Days)	pH	Activity (Units/ml)	Per Cent of Activity at Zero Time
0	8.0	0.46	100.0
	8.5	0.47	100.0
	9.0	0.47	100.0
	9.5	0.47	100.0
	10.0	0.47	100.0
	10.8	0.50	100.0
	C[1]	0.46	100.0
0.25	8.0	0.61	131.1
	8.5	0.64	136.3
	9.0	0.62	132.3
	9.5	0.63	134.8
	10.0	0.62	131.7
	10.8	0.63	124.4
	C	0.61	131.8
0.50	8.0	0.52	112.1
	8.5	0.52	112.0
	9.0	0.53	113.1
	9.5	0.52	111.3
	10.0	0.52	111.3
	10.8	0.53	105.4
	C	0.52	112.1
1.0	8.0	0.44	94.1
	8.5	0.45	96.9
	9.0	0.46	97.9
	9.5	0.45	97.3
	10.0	0.45	95.2
	10.8	0.46	91.1
	C	0.46	99.7
1.5	8.0	0.44	95.8
	8.5	0.45	96.9
	9.0	0.46	98.3
	9.5	0.45	96.8
	10.0	0.46	98.6
	10.8	0.46	91.1
	C	0.45	98.3

TABLE XVI (Continued)

Time (Days)	pH	Activity (Units/ml)	Per Cent of Activity at Zero Time
3.25	8.0	0.44	95.8
	8.5	0.45	96.2
	9.0	0.44	93.5
	9.5	0.44	94.9
	10.0	0.43	92.5
	10.8	0.46	91.7
	C	0.45	98.3
3.5	8.0	0.45	98.3
	10.8	0.45	90.2
	C	0.45	96.5
4.0	8.0	0.47	101.7
	10.8	0.47	92.7
	C	0.46	98.6
4.25	8.0	0.60	129.1
	8.5	0.59	125.7
	9.0	0.60	128.2
	9.5	0.59	125.9
	10.0	0.59	126.6
	10.8	0.58	115.9
	C	0.58	126.0
4.5	8.0	0.47	101.4
	10.8	0.46	91.4
	C	0.45	97.6
5.25	8.0	0.52	113.1
	10.8	0.53	105.1
	C	0.51	110.7
5.5	8.0	0.48	103.1
	10.8	0.49	96.5
	C	0.48	102.8
6.25	8.0	0.55	118.3
	10.8	0.54	107.3
	C	0.52	112.8
6.5	8.0	0.47	102.1
	10.8	0.47	93.3
	C	0.46	99.0
7.25	8.0	0.44	94.5
	10.8	0.42	83.8
	C	0.43	92.4
7.5	8.0	0.45	97.2
8.5	8.0	0.43	93.1

TABLE XVI (Continued)

Time (Days)	pH	Activity (Units/ml)	Per Cent of Activity at Zero Time
9.25	8.0	0.48	103.5
	C	0.45	96.5
10.25	8.0	0.51	111.1
	C	0.47	102.4
11.25	8.0	0.46	99.3
	C	0.40	87.2
12.25	8.0	0.46	99.0
	C	0.40	86.5
13.25	8.0	0.56	121.1
	C	0.48	103.5
14.25	8.0	0.42	90.7
	C	0.37	79.8
14.5	8.0	0.44	94.1
	C	0.36	77.0
15.25	8.0	0.38	81.3
	C	0.32	68.9
16.0	8.0	0.36	78.5
	C	0.33	70.6
16.5	8.0	0.36	78.9
	C	0.35	75.1

[1]C is the control at pH 8.0 in 100 mM Tris-HCl buffer.

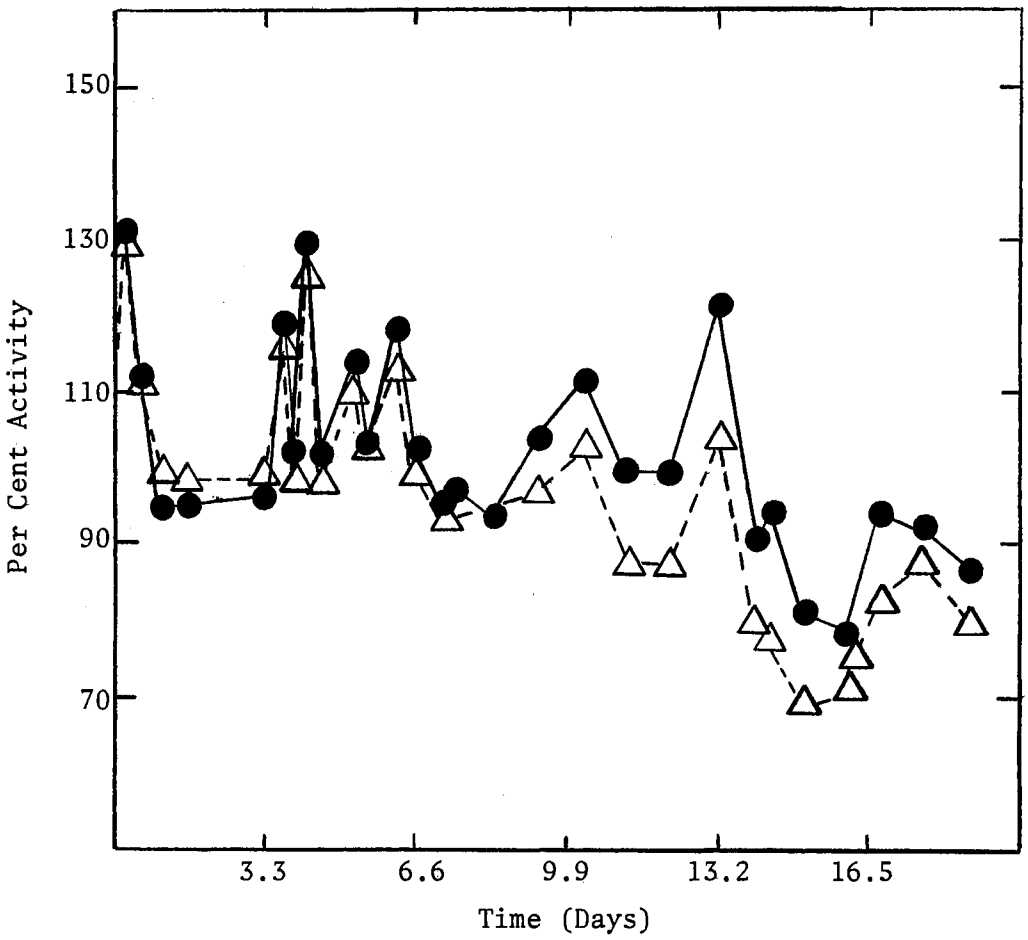


Figure 14. pH Stability of UDP-Glucose Pyrophosphorylase in Piperazine-Glycylglycine, pH 8.0

Experiment details are presented in the text. 100% activity equals 0.46 I.U./ml. ●—●, piperazine-glycylglycine buffer, pH 8.0; Δ--Δ, control.

DEAE-cellulose chromatography. As discussed in Chapter III, the stability does suggest other purification techniques may be used to advantage. The rapid decrease in enzymatic activity followed by a relatively stable period for several days which occurs below pH 4.4 in borate-citrate-phosphate buffer suggests two forms of enzyme with different pH stabilities similar to the two forms with different stabilities in Tris and Tricine buffers reported for the slime mold D. discoideum (33).

Effect of EDTA, Sulfhydryl Reagents, and Sucrose

EDTA does not affect the enzyme until it sufficiently complexes Mg^{2+} ion. The enzyme preparation, purified through Step VII, either contained no inhibitory heavy metals or was not affected by them. The EDTA solution was adjusted to pH 8.0 before addition to the reaction mixture. Addition of the sulfhydryl reagents, 10 mM mercaptoethanol or 1 mM dithiothreitol did not affect either the activity or the storage of the enzyme.

Sucrose was found to inhibit UDP-glucose pyrophosphorylase activity. The effect on the UDP-glucose dehydrogenase in the assay was determined by omitting the substrates, Glc-1-P and UTP, and adding 0.2 mM UDP-glucose. The UDP-glucose dehydrogenase was not inhibited. The results are summarized in Table XVII. A 1% sucrose solution is equal to 0.292 M.

TABLE XVII

EFFECT OF SUCROSE ON UDP-GLUCOSE PYROPHOSPHORYLASE ACTIVITY

Percent Sucrose	UDP-Glucose Pyrophosphorylase		UDP-Glucose Dehydrogenase	
	I.U.	Per Cent Activity	I.U.	Per Cent Activity
0	1.22	100	0.0096	100
1	1.14	93.5	-	-
2	0.66	54	0.0096	100

Molecular Weight

Molecular weight determinations were performed by 5-20% sucrose density gradient sedimentation according to Martin and Ames (100). The best estimation obtained by this method gave a molecular weight of 500,000 (Figure 15C) at 20°C. Catalase was used as a marker and its molecular weight was taken as 244,000 (110). The UDP-glucose pyrophosphorylase activity peak moved 57% of the way down the centrifuge tube. The activity peak is symmetrical and showed no evidence of another active species. Levine et al. (73) have shown four species upon sucrose gradient sedimentation of an aged bovine liver preparation.

To test the effect of temperature on possible association or dissociation and the effect of different purification procedures upon the enzyme, three more gradients were run at 2°C. Aliquots of the enzyme solution prepared through Step II and Step IV (the heat step) were applied. To a third gradient was applied a desalted sample off a

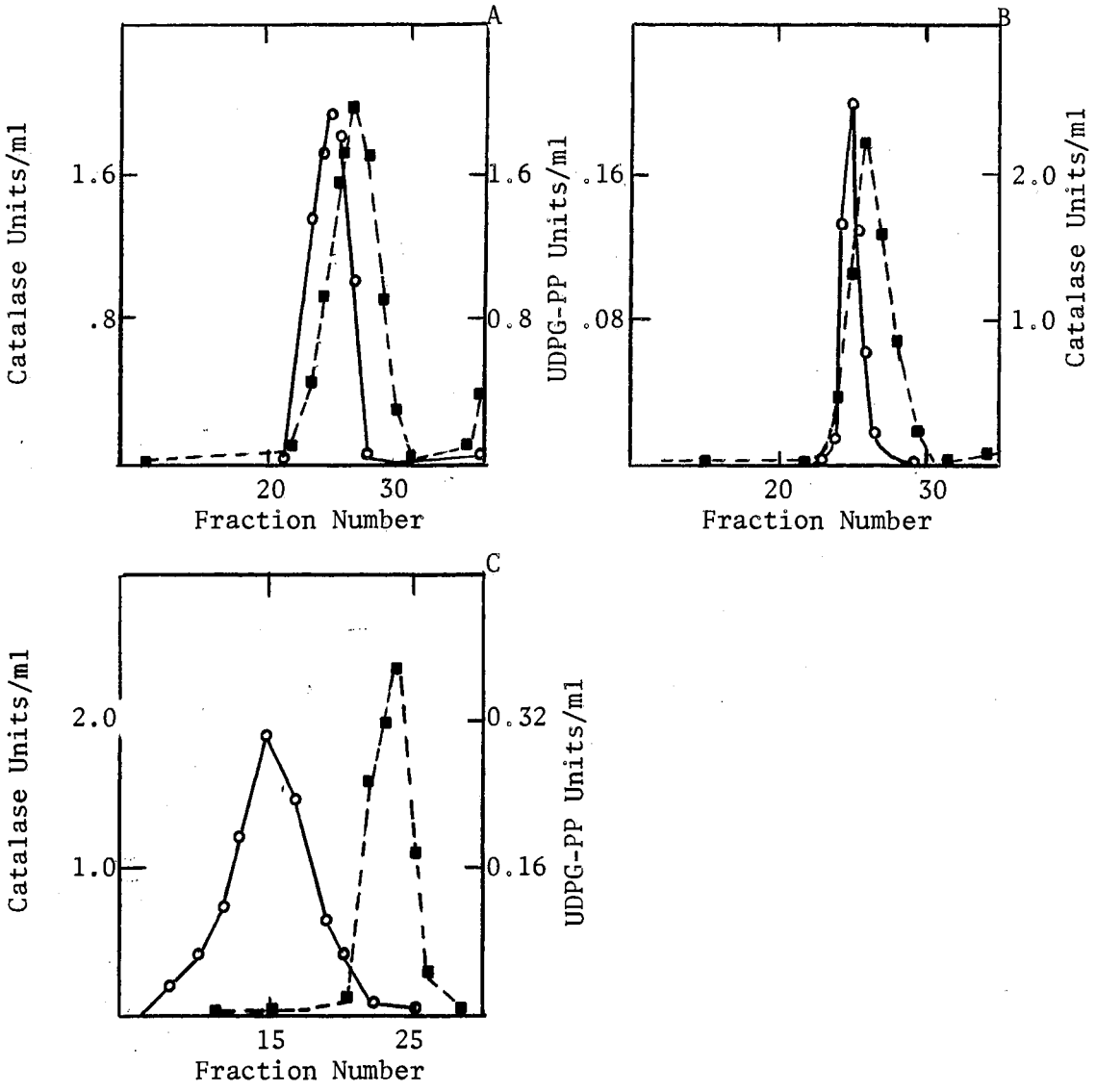


Figure 15. Analytical Sucrose Density Gradient Centrifugation of UDP-Glucose Pyrophosphorylase

5-20% sucrose density gradients were centrifuged at 65,000 RPM for 90 minutes at 2°C (Gradients A and B), or at 20°C (Gradient C). Gradient A contained 60 µl of a UDP-glucose pyrophosphorylase (UDPG-PP) solution purified through Step IV, 25 µl catalase, and 20 mM potassium phosphate, pH 8.0, throughout the gradient; [B] 25 µl desalted UDP-glucose pyrophosphorylase from the active fraction off a DEAE-cellulose column, pH 10.0, 25 µl catalase, and 20 mM potassium phosphate, pH 8.0, throughout the gradient; [C] 25 µl UDP-glucose pyrophosphorylase purified through Step VII, 25 µl catalase, and 150 mM KCl, 5 mM EDTA, and 5 mM $MgCl_2$, pH 7.5, throughout the gradient. O—O, UDP-glucose pyrophosphorylase activity; ■---■, catalase activity.

DEAE-cellulose column at pH 10.0. The sample after heat treatment and the material off the DEAE-cellulose column when measured relative to catalase gave a molecular weight of 308,000 and 291,000, respectively. The runs are shown in Figures 15A and 15B. Again the activity peaks show no signs of heterogeneity. However, since the protein migrated only 30% of the distance down the tube and was not well separated from catalase, the calculated values are subject to greater experimental error than the experiment at 20°C. These values compare with a molecular weight of 450,000 obtained by Fitzgerald and Ebner (74) and 350,000 by Albrecht et al. (31), using the same technique. Unfortunately, the sample before the heat step was spilled after centrifugation. Since the values for the molecular weight obtained from the material after heat treatment (Step IV) and from the desalted DEAE-cellulose material did not show a clear-cut dissociation and were similar to the values obtained by other workers (74,31), the experiment was not repeated.

The best molecular weight value obtained for a UDP-glucose pyrophosphorylase enzyme is 480,000 reported by Levine et al. (73) for bovine liver. It was determined by sedimentation velocity and sedimentation equilibrium studies. Thus a dimer would have a molecular weight of 240,000 and would be expected to migrate simultaneously with or slightly behind catalase.

Attempts to determine the molecular weight of the bovine mammary gland enzyme on Sephadex G-200 (105) and Bio-Gel P-300 gave only approximations of greater than 400,000 because the enzyme was in the non-linear portion of the elution pattern.

CHAPTER V

KINETIC ANALYSIS OF UDP-GLUCOSE PYROPHOSPHORYLASE

Experimental Procedure

Materials and Methods

Glc-1-P, UDP-glucose, $NADP^+$, phosphoglucomutase (EC 2.7.5.1, crystalline suspension in ammonium sulfate from rabbit muscle), and Glc-6-P dehydrogenase (EC 1.1.1.49, type XV from Bakers' yeast) were purchased from Sigma Chemcial Co.; quinine sulfate (N. F. grade), triethanolamine (certified), $MgCl_2$, and sodium pyrophosphate from Fisher Chemical Co.; and UTP from P-L Biochemicals. All other regeants were of reagant grade.

For kinetic studies in the direction of UDP-glucose synthesis, UDP-glucose pyrophosphorylase from bovine mammary tissue was purified through Step VII, chromatographed on DEAE-cellulose at pH 10.0, precipitated by ammonium sulfate, dissolved in a minimal volume of 0.2 M potassium phosphate, and desalted on a Bio-Gel P-10 column (1.15 x 22.5 cm) equilibrated with 20 mM triethanolamine, pH 8.0.

For kinetic studies in the direction of Glc-1-P formation, UDP-glucose pyrophosphorylase from bovine mammary tissue was purified through Step VII, chromatographed on DEAE-cellulose at pH 9.7, precipitated by ammonium sulfate, dissolved in a minimal volume of 0.2 M glycine, pH 9.7, chromatographed on a Sephadex G-200 column (3 x 100 cm),

precipitated by ammonium sulfate and dissolved in a minimal volume of 20 mM triethanolamine, pH 8.5. Because dilutions of 1:10,000 or greater were usually used in these kinetic analyses, the enzyme preparation was not desalted.

For kinetic studies using bovine liver UDP-glucose pyrophosphorylase, the enzyme was supplied by Dr. R. G. Hansen, Department of Chemistry, Utah State University, Logan, Utah, as a suspension in ammonium sulfate. The suspension contained 12.7 mgs of protein per ml and had a specific activity of 103 units per mg protein.

Estimation of Substrate Concentrations

The concentration of Mg^{2+} ion in a 0.5 M $MgCl_2$ stock solution was estimated by atomic absorption spectrometry and by passing measured amounts of $MgCl_2$ solution through a Dowex 50 column (111). The acid effluent from the Dowex 50 column was then titrated with standard base. The atomic absorption spectrometry was performed at 286.4 nm with a flow rate of 4.6 ml per minute on a Perkin-Elmer Model 303 atomic absorption spectrometer with a DCR1 readout. Commercial Mg^{2+} standards were used. Both methods gave comparable results.

The concentration of PP_i was estimated by the method of Fiske and Subbarow (56) or Lowry and Lopez (112). To determine free inorganic phosphate in the pyrophosphate solution the method of Lowry and Lopez (112) was used.

UDP-glucose concentration was estimated by its absorbance at 262 nm and by measuring the amount of NADH present after incubation with UDP-glucose dehydrogenase (53). UTP and Glc-1-P were prepared by weighing calculated amounts and dissolving them in water.

Spectrophotometric Assay for UDP-Glucose Pyrophosphorylase

Assays for UDP-glucose synthesis were similar to the assay described in Chapter III with the exception that 100 mM triethanolamine, pH 8.0, was used in place of Tris-HCl. $MgCl_2$, UTP, and Glc-1-P were varied according to the experiment.

Fixed-Time Assay for UDP-glucose Pyrophosphorylase

Reaction mixtures contained, in a total volume of 1.0 ml: 100 mM triethanolamine at pH 8.0, UTP and sodium pyrophosphate at the desired concentration, and sufficient $MgCl_2$ to maintain the concentration of Mg^{2+} at 1 mM (see Results). Before the addition of enzyme the tubes were incubated for 5 minutes at 30°C. The enzyme was added with a Hamilton microliter syringe with a stop. All assays were run for at least four different time intervals to ensure that initial velocities were measured. The reaction was stopped by adding 0.10 ml of 1.0 N HCl. After 2.5 minutes 0.05 ml of 2.0 N KOH was added to return the pH to 8.0. Either Eppendorf or Oxford micropipets were used for the addition of acid and base. Blank values were obtained by adding acid, immediately followed by enzyme, and neutralizing 2.5 minutes later with base.

Glc-1-P was estimated by using the reactions of phosphoglucomutase and Glc-6-P dehydrogenase. To each of the reaction mixtures were added 0.1 ml of the coupling system containing 0.1 mM EDTA, 10 mM triethanolamine, pH 8.0, 6 mM $NADP^+$, 0.002 ml of phosphoglucomutase, and 0.001 ml of Glc-6-P dehydrogenase. NADPH was estimated by measuring the absorbance at 340 nm or by measuring the natural fluorescence of NADPH (excitation at 350 nm, emission at 460 nm) (49).

Absorbance measurements were performed on a Beckman DU

spectrophotometer. Fluorescence measurements were performed on an Aminco-Bowman Spectrophotoflurimeter equipped with a water-jacketed sample chamber with thermoregulation at 25°C. A Xenon lamp was used as the light source. The detector was an IP-128 photomultiplier tube. Slit program number 5 as described in the Aminco-Bowman instruction manual was used to obtain maximum sensitivity. NADPH concentrations as low as 10^{-7} M were easily detected using standard 3 ml quartz cuvettes. The instrument was standardized with a 0.01 µg per ml solution of quinine sulfate. The fluorescence of this solution was checked after each reading and minor adjustments of the instrument made as required. The quinine sulfate solution was stored in the dark at 0 to 4°C.

All glassware was acid washed in a solution of one volume concentrated nitric acid and two volumes concentrated sulfuric acid, followed by repeated rinsing with distilled water and a final rinse in deionized water. The quartz cuvettes were cleaned by boiling in concentrated nitric acid.

Analysis of Data

The type of curve obtained was determined by graphical analysis of the data which were then analyzed by an appropriate computer program (113,114). Linear intersecting initial velocity plots were fitted to Equation 1; linear noncompetitive inhibition plots were fitted to Equation 2.

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \quad (1)$$

$$v = \frac{VA}{K(I + \frac{I}{K_{is}}) + A(1 + \frac{I}{K_{ii}})} \quad (2)$$

Results

Kinetic Analysis of Bovine Mammary UDP-Glucose Pyrophosphorylase

A large amount of indirect evidence indicates that the role of Mg^{2+} ion in the UDP-glucose pyrophosphorylase reaction is to participate as the $MgUTP^{2-}$ and $MgPP_i^{2-}$ complexes. The stability constants for $MgPP_i^{2-}$, $MgUTP^{2-}$ and MgGlc-1-P at pH 8.0 are considered to be 250,000 (77), 70,000 (75,76), and 20 (79,80), respectively. Although 70,000 is the stability constant determined for $MgATP^{2-}$ (75), $MgUTP^{2-}$ has been shown to have the same stability constant by a different technique under different conditions (76). The stability constants of UDP-glucose and NAD^+ are unknown, but they might be expected to be similar to ADP which has a stability constant of 4×10^3 (75). Since the stability constants for UTP and PP_i are so large, in solution they will essentially exist as their Mg^{2+} complexes. Glc-1-P and UDP-glucose were assumed to react in a noncomplexed form. During this analysis free Mg^{2+} ion was held constant at 1 mM so that any effect of free Mg^{2+} ion on enzyme was constant. Binding of Mg^{2+} ion by the coupling reagents, NAD^+ and UDP-glucose dehydrogenase, as well as by Glc-1-P, UDP-glucose, and triethanolamine was considered negligible at the concentrations used in the assays. Because triethanolamine binds only negligible amounts of Mg^{2+} (75), it was chosen as the buffer.

Steelman and Ebner (54) in the determination of the apparent K_m values for UDP-glucose pyrophosphorylase from bovine mammary tissue indicated substrate inhibition above 0.5 mM UTP. No preincubation of the assay mixtures preceeded assaying. Therefore, two explanations in addition to substrate inhibition were possible. First, if UTP was kept in

an ice-bath as it usually is, the temperature of the reaction mixture may have dropped when large volumes of UTP were added to the mixture. Second, since total magnesium was held constant and the concentration of UTP was varied, what might have been seen at higher concentrations of UTP was a decrease in the free Mg^{2+} ion resulting in a reduction in rate.

Figure 16 shows that by preincubating and varying $MgUTP^{2-}$ no substrate inhibition can be observed up to 2 mM UTP. The effect of 10-minute preincubation is seen to increase the rate especially at high concentrations of $MgUTP^{2-}$ and to completely eliminate the apparent substrate inhibition. Hence all further experiments were conducted by preincubating the entire reaction mixture except for enzyme for 10 minutes at 30°C. Thirty degrees was chosen because it is the temperature at which the stability constant for $MgUTP^{2-}$ has been most accurately measured (75,76). Controls showed that the assay reagents were stable during the time of preincubation.

Three initial velocity plots were run. Two of them are presented in Figures 17 and 18. The experimental points are plotted and the lines are drawn using a statistical fit to Equation 1. It is assumed that the variance is constant throughout the experiment and thus a weighting factor of v^4 was used. The lines are intersecting indicating a sequential mechanism (ordered, Theorell-Chance, or rapid equilibrium random) (83). Secondary plots of the slope and intercept are linear. The lines intersect above the abscissa which indicates that $K_{ia} > K_a$. In the only other study of initial velocity patterns on a UDP-glucose pyrophosphorylase the lines crossed below the abscissa indicating that $K_{ia} < K_a$. Thus the difference between the mechanism of the human

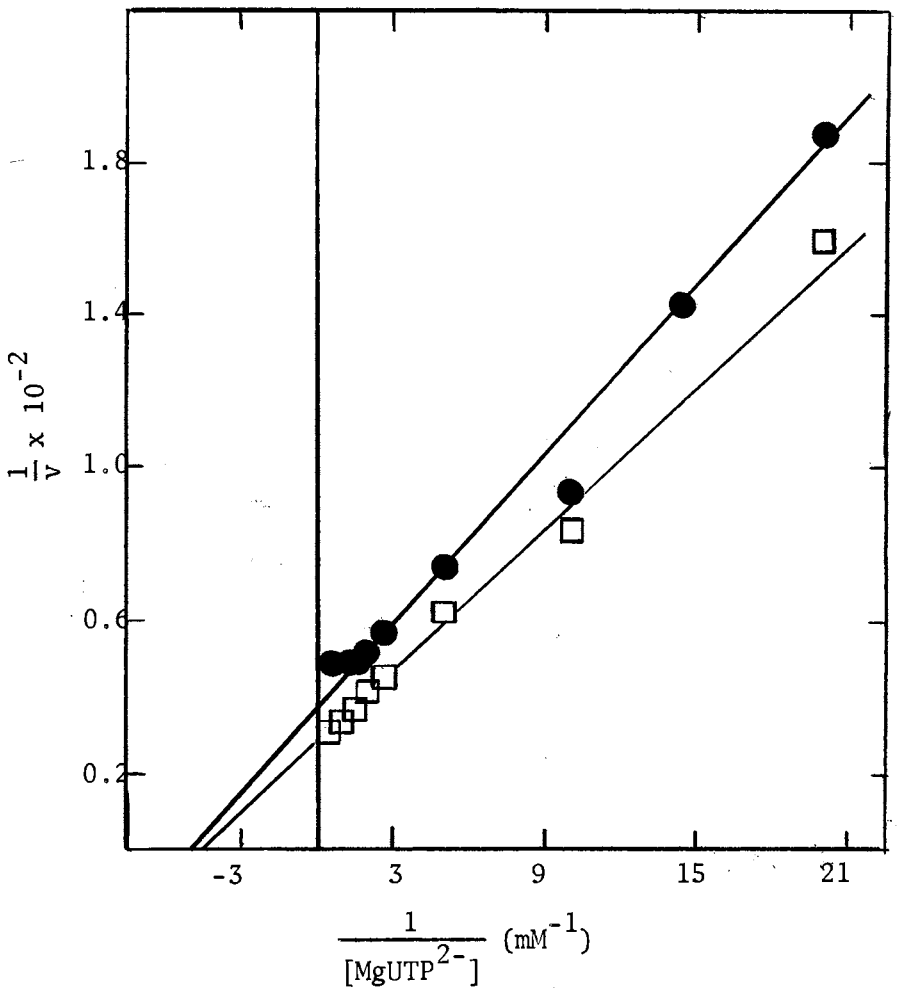


Figure 16. Effect of Preincubation on the Rate of UDP-Glucose Synthesis

Reaction mixtures contained 2.2 µg enzyme, $MgUTP^{2-}$ as indicated, Glc-1-P at 50 µM, free Mg^{2+} ion at 1 mM, and 100 mM triethanolamine at pH 8.0. v, initial rate of UDP-glucose formation in micromoles per ml per min. ●, no preincubation; □, reaction mixture preincubated for 10 minutes at 30°C.

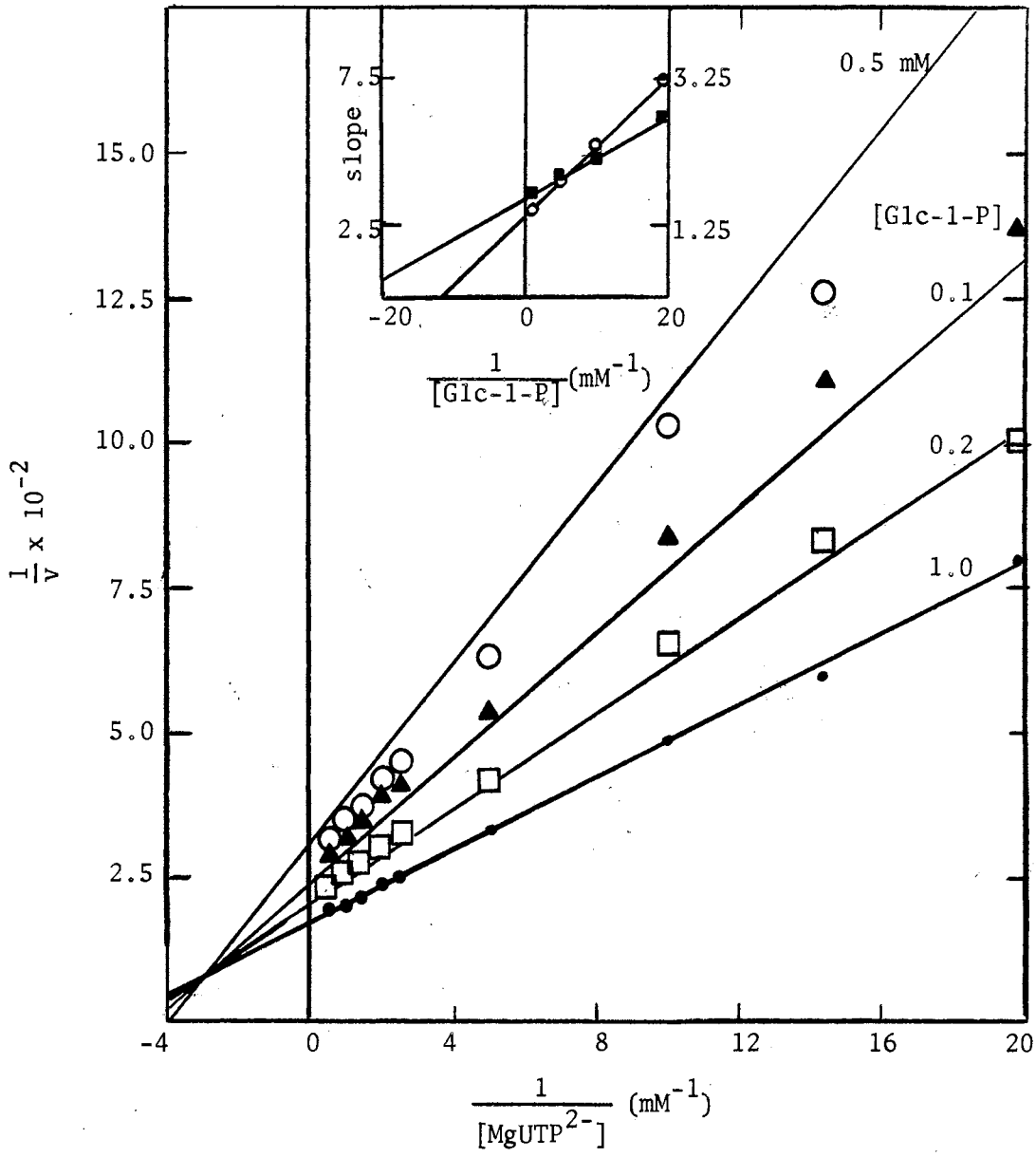


Figure 17. Double Reciprocal Plot With $MgUTP^{2-}$ as the Variable Substrate at Fixed Concentrations of Glc-1-P

Reaction mixtures contained 2.2 µg enzyme, $MgUTP^{2-}$ and Glc-1-P as indicated, free Mg^{2+} ion at 1 mM, and 100 mM triethanolamine at pH 8.0. v, initial rate of UDP-glucose formation in micromoles per ml per minute. Inset, replot of slopes and intercepts against the reciprocal of Glc-1-P; O, slope; ■ , intercept.

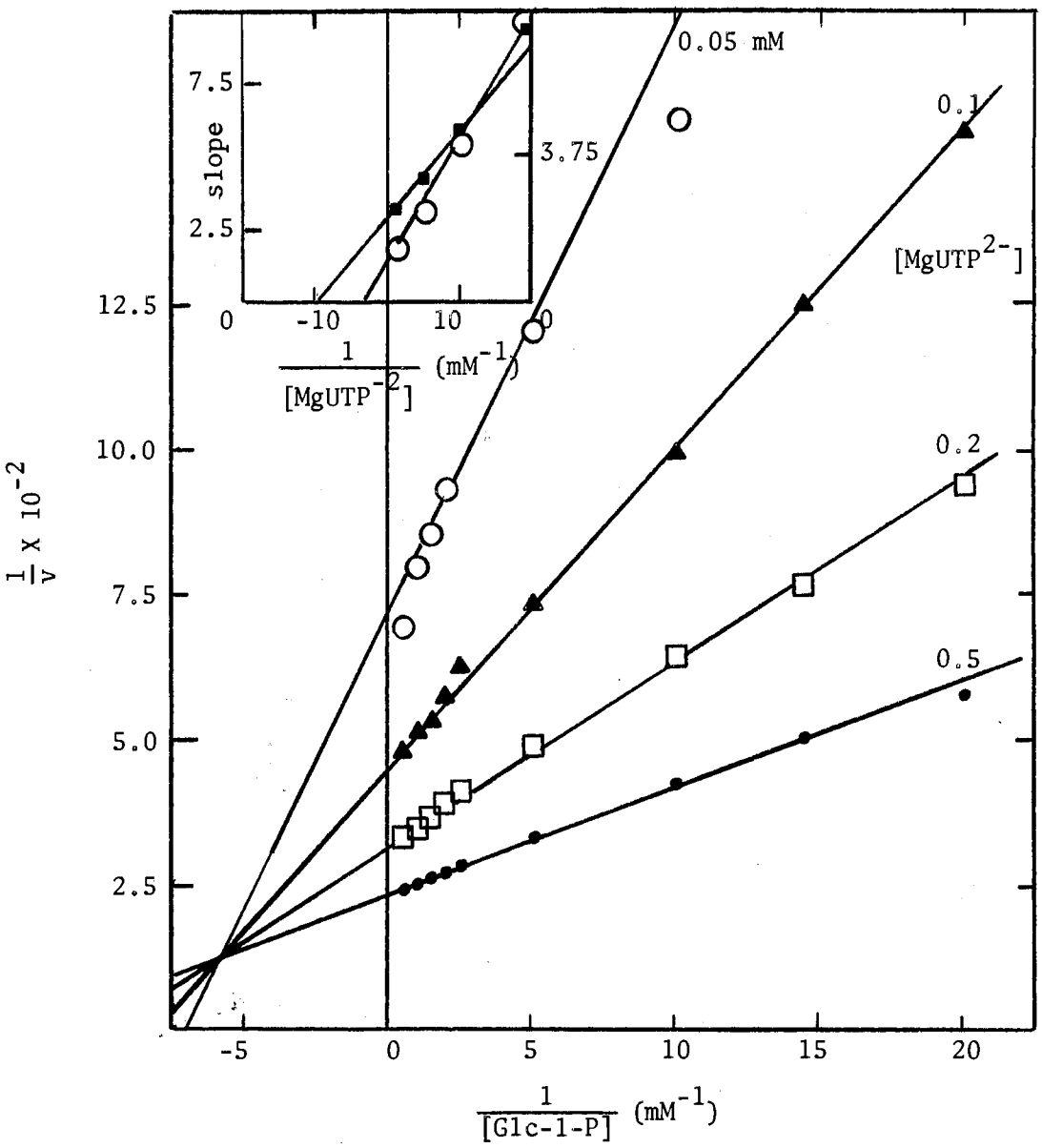


Figure 18. Double Reciprocal Plot With Glc-1-P as the Variable Substrate at Fixed Concentrations of $MgUTP^{2-}$

Reaction mixtures contained 2.2 µg enzyme, $MgUTP^{2-}$ and Glc-1-P as indicated, free Mg^{2+} ion at 1 mM, and 100 mM triethanolamine at pH 8.0. v, initial rate of UDP-glucose formation in micromoles per ml per minute. Inset, replot of slopes and intercepts against the reciprocal of $MgUTP^{2-}$; ○, slope; ■, intercept.

erythrocyte enzyme and the bovine mammary gland enzyme is quantitative. Qualitatively, they both indicate a sequential mechanism. Since the lines on the double reciprocal plots and the secondary plots are linear, sigmoidal kinetics are not occurring.

The kinetic constants from the initial velocity studies are presented in Table XVIII. A comparison of this table with the apparent K_m values in Table II, indicates very good agreement between the constants for bovine liver and bovine mammary tissue. However, the true constants obtained for the human erythrocyte enzyme are significantly different. Comparison of the constants of the three enzymes indicates that $K_{Glc\text{-}1\text{-}P} < K_{MgUTP^{2-}}$.

TABLE XVIII

KINETIC CONSTANTS FOR UDP-GLUCOSE SYNTHESIS FROM INITIAL VELOCITY STUDIES

Kinetic Constant	Experiment I	Experiment II	Weighted Mean
V	69.9 ± 1.71	73.0 ± 2.63	70.8 ± 0.014
$K_{MgUTP^{2-}}$	0.153 ± 0.010	0.167 ± 0.022	0.156 ± 0.009
$K_{iMgUTP^{2-}}$	0.417 ± 0.116	0.342 ± 0.12	0.417 ± 0.084
$K_{Glc\text{-}1\text{-}P}$	0.053 ± 0.009	0.042 ± 0.007	0.046 ± 0.006
$K_{iGlc\text{-}1\text{-}P}$	0.168 ± 0.021	0.085 ± 0.026	0.135 ± 0.016

Weighted mean and the standard error (S.E.) of the mean were calculated using the following formulas (115):

$$\text{Mean} = \frac{\Sigma x_i w_i}{\Sigma w_i}$$

$$\text{S.E.} = \frac{1}{\sqrt{\Sigma w_i}} \quad \text{where } w_i = \frac{1}{(\text{S.E. for each experiment})^2} \quad .$$

Figures 19 and 20 show inhibition by the product, magnesium pyrophosphate, with respect to $MgUTP^{2-}$ and Glc-1-P, respectively. The data are plotted in the same manner as for the initial velocity patterns with the points being the experimental data and the lines representing the best fit to Equation 2. Both inhibitions are noncompetitive. Secondary plots of slope and intercept for the inhibition by magnesium pyrophosphate with $MgUTP^{2-}$ as the variable are linear. Although the secondary plots of slope and intercept when Glc-1-P is varied could be drawn as hyperbolic, there is insufficient data to justify this, and therefore this plot is also considered to be linear. Since both magnesium pyrophosphate inhibition plots yield secondary plots that appear linear, there is no evidence for dead-end complexes. The noncompetitive inhibition by magnesium pyrophosphate shown with both Glc-1-P and $MgUTP^{2-}$ suggests that the reaction is ordered and that $MgPP^{2-}$ is the first product to be release. However, the data cannot distinguish the order of addition of $MgUTP^{2-}$ and Glc-1-P. The data are not consistent with a Theorell-Chance or rapid equilibrium random mechanism. The quantitative constants obtained from the initial velocity patterns and product inhibition patterns are shown in Table XIX with their associated standard errors.

In an attempt to study product inhibition and initial velocity patterns in the reverse direction, a modification of the assay first described by Munch-Petersen (12) was used. It was decided that to assure better free Mg^{2+} ion control in solution that the number of possible complexing agents should be reduced. Therefore, a fixed time assay was used to eliminate any effect of the coupling system, composed of $NADP^{+}$, phosphoglucomutase and glucose-6-P dehydrogenase.

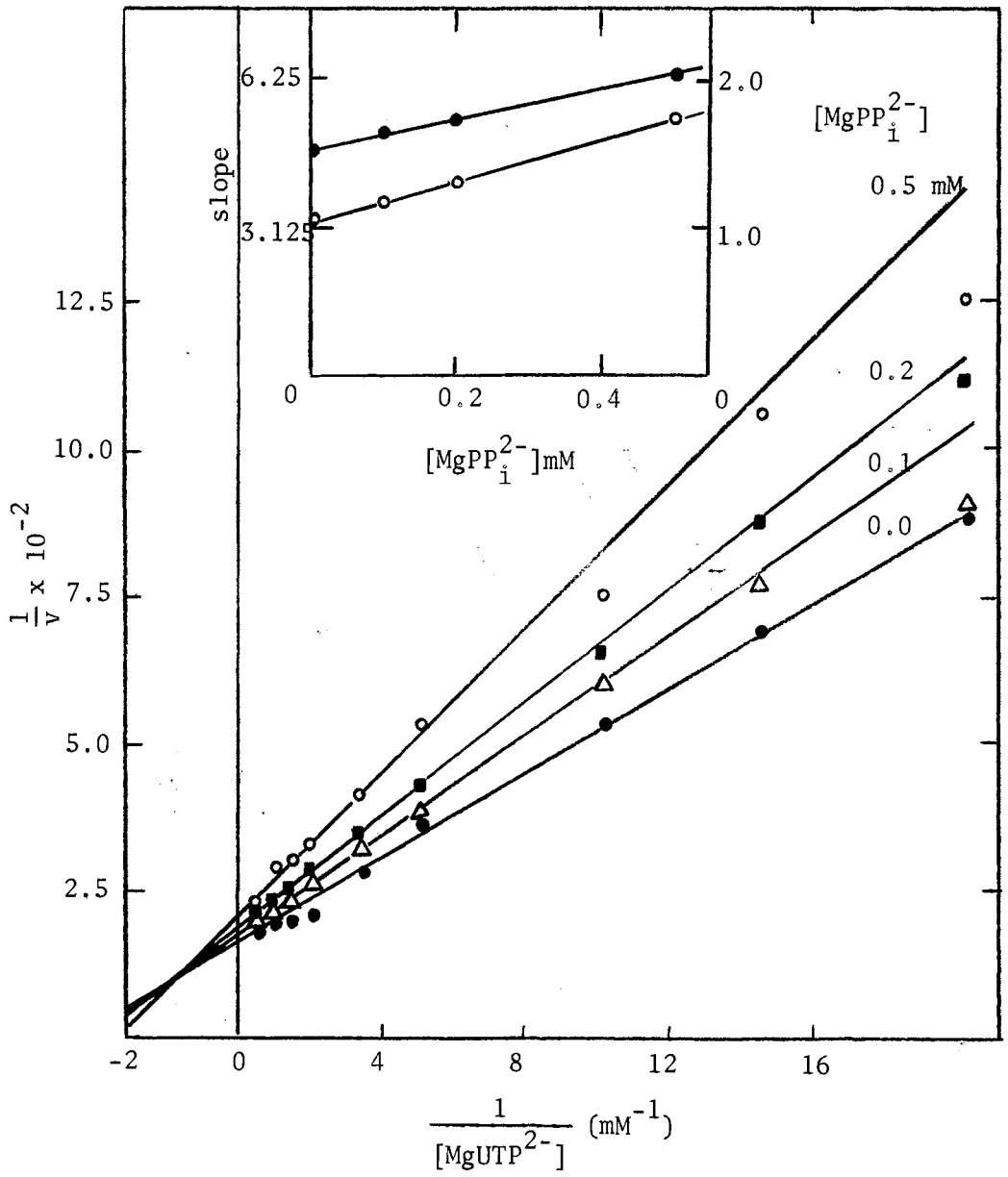


Figure 19. Inhibition of UDP-Glucose Synthesis by $MgPP_i^{2-}$ With $MgUTP^{2-}$ as the Variable Substrate

Reaction mixtures contained 2.2 µg enzyme, $MgPP_i^{2-}$ and $MgUTP^{2-}$ as indicated, free Mg^{2+} ion and Glc-1-P at 1 mM, and 100 mM triethanolamine at pH 8.0. v, initial rate of UDP-glucose formation in micromoles per ml per minute. Inset, replot of slopes and intercepts against $MgPP_i^{2-}$ concentration; O, slope; ●, intercept.

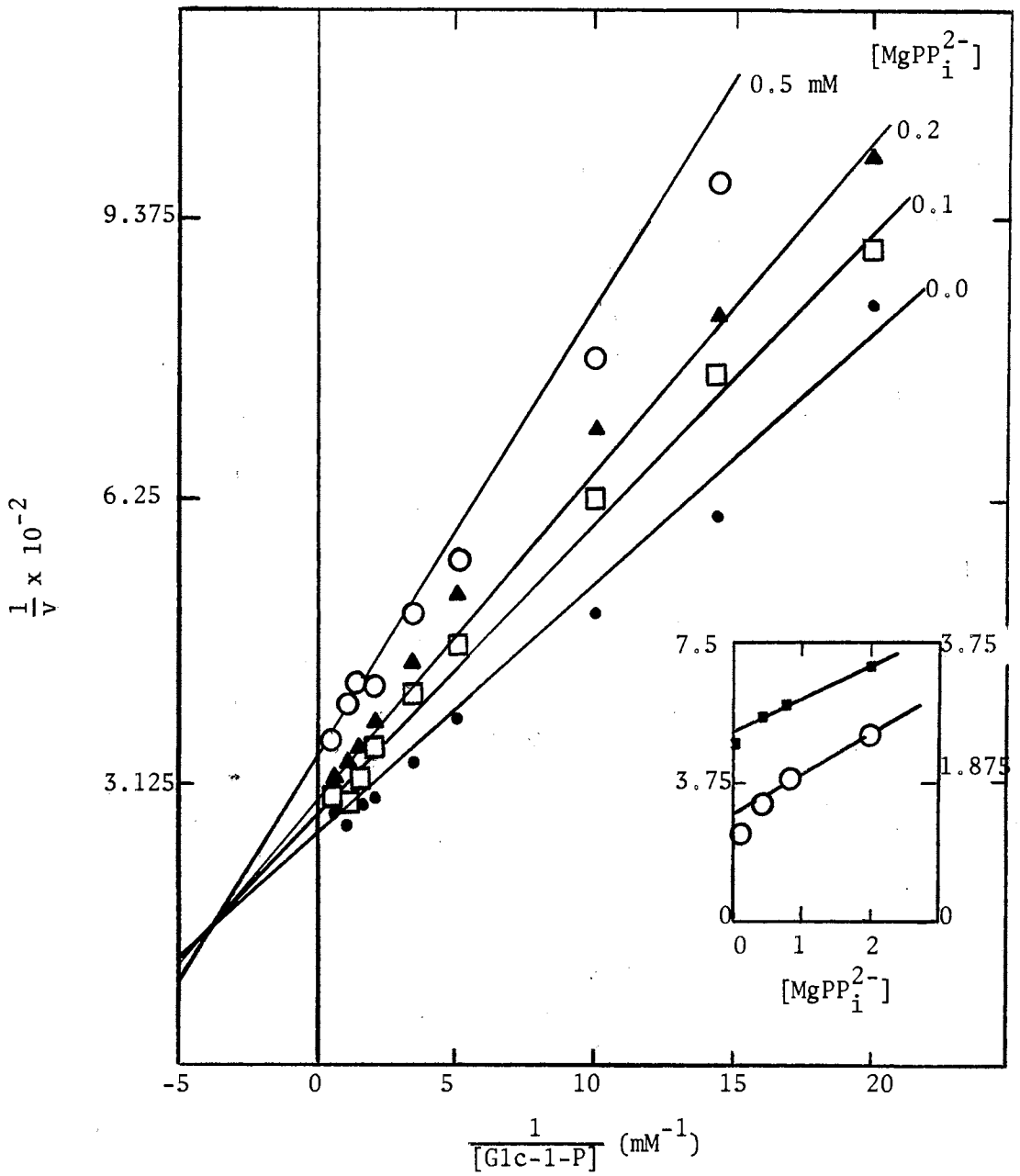


Figure 20. Inhibition of UDP-Glucose Synthesis by $MgPP_i^{2-}$ With Glc-1-P as the Variable Substrate

Reaction mixtures contained 2.2 µg enzyme, $MgPP_i^{2-}$ and Glc-1-P as indicated, free Mg^{2+} ion and $MgUTP^{2-}$ at 1 mM, and 100 mM triethanolamine at pH 8.0. v, initial rate of UDP-glucose formation in micromoles per ml per minute. Inset, replot of slopes and intercepts against $MgPP_i^{2-}$ concentration; ○, slope; ■, intercept.

TABLE XIX

APPARENT KINETIC CONSTANTS FOR PRODUCT INHIBITION OF UDP-GLUCOSE PYROPHOSPHORYLASE BY $MgPP_i^{2-}$

Apparent Kinetic Constant	Variable Substrate Glc-1-P	Variable Substrate $MgUTP^{2-}$
K	0.108 ± 0.008[1]	0.253 ± 0.017
K_{is}	0.633 ± 0.172	0.766 ± 0.193
K_{ii}	1.46 ± 0.24	1.44 ± 0.27

[1]Values for the kinetic constants with the standard errors.

Phosphoglucomutase is known to be sensitive to Mg^{2+} ion with 1 mM giving optimum activity (116).

Attempts to stop the reaction by heating for one minute in a boiling water bath, produced a turbid precipitate that could not be assayed by conventional spectrophotometric analysis. By heating various combinations of the reactants together it was found that $MgCl_2$ and inorganic pyrophosphate formed the precipitate. The precipitate was frequently difficult to observe visually. Although centrifugation of the sample before analysis would have eliminated the problem, the time required to centrifuge the large number of assays needed to produce an accurate kinetic plot did not favor this possibility.

Making the reaction mixture 0.1 M in HCl, denatured the enzyme and reduced the pH to approximately 1.4. The reaction mixture had to remain at this pH for two minutes or longer. If the pH was immediately readjusted to pH 8.0 by an equivalent amount of potassium hydroxide, the

reaction would proceed at an extremely slow but significant rate. The linearity of the coupling system to Glc-1-P is shown in Figure 21. The absorbance measured at 340 nm was stable over a period of one day or more. UDP-glucose can be hydrolyzed by base to UMP and Glc-1-P (8), and by acid to form UDP and glucose (117). Since triethanolamine at 0.1 M (the concentration in the assay) has strong buffering capacity at pH 8.0, it is unlikely that an overaddition of base would be great enough to make base hydrolysis a problem. On the other hand, acid hydrolysis would not produce interfering products. Nonetheless, the acid level needed to be maintained below the concentration that would produce hydrolysis of Glc-1-P. Controls showed that hydrolysis of either UDP-glucose or Glc-1-P was not a problem.

The linearity of the assay with time is shown in Figure 22. It can be seen that under the assay conditions linearity exists for approximately 0.1 change in absorbance at 340 nm. Since absorbance changes lower than this are probably not of sufficient accuracy, lower substrate concentrations are not practical. Figure 22 also indicates that the rate is proportional to enzyme concentration over at least a five fold concentration range.

An initial velocity plot using this assay is shown in Figure 23. Although the parallel lines suggest a ping-pong mechanism (83), this cannot be true because the kinetic analysis in the reverse direction indicated an ordered mechanism. Thus, either $K_{ia}K_b/B$ is very small relative to K_a, or K_{ia} is very small relative to the range over which A is being varied.

In both cases the lines do intersect but the data are not of sufficient accuracy to detect a change in slope. However, if either

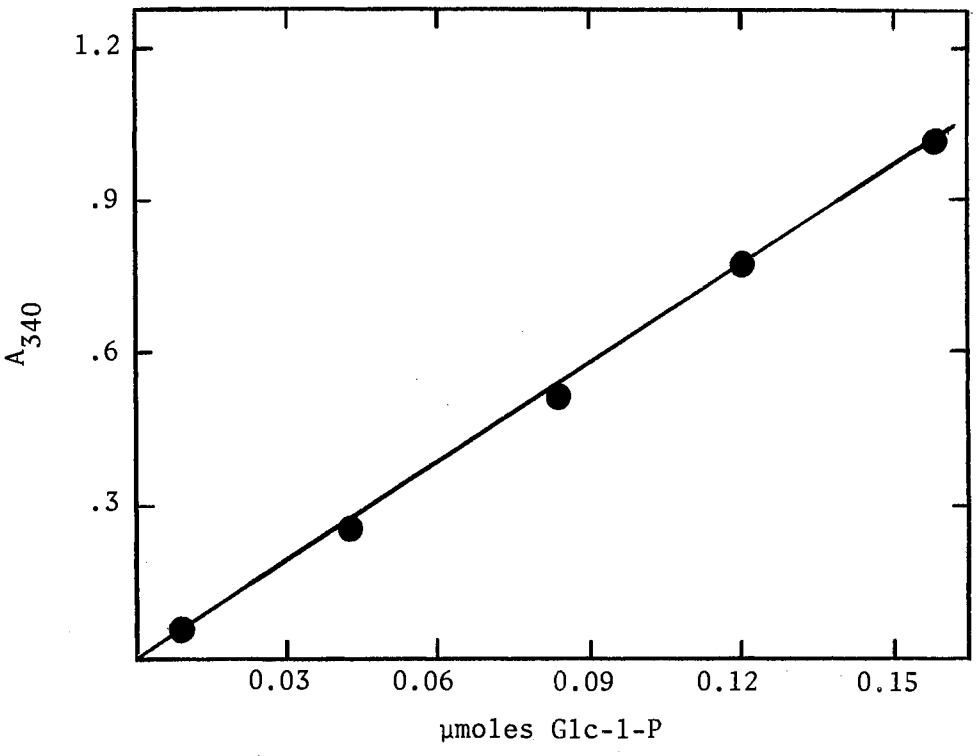


Figure 21. Linearity Between Glc-1-P Concentration and the Absorbance at 340 nm

Various concentrations of Glc-1-P were incubated with the coupling system and 100 mM triethanolamine at pH 8.0 for 30 minutes in a 1.1 ml reaction mixture at 30°C.

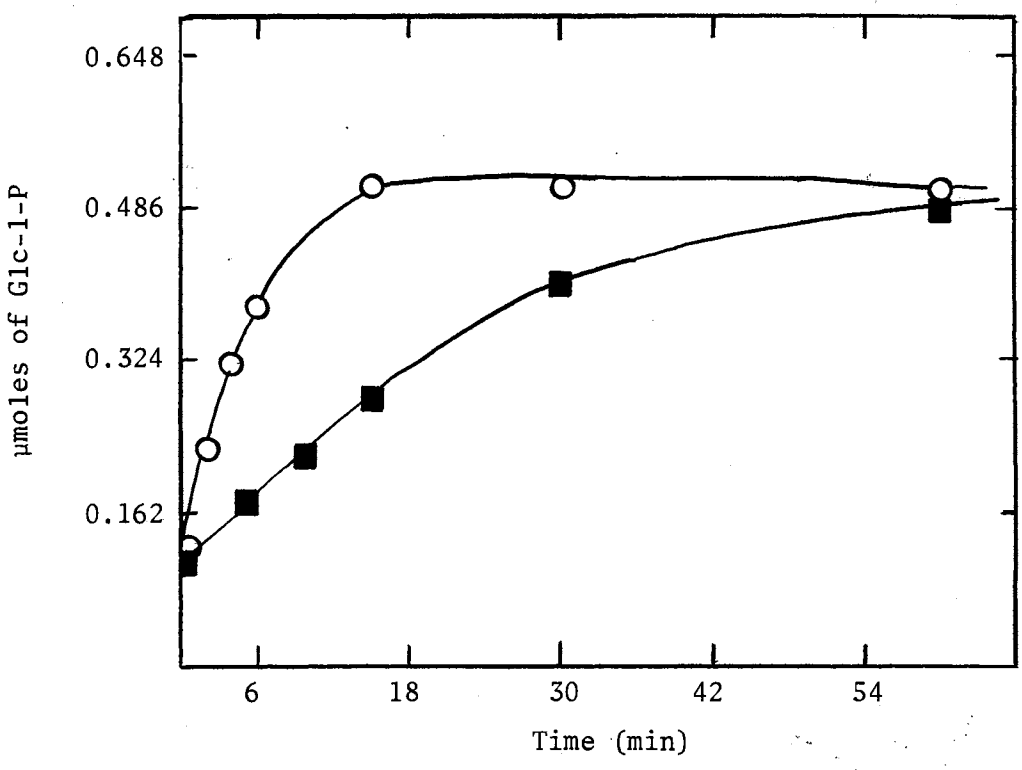


Figure 22. Linearity of the Rate of Glc-1-P Formation Using a Fixed Time Absorbance Assay at Two Different Enzyme Concentrations

Reaction mixtures contained enzyme, 0.2 mM $MgPP_i^{2-}$, 0.1 mM UDP-glucose, free Mg^{2+} ion at 1 mM, and 100 mM triethanolamine at pH 8.0. ■, 0.01 ml of a 1:10 dilution of enzyme solution (rate: 0.010 µmoles/min); ○, 0.05 ml of a 1:10 dilution of enzyme solution (rate: 0.050 µmoles/min).

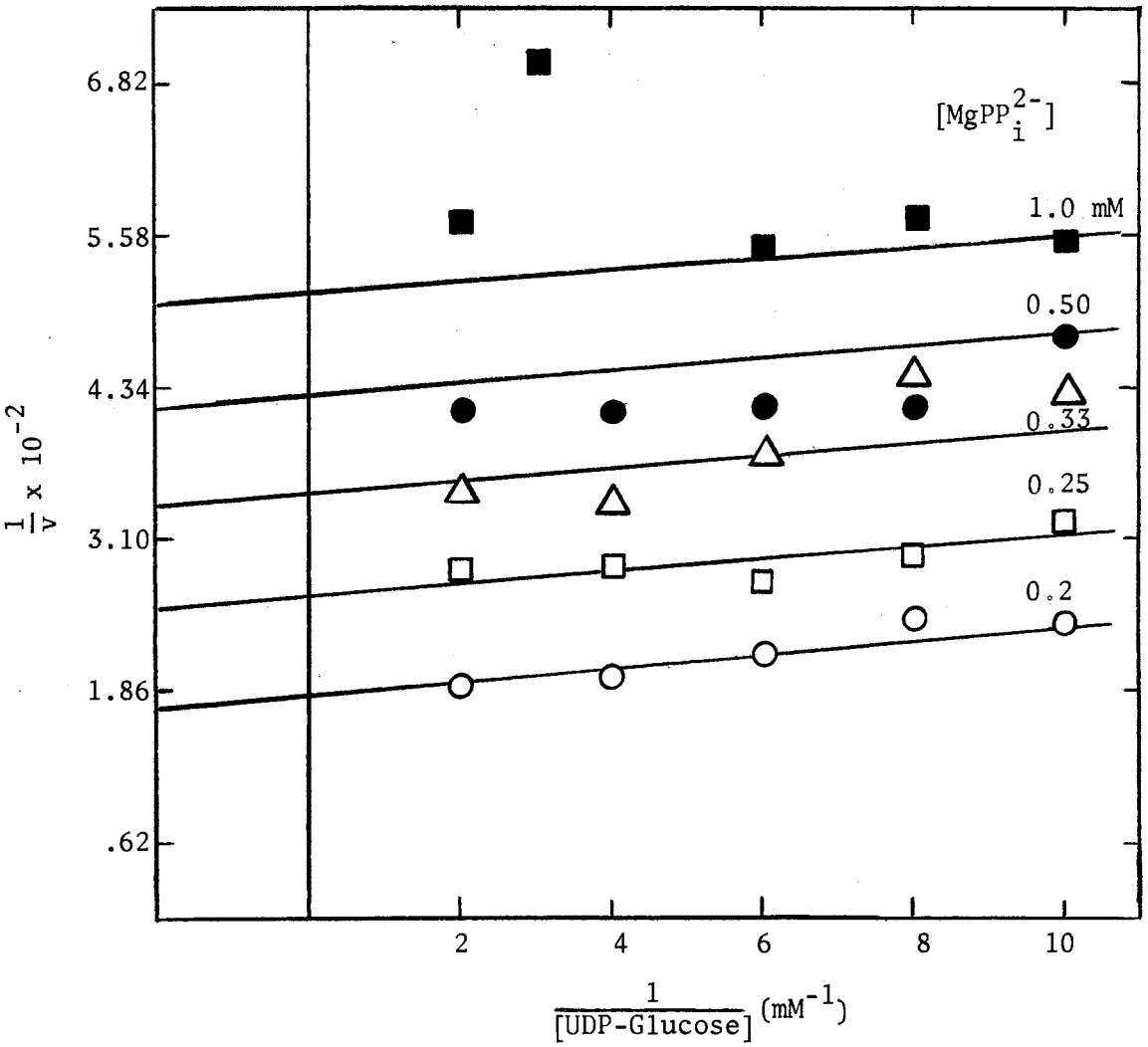


Figure 23. Double Reciprocal Plot With UDP-Glucose as Variable Substrate at Fixed Concentrations of $MgPP_i^{2-}$ Using a Fixed Time Absorbance Assay

Reaction mixtures contained 0.01 ml of a 1:10 dilution of enzyme, $MgPP_i^{2-}$ and UDP-glucose as indicated, free Mg^{2+} ion at 1 mM, and 100 mM triethanolamine at pH 8.0. v, initial rate in micromoles per ml per minute.

substrate concentration were lowered, the lines should become nonparallel. A statistical analysis using Equation 1 indicates that the data are not of sufficient accuracy to yield reliable constants (Table XX).

Three means of sufficiently increasing the sensitivity of the measurement of product are to use a radioactive substrate, to use absorption spectroscopy with a 3 cm or longer light path, or to use fluorometric measurements of the reduced pyridine nucleotide as described by Lowry et al. (49). If a cuvette with a longer light path were used, the reaction mixture would need to be increased and hence the cost would increase. A radioactive assay would mean finding a suitable radioactive substrate and testing out methods of separating the radioactive product from substrate. Fluorometric analysis appeared the best choice since the measurements would yield the required sensitivity and proper instrumentation was available.

The proportionality between Glc-1-P and fluorescence is shown in Figure 24. Because fluorescence is subject to quenching, this curve was run under nearly identical conditions to those in a regular assay. Triethanolamine, $MgCl_2$, UDP-glucose, and PP_i were added at the concentration to be used in an assay. Glc-1-P was then added at the desired concentration and an equivalent concentration of UTP added. For example, when 1 nmole of Glc-1-P was added to the reaction mixture, one nmole of UTP was added; when 5 nmoles Glc-1-P were added, 5 nmoles of UTP were added. Hydrochloric acid, 0.1 ml of 1 N, was then added, followed immediately by enzyme. After 2.5 minutes, 0.5 ml of 2 N potassium hydroxide was added and then the coupling system. Thus the solutions for the standard curve of Glc-1-P nearly mimicked the solutions used in a normal assay. As Figure 24 shows, a linear relationship was

TABLE XX

KINETIC CONSTANTS FOR PYROPHOSPHOROLYSIS FROM INITIAL VELOCITY STUDIES

Assay	$K_{UDP\text{-}glucose}$ (μM)	$K_{iUDP\text{-}glucose}$ (μM)	$K_{MgPP_i^{2-}}$ (μM)	$K_{iMgPP_i^{2-}}$ (μM)
BOVINE MAMMARY				
Absorbance UDP-glucose (0.1 to 0.5 mM) $MgPP_i^{2-}$ (0.2 to 1.0 mM)	81.9±37.7[1]	-8 ±16	893 ±220	-93 ±154
Fluorescence UDP-glucose (4.08 to 20.4 μM) $MgPP_i^{2-}$ (0.216 to 1.08 mM)	21.3± 5.6	6.7± 2.6	608 ±185	191 ± 64.7
BOVINE LIVER				
Fluorescence UDP-glucose (4.08 to 20.4 μM)	27.9± 4.1	-1.7± 1.4	298 ± 67	-18.3± 16.2
Fluorescence UDP-glucose (20.0 to 420 μM) $MgPP_i^{2-}$ (20.0 to 420 μM)	9.4± 3.5	18.8±10.7	24.2± 5.5	48.4± 32.6

[1]Values for the kinetic constants with the standard errors.

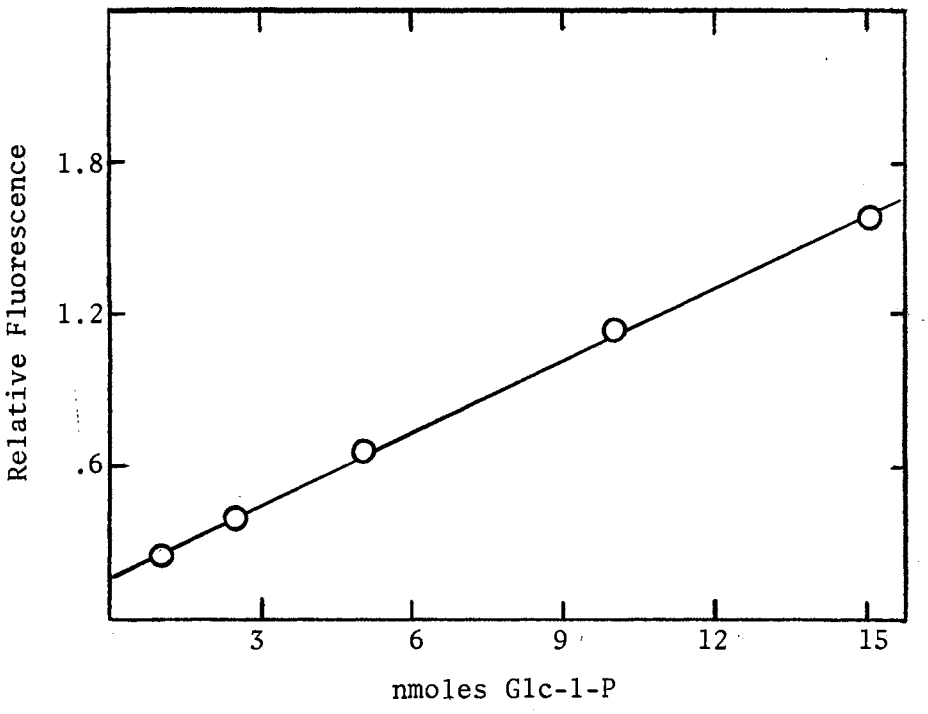


Figure 24. Linearity Between Glc-1-P and the Intensity of Fluorescence

Reaction mixtures (1.1 ml) contained Glc-1-P at the concentrations indicated, UTP at a concentration equal to Glc-1-P, free Mg^{2+} ion at 1 mM, 0.25 mM $MgPP_i^{2-}$, 0.06 mM UDP-glucose, coupling system, and 100 mM triethanolamine, pH 8.0. Incubations were for 30 minutes at 30°C.

obtained. Quenching occurred only above 20 μM, the concentration at which NADPH has been shown to quench (118).

Different preparations of UDP-glucose and $NADP^+$ showed differing amounts of background fluorescence. UDP-glucose frequently would vary in its fluorescence only in the presence of coupling enzymes. A few preparations gave insignificant background fluorescence.

Several initial velocity plots were performed using nine points (three lines with three points per line) to determine the correct concentration range. All the plots intersected near the abscissa. A large experiment was then run as shown in Figures 25 and 26 which illustrates many of the problems encountered. Figure 25 shows the time course of several assays at a fixed $MgPP_i^{2-}$ concentration and at five different levels of UDP-glucose. Other levels of $MgPP_i^{2-}$ gave similar results. All of the experimental blank values were the same. The change in velocity with time was linear but the lines do not extrapolate to the experimental blank value. Previous experiments had shown both this behavior and also the case where the lines do extrapolate to the experimental blank value. In those cases in which the experimental blank and extrapolated blank were different, it was assumed that a fluorescent contaminant was present. However, in this experiment the lines can be extrapolated to a common intersection point which is the experimentally determined blank, but the intersection occurs at negative time. The only explanation that appears to fit this data is that one enzymatic form is being converted to a slower enzymatic form within the first minute of incubation.

A time course experiment at the same enzyme concentration and at one-half the enzyme concentration is shown in Figure 27. Two distinct

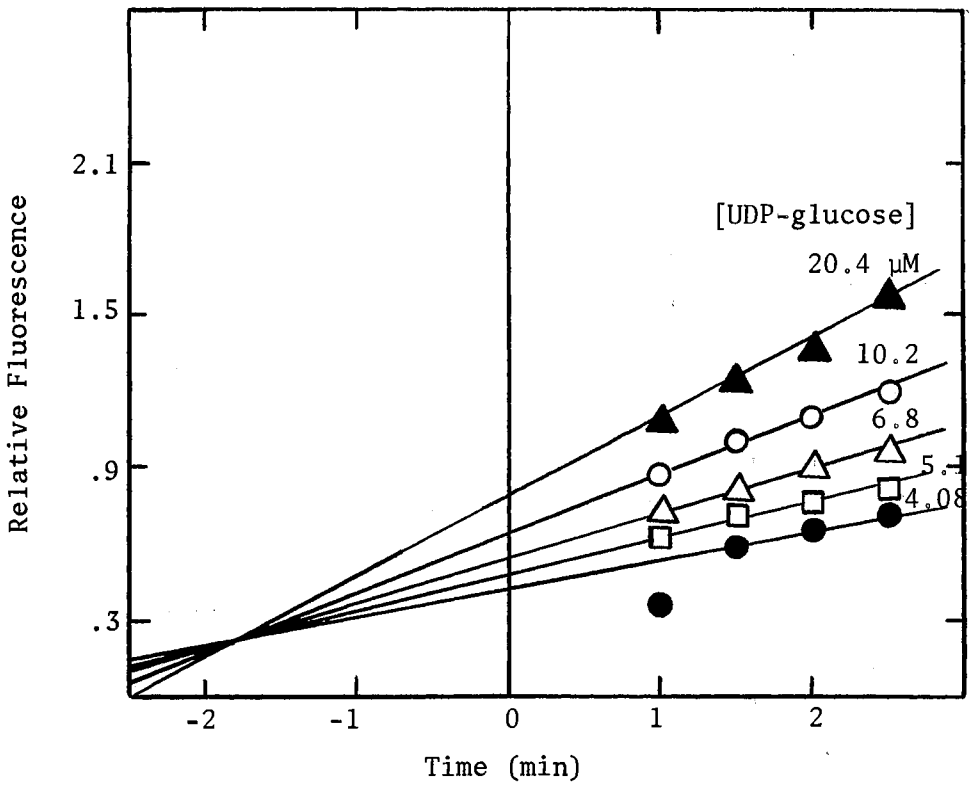


Figure 25. Assays of Pyrophosphorolysis at a Fixed $MgPP_i^{2-}$ Concentration and Variable UDP-glucose Concentrations

Reaction mixtures contained 0.002 ml of 1:10 dilution of enzyme, 0.36 mM $MgPP_i^{2-}$, Mg^{2+} at 1 mM, 100 mM Triethanolamine, pH 8.0, and UDP-glucose at 4.08 (●), 5.1 (□), 6.8 (△), 10.2 (○), or 20 µM (▲).

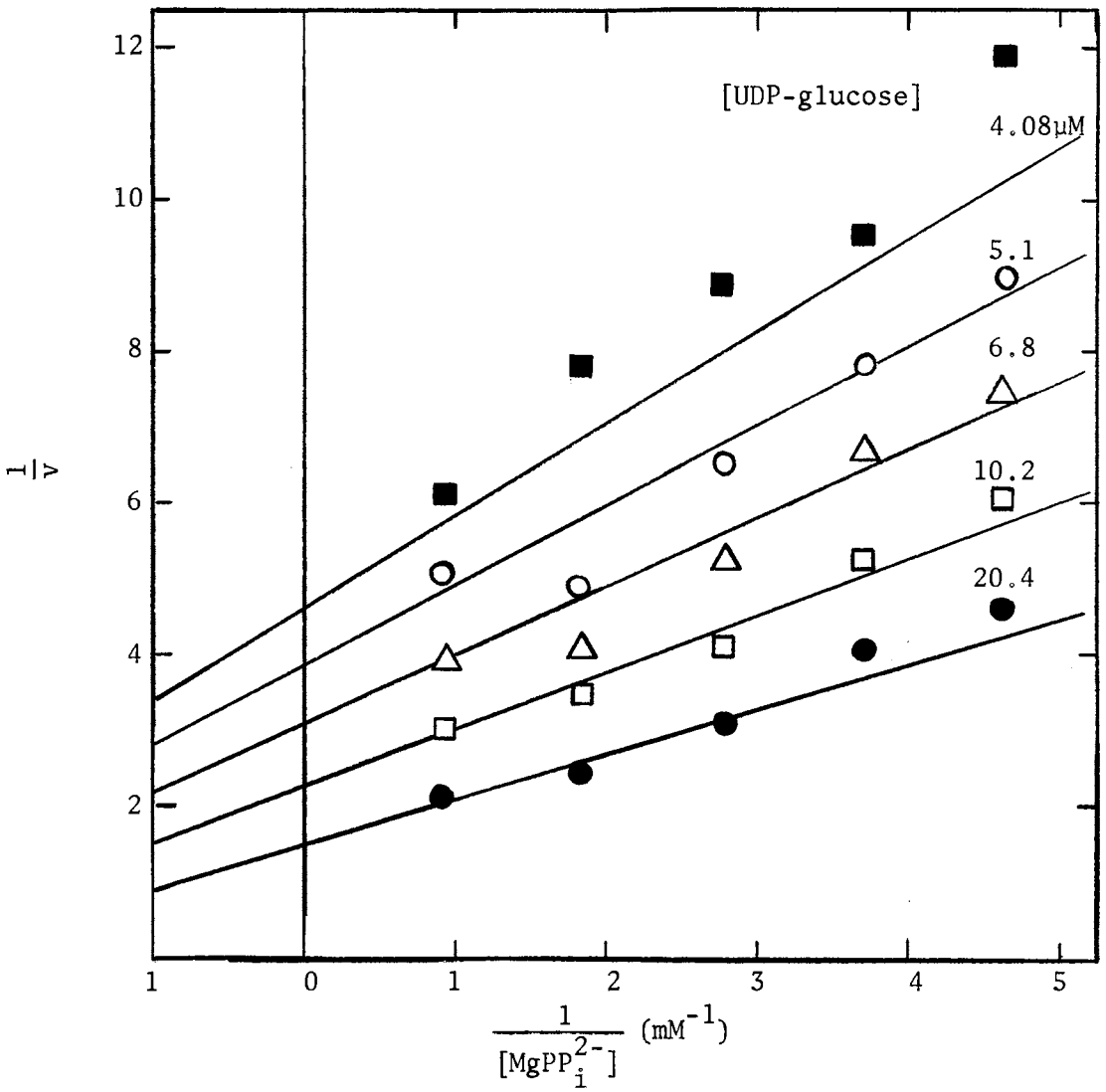


Figure 26. Double Reciprocal Plot With $MgPP_i^{2-}$ as Variable Substrate at Fixed Concentrations of UDP-Glucose Using a Fluorescence Assay

Reaction mixtures contained 0.002 ml of a 1:10 dilution of enzyme, $MgPP_i^{2-}$ and UDP-glucose as indicated, Mg^{2+} ion at 1 mM, and 100 mM triethanolamine at pH 8.0. v, initial rate in change in relative fluorescence per minute.

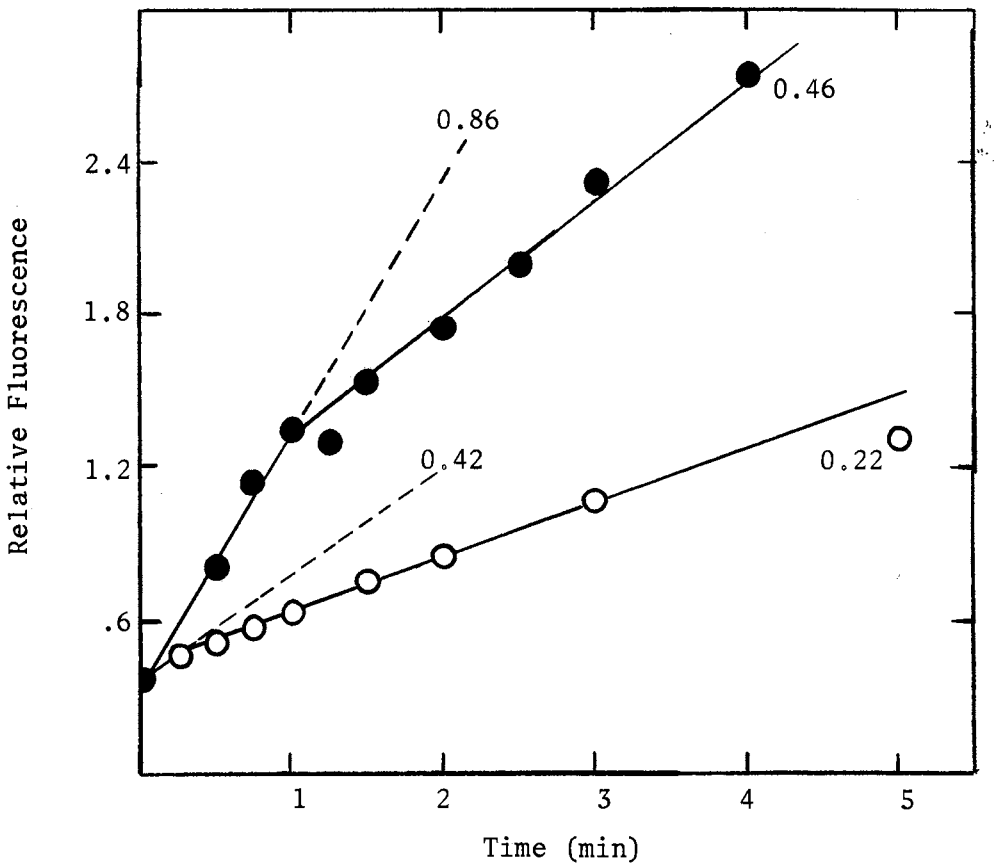


Figure 27. Biphasic Time Course of the Fluorescence Assay at Two Enzyme Concentrations

Reaction mixtures contained 0.36 mM $MgPP_i$, 20.4 μM UDP-glucose, Mg^{2+} ion at 1 mM, 100 mM triethanolamine at pH 8.0, and enzyme. ○, .001 ml of 1:10 dilution of enzyme; ●, 0.002 ml of 1:10 dilution of enzyme. Rates are indicated on the figure and are expressed as the change in fluorescence per minute.

slopes with a sharp breaking point can be seen at the original enzyme concentration in support of the two enzyme-form supposition. The concentration of NADPH was within the linear portion of the fluorescence calibration curve so that quenching should not have occurred. Controls with the complete assay system minus UDP-glucose and $MgPP_i^{2-}$ were equal to the experimental blank. Thus contaminating enzymes utilizing either substrate separately may be ruled out. The time course at one-half the original enzyme concentration can be interpreted in a similar manner but the data are not sufficient. The concentration of both forms of the enzyme appears to be proportional to velocity. Therefore, it seems justifiable to treat the rates obtained by extrapolation to a negative time in Figure 25 as if they were initial velocity rates. An initial velocity plot using this data is shown in Figure 26. The fit is reasonable but a large amount of error is present as indicated by the large standard errors in Table XX. In addition, the error is not random. Attempts to further study the nature of the biphasic time course could not be done because this property was lost. Figure 28 shows that the enzyme may be unstable at high dilution and that PP_i may be required for optimum stability as shown by Newell and Sussman (33) in the slime mold D. discoideum. In the dilution experiment the total amount of enzyme present in each assay was the same, but the enzyme had been diluted to different concentrations and incubated for five minutes at 30°C prior to addition to the assay mixture. Although the rates varied at different dilutions of the stock solution, all the rates extrapolated to the experimentally determined blank.

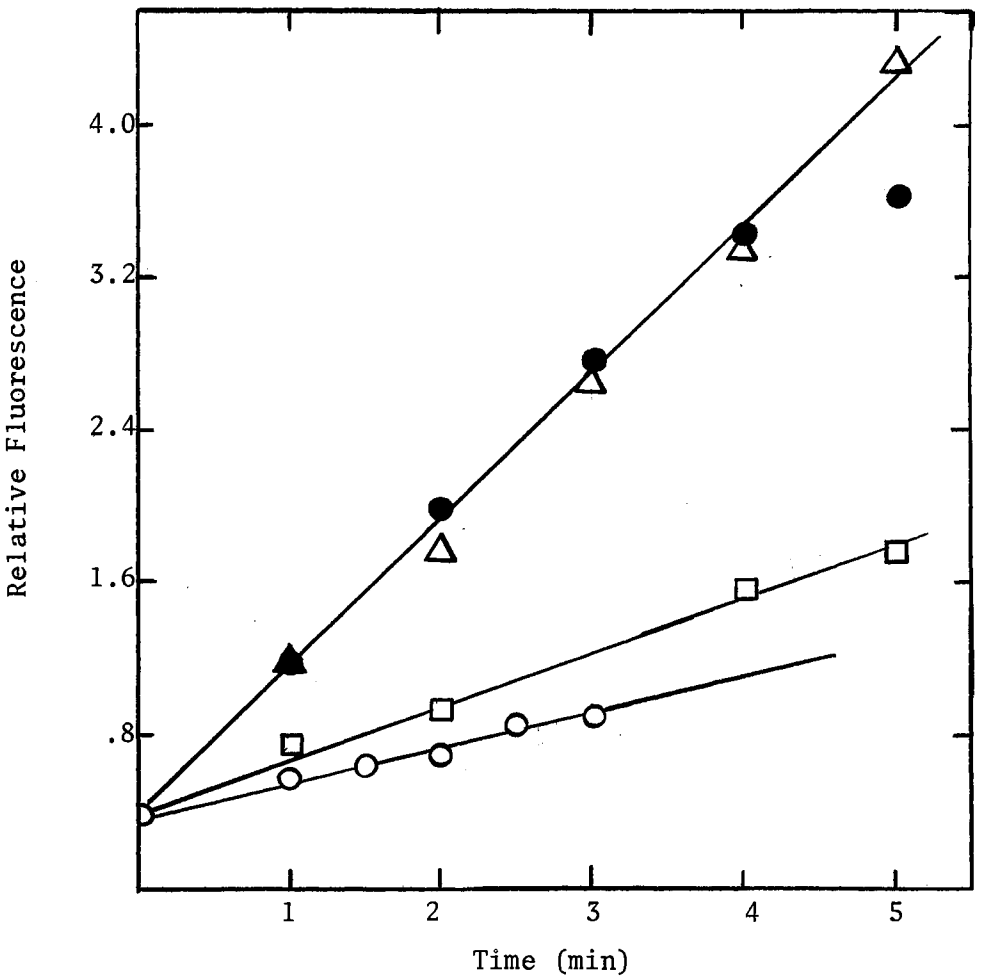


Figure 28. Dilution Stability of UDP-Glucose Pyrophosphorylase

Reaction mixtures contained 0.36 mM $MgPP_i^{2-}$, 20.4 μM UDP-glucose, Mg^{2+} ion at 1 mM, 100 mM triethanolamine at pH 8.0, and enzyme at 0.002 ml of a 1:10 dilution or the equivalent. 0, 0.002 ml of 1:10 dilution of enzyme added to the incubation mixture and preincubated at 30°C; reaction started by addition of $MgPP_i^{2-}$. The remaining assays were started with the addition of enzyme, but the enzyme was added as 0.20 ml of a 1:1000 dilution (□), 0.02 ml of a 1:100 dilution (△), or 0.002 ml of a 1:10 dilution (●). Preincubations were for 5 minutes.

Kinetic Analysis of Bovine Liver UDP-Glucose Pyrophosphorylase

Because kinetic analysis of the enzyme from bovine mammary tissue did not appear simple and to conserve the purified enzyme for later use, attention was directed toward the enzyme from bovine liver.

No reproducible loss in activity was found when various dilutions of enzyme were assayed. To test the stability of the products, six assays were performed simultaneously, but the coupling system was added at one hour intervals. The range in variation of the rates determined for the six assays with five points per assay was plus or minus 2.6% of the mean. In the same experiment the stability of the fluorescence of the assay mixture was determined. The maximum variation was $\pm$ 0.7% of the mean after one hour, $\pm$ 2.0% after two hours, $\pm$ 2.3% after three hours, $\pm$ 3.3% after four hours, and $\pm$ 5% after five hours. Thus, both the Glc-1-P after enzyme inactivation and the fluorescence of NADPH were stable. Certain experiments did give considerable error which was considered to be due to contaminants and variation in the addition of a "constant" amount of enzyme. The fluorescence slowly decreased with time which resulted in the wider range of variation after five hours. This is in accord with the finding of Lowry et al. (49) that the fluorescence of reduced pyridine nucleotides decreases slightly with time in the presence of Mg^{2+} ion. Attempts to further stabilize the fluorescence by adding EDTA to the reaction mixtures after the coupling system had reached completion did not have any noticeable effect.

As with the mammary gland enzyme, the increase in fluorescence is proportional to time. The very good proportionality between rate and enzyme concentration is shown in Figure 29. Preliminary experiments to determine the proper concentration range for an initial velocity plot

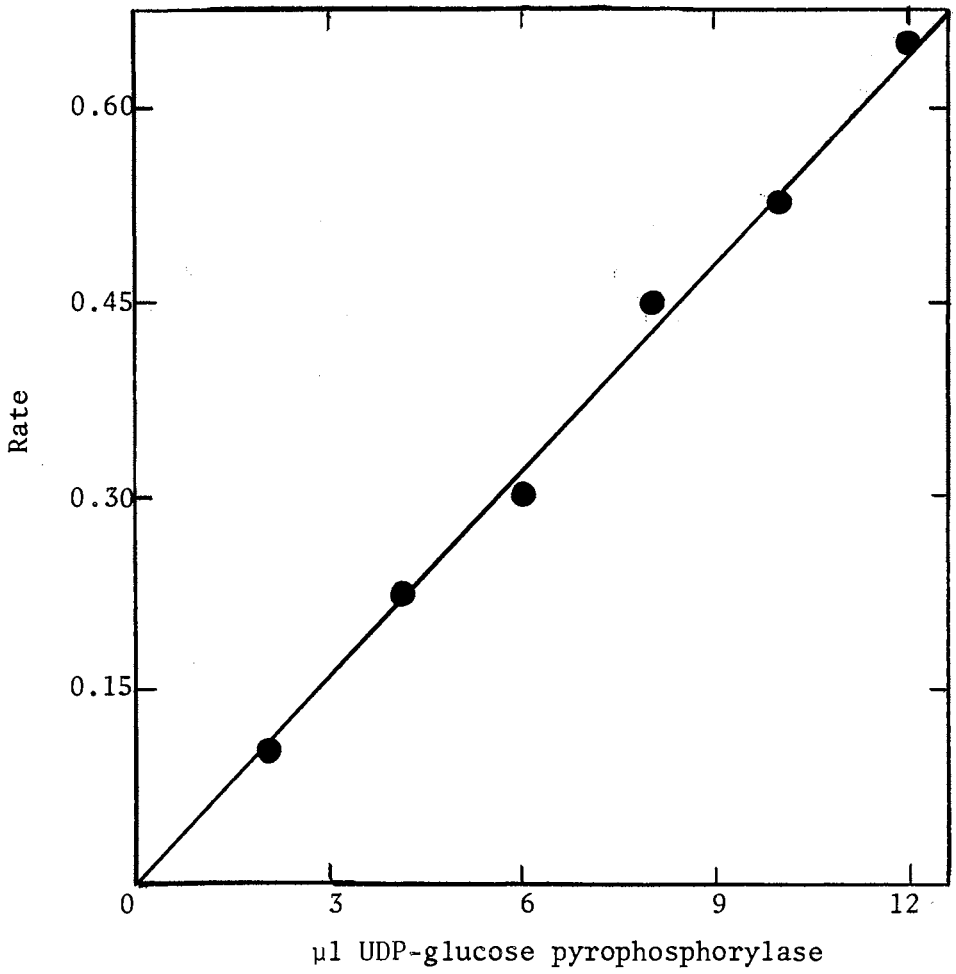


Figure 29. Proportionality Between Rate and Bovine Liver UDP-Glucose Pyrophosphorylase Using a Fluorescence Assay

Reaction mixtures contained 0.25 mM $MgPP_i^{2-}$, 60 µM UDP-glucose, Mg^{2+} ion at 1 mM, 100 mM triethanolamine at pH 8.0 and bovine liver UDP-glucose pyrophosphorylase as indicated at a 1:100 dilution. Rate is expressed as the change in relative fluorescence per minute.

indicated an intersecting pattern. An experiment, performed at about the K_m values obtained from the initial velocity plots for the bovine mammary tissue, showed that the substrate concentrations were much too high. The statistical data are presented in Table XX.

Figures 30 and 31 show that a good line on a double reciprocal plot can be obtained when either substrate is fixed and the other varied. However, an initial velocity plot run in the appropriate concentration range yielded very poor results with large standard deviations as shown in Table XX. In this experiment the experimental blanks varied greatly while the extrapolated blanks appeared to give the same values for the same levels of PP_i but varied with UDP-glucose. Thus an interaction or impurity in the reagents was suspected. The concentration of the stock solution of UDP-glucose was determined to be 10.8 mM by its absorbance at 262 nm but only 8.0 mM by enzymatic conversion. Therefore this batch of UDP-glucose was probably only 80 to 90% pure.

A large amount of variation was frequently found in the fluorescence assay with enzyme from either bovine mammary gland or liver. This could usually be attributed to two sources of error. The first was variation in the pipeting of the enzyme. It was found that a Hamilton PB 600-1 repeating dispenser, microliter syringe unit, was the most accurate and reliable way of enzyme addition. However, this type of syringe was only available for a few experiments and in its absence a Hamilton microliter syringe with a stop was used. The error here probably occurred when the syringe was refilled and wiped carefully after each addition to an assay mixture to remove traces of solution from the needle tip. The tip of the needle should be cut at a right angle as opposed to a beveled tip for ease in wiping.

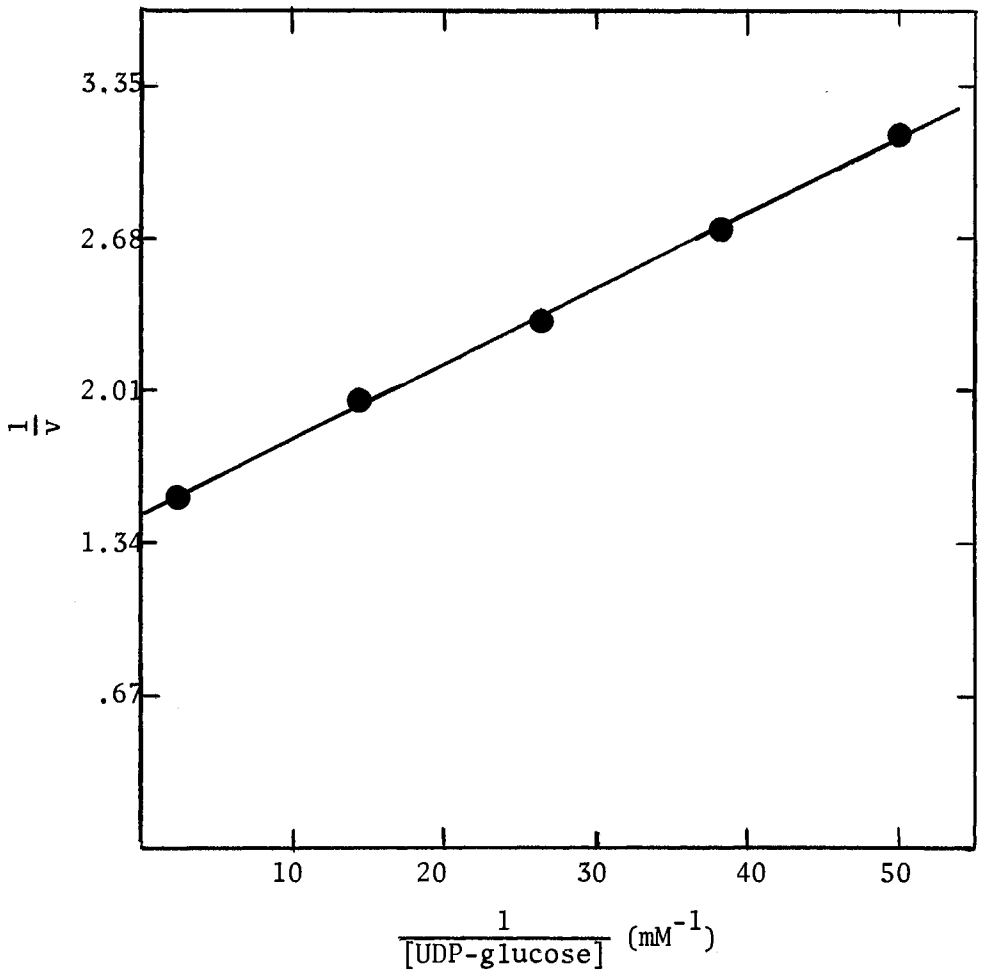


Figure 30. Lineweaver-Burk Plot for Bovine Liver UDP-glucose Pyrophosphorylase With UDP-Glucose as the Variable Substrate

Reaction mixtures contained 0.002 ml of a 1:700 dilution of bovine liver UDP-glucose pyrophosphorylase, 38.2 μM $MgPP_i^{2-}$, UDP-glucose as indicated, Mg^{2+} ion at 1 mM, and 100 mM triethanolamine at pH 8.0. v is expressed as the change in relative fluorescence per minute.

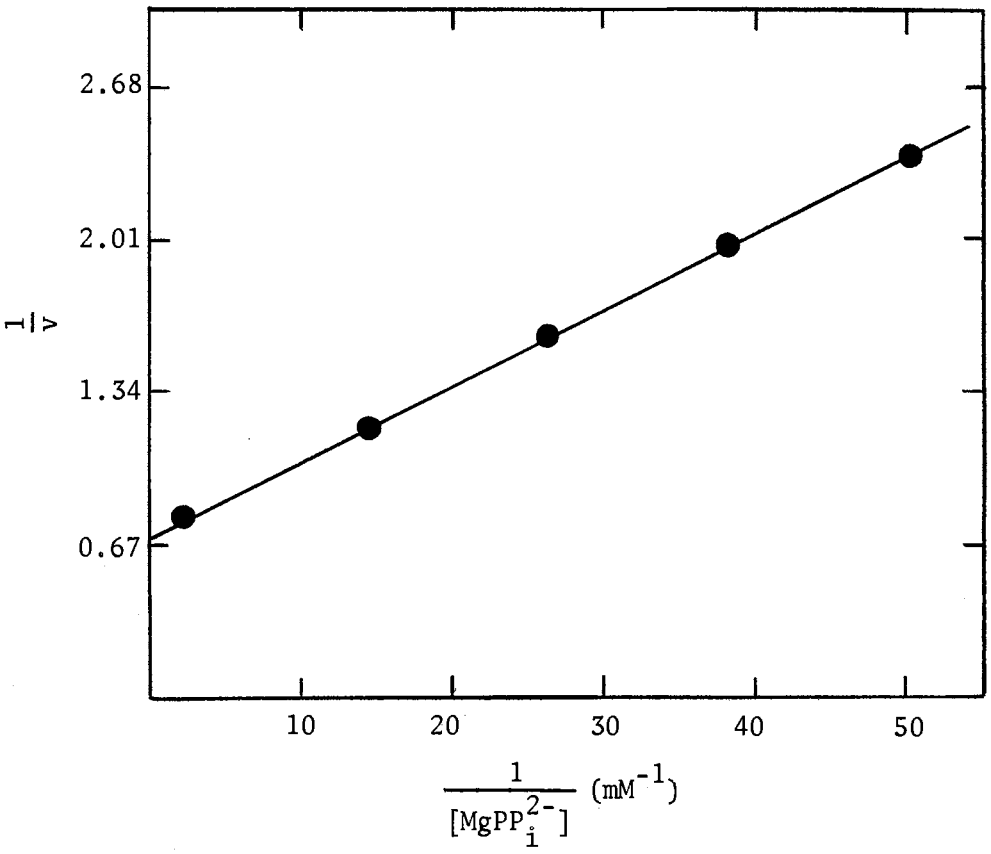


Figure 31. Lineweaver-Burk Plot of Bovine Liver UDP-Glucose Pyrophosphorylase With $MgPP_i^{2-}$ as the Variable Substrate

Reaction mixtures contained 0.002 ml of a 1:700 dilution of bovine liver UDP-glucose pyrophosphorylase, $MgPP_i^{2-}$ as indicated, 60 μM UDP-glucose, Mg^{2+} ion at 1 mM, and 100 mM triethanolamine at pH 8.0. v is expressed as the change in relative fluorescence per minute.

The second major source of error was the sensitivity of the coupling system to metals. Phosphoglucomutase is extremely sensitive to heavy metals and its efficiency could be blocked by using a Cornwall automatic syringe or by other unidentified sources. The inhibition by heavy metals could be alleviated by adding EDTA to a final concentration of 10^{-5} M, before or during addition of the coupling system.

To protect the enzyme against possible surface denaturation, 0.2% bovine serum albumin was included in the assay mixture. The bovine serum albumin protected the enzyme against acid denaturation and the acid concentration needed to be doubled before the reaction was stopped. At this concentration of acid some Glc-1-P must also have been hydrolyzed since the amount of fluorescence fell to 70% of the amount obtained when no bovine serum albumin was added.

To use this assay in further experiments in which both substrates are varied will require the determination of the purity of the substrates and the possible interactions between substrates, $NADP^+$, NADPH, and coupling enzymes which may change the fluorescence of the solution. Until these facts are known, reliable constants probably cannot be obtained. As discussed previously, the kinetics of the reaction in the direction of pyrophosphorolysis could possibly be determined by absorbance methods in a 3 cm or longer light path or by a radioactive assay. The sensitivity and the excellent proportionality between fluorescence and enzyme concentration suggest this assay can be used for determining very small quantities of the enzyme.

CHAPTER VI

DISCUSSION

UDP-glucose pyrophosphorylase was purified from an acetone powder of bovine mammary tissue by ammonium sulfate fractionation, heat treatment, DEAE-cellulose chromatography and Sephadex G-200 chromatography. The enzymatic preparation represented a 500 fold purification over the crude extract and had a specific activity of approximately 16 I.U./1.0 A_{280}. Assayed in the reverse direction, i.e., the pyrophosphorolysis reaction, the specific activity would be approximately 50 I.U./1.0 A_{280}. If the bovine mammary gland enzyme has the same specific activity as the crystalline bovine liver enzyme, i.e., 240 I.U./mg of protein, the enzyme from mammary gland is at least 20% pure. The purity of enzymatic protein may be much higher depending upon the similarity of liver and mammary gland specific activities, the method of protein estimation, and the amount of denatured enzymatic protein. Calculations, based on the specific activity for bovine liver and the activity of the crude extract from mammary gland acetone powder, indicate that the crude extract from 50 grams of acetone powder contains less than 8.5 mg of enzymatic protein. A very high yield of 10% during a classical purification procedure would yield a maximum of 850 µg of enzymatic protein. Thus, the principle obstacle in the purification of UDP-glucose pyrophosphorylase from bovine mammary tissue is the lack of sufficient enzymatic protein. Three methods could possibly be used to increase the amount

of enzymatic protein. First, more enzymatic protein could be used initially by simply increasing the quantity of acetone powder or by carefully selecting a healthy lactating animal at the appropriate stage of lactation. The first alternative would require a very large scale up and is severely limited by centrifuging capacity of the currently available equipment.

The second method assumes that one or more forms of UDP-glucose pyrophosphorylase is labile in Tris-HCl buffer. Newell and Sussman (33) found that this was the case in the slime mold, D. discoideum. The labile form was stabilized by substituting Tricine for Tris-HCl. A similar procedure could be tried for the extracts from bovine mammary gland.

The third method is to use a trapping system to concentrate the enzymatic protein that is present initially. To be most effective, this type of procedure should be performed at the earliest possible step. The most promising method appears to be affinity chromatography in which a ligand that binds to UDP-glucose pyrophosphorylase is attached to a solid support. Cuatrecasas (119) has developed several procedures for the attachment of ligands to agarose beads or Bio-Gel P-300 when the ligand contains an amino, carboxyl, phenolic, or imidazole group. In addition the distance of the ligand from the solid support can be varied by varying the length of the hydrocarbon chain connecting the ligand to the support. This is very important for a large molecule such as UDP-glucose pyrophosphorylase which may require a sterically unhindered form of the ligand for binding. Agarose gels would appear to be best as a solid support because they are more porous gels and would give the UDP-glucose pyrophosphorylase molecule freer access to

the gel derivative than Bio-Gel P-300 which only slightly retards the enzyme (Figure 9). A drawback to agarose gels is the apparent binding which occurs when UDP-glucose pyrophosphorylase is passed through a Bio-Gel A-5m column. This may not be a problem if Sepharose is used which contains agarose and a dextran polymer rather than agarose and polyacrylamide. As discussed in Chapter III, Bio-Gel A-5m may possibly be used as an affinity column without covalently binding ligands. It may also be possible to chemically modify the galactosyl moieties of agarose directly; and in this manner provide a binding ligand, assuming steric hinderance is not a problem.

Since glucose-1-P, glucosamine-1-P, and galactose-1-P bind to the enzyme while glucose and galactose do not, it appears that phosphate in the C-1 position of the sugar is essential. TDP-glucose is a good competitive inhibitor of the E. coli enzyme (14), and hence either TDP-glucose or TTP might bind tightly to the enzyme. Therefore, a support containing Glc-1-P, TDP-glucose, TTP, or an appropriate derivative of these ligands may effectively adsorb UDP-glucose pyrophosphorylase from a mixture of proteins. Based on an ordered mechanism with Glc-1-P as the second substrate to react, an agarose column derivatized by attaching Glc-1-P while leaving the free phosphate group in the C-1 position, would be expected to bind enzyme in the presence of $MgCl_2$ and UTP. Removal of $MgCl_2$ and UTP would then elute the enzyme. A column containing TDP-glucose or TTP, on the other hand would be expected to retard the enzyme without addition of substrates. Although Mg^{2+} ion might be required for TTP, Gillett et al. (86) have reported that UTP will bind strongly to bovine liver UDP-glucose pyrophosphorylase in the absence of metal.

Hydroxylapatite columns at pH 8.0, 8.5, 9.0 and 9.6 bound the enzyme but did not give significant purification. Because each substrate of the UDP-glucose pyrophosphorylase reaction contains at least one phosphate group, binding of the enzyme would be expected with the calcium phosphate polymer acting as a substrate analog. Binding by carboxyl groups not necessarily located at the active site may also be important (120).

The observation that the enzyme can be stable over a wide range of temperature and pH would give great latitude in choosing elution conditions for an affinity column. In addition, pH and CM-cellulose chromatography could possibly be used to advantage. The observed loss in enzymatic activity in crude fractions at slightly acid pH could be due to proteolysis. In this case, a proteolytic inhibitor such as phenylmethyl sulfonyl fluoride could be added to the solution.

Disc gel electrophoresis at pH 9.8 of samples from Steps I through VII shows that a slower moving, inactive component is always present. This component at Step VII is the major protein band and after a second DEAE-cellulose chromatography step at pH 10.0 is the only band observed. Molecular weight determinations of this component by sedimentation velocity centrifugation, Bio-Gel P-300 chromatography and Sephadex G-200 chromatography give estimated values of 150,000, 200,000, and 210,000, respectively, The component was removed by sucrose density gradient centrifugation and gel filtration. The component could be an inactive form of the native enzyme. If this were the case the native enzyme would have to be irreversibly cleaved since no activity was ever observed in the 200,000 molecular weight region after sucrose density gradient centrifugation or gel filtration. The component could be

formed during the purification; or it could be the end product of a Tris labile form of UDP-glucose pyrophosphorylase, arising during extraction of the protein from the acetone powder.

Disc gel electrophoresis was not used to analyse the purity of the preparation because only a few µg of the highest purity material was isolated. In addition, denatured and active protein as well as polymeric species could give ambiguous results which would require experiments and milligram quantities of the material to give a good indication of purity.

Sucrose density gradient centrifugation and gel filtration indicate that the molecular weight of bovine mammary tissue UDP-glucose pyrophosphorylase is between 300,000 and 500,000. The best sucrose density gradient estimation was 500,000 which agrees very well with 480,000 (31) obtained for the bovine liver enzyme by ultracentrifugal analysis. The concentration of sucrose in the 5 to 20% gradients is sufficient to completely inhibit the activity of the enzyme (Table XVII). Although the mechanism of sucrose inhibition is not known, a large structural change probably does not occur since the estimated molecular weight is in good agreement with the bovine liver enzyme and is in accord with the gel filtration data. In fact it would be expected that the bovine liver and bovine mammary tissue enzymes are nearly identical. No evidence has been obtained during this study to indicate that they are not.

The UDP-glucose pyrophosphorylase from bovine mammary tissue and from liver is stable at 50°C for at least one hour in 50% ammonium sulfate at pH 8.0. The mammary tissue enzyme cannot be stored at -10°C or at 22°C for more than two days without loss of activity. The enzyme is stable from pH 5.0 to 10.8 after purification through Step VII.

Sulfhydryl reagents, mercaptoethanol and dithiothreitol, have no effect on the enzyme. These facts suggest that the UDP-glucose pyrophosphorylase molecule is held in an active conformation by a variety of ionic and hydrophobic bonds without a single, critical bond confering catalytic activity.

Although there is no single experiment which conclusively indicates two forms of UDP-glucose pyrophosphorylase, all of the evidence together suggests that there may be. The first indication of two forms of UDP-glucose pyrophosphorylase is the multiple activity peaks (usually two) which were observed upon DEAE-cellulose column chromatography with linear gradient elution or upon hydroxylapatite column chromatography with elution by a single concentration of buffer. The number and concentration ratio of the multiple activity peaks varied with each preparation. In only a few cases was a single activity peak found.

The pH stability studies could be interpreted as resulting from two forms. Of particular interest is the rapid drop in activity, which occurred within two minutes at pH 3.0. While the activity initially dropped to 20% of the control value at pH 8.0, it required 86 days before the remaining activity decreased to a level below detection. Thus, a pH 3.0 labile form could contribute to about 80% of the activity and a pH 3.0 stable form to about 20%. A similar type of behavior was observed when the enzyme was acid killed during the fixed time assays. If the pH was dropped to 1.4 and then immediately readjusted to 8.0, a small but detectable amount of activity remained. On the other hand, if the pH was held at 1.4 for 2.5 minutes no activity was detected. pH 2.0 did not sufficiently inactivate the fixed time assay after 2.5 minutes.

Some data during the kinetic analysis suggest two catalytically different forms of the enzyme. The biphasic curves which occurred during the time course of the fixed time assay for bovine mammary tissue UDP-glucose pyrophosphorylase, indicated that one form is converted to a second in the presence of substrates, Mg^{2+} ion, and triethanolamine, pH 8.0, or that one form is labile in the presence of the assay medium. The transient nature of this phenomena could be explained by an irreversible conversion of one form to another or by coincidental technical difficulties over several experiments. Some evidence also suggested that the UDP-glucose pyrophosphorylase activity from bovine mammary tissue was unstable in very dilute solution.

In support of a single active form, only one activity band was observed upon disc gel electrophoresis. However, the sample was a pooled fraction of a single activity peak off DEAE-cellulose chromatography. A second peak with a relatively small amount of activity eluted from this column and may have shown a different activity band. Only a single symmetrical activity peak was observed upon sucrose density gradient centrifugation. A few gel filtration columns have had the peak tube drop in activity so as to produce two apparent activity peaks. However, this always involved only the one tube; and, although the activity elution profile was repeated twice, with different aliquots of the same sample (Figure 11C), the best interpretation is probably only a single activity peak.

If different forms do occur, they could be aggregated species, isozymes, or non-specific sugar nucleotide pyrophosphorylase contaminants. The evidence would indicate that the multiple forms of the enzymes would have very similar properties or are intercovertible.

Another possibility is that a protein in the crude homogenate has a high affinity for UDP-glucose pyrophosphorylase and remains associated with the enzyme throughout the course of purification. As discussed above there does appear to be a component which is always associated with the preparation through the DEAE-cellulose steps.

Kinetic analysis of the UDP-glucose pyrophosphorylase reaction in the direction of synthesis is consistent with the mechanism in Figure 1, namely, an ordered mechanism with $MgUTP^{2-}$ and UDP-glucose being the outside pair of reactants. Initial velocity patterns intersected above the abscissa and indicated a sequential mechanism. Product inhibition using $MgPP_i^{2-}$ yielded noncompetitive inhibition with respect to both $MgUTP^{2-}$ and Glc-1-P as the variable substrate. For a steady-state ordered mechanism this means that $MgPP_i^{2-}$ must be the first product to be released. However, the order of addition of $MgUTP^{2-}$ and Glc-1-P cannot be established. Since the lines on the primary and secondary plots were all linear, no dead-end complexes or sigmoidal kinetics were indicated. The variable substrate was usually varied over a 20-fold range. An ordered mechanism has also been proposed for adenylate pyrophosphorylase (adenosine monophosphate: pyrophosphate phosphoribosyltransferase, EC 2.4.2.7) and for nicotinamide mononucleotide pyrophosphorylase (nicotinate phosphoribosyl transferase, EC 2.4.2.11) (121,122). In both cases phosphoribosyl pyrophosphate has been suggested as the first substrate to add and PP_i the first product to be released.

Quantitatively the mechanism is different from the human erythrocyte enzyme. This is immediately obvious when comparing the double reciprocal plots of the initial velocities. The plots for the bovine mammary tissue enzyme intersect above the abscissa and the human

erythrocyte enzyme intersects below the abscissa, suggesting that $K_{ia} > K_a$ and $K_{ia} < K_a$, respectively. Comparison of the true Michaelis and inhibition constants for the human erythrocyte and bovine mammary tissue enzymes indicates that $K_{Glc\text{-}1\text{-}P} < K_{MgUTP^{2-}}$ in both cases. The kinetic constants for bovine mammary gland and bovine liver (31) enzymes appear to be the same.

The sensitivity of the bovine mammary tissue and human liver enzymes to sulfhydryl reagents also differs. The bovine mammary tissue enzyme was unaffected by dithiothreitol or mercaptoethanol while one of these reagents was required for stability of the human erythrocyte enzyme. On the basis of sulfhydryl sensitivity and kinetic analysis, it would appear that human erythrocyte, human liver, human brain, and mung bean enzymes are similar, and that the bovine mammary tissue and bovine liver enzymes are similar.

Analysis of the pyrophosphorolysis reaction of UDP-glucose pyrophosphorylase has proved very difficult. The kinetic constants are in the micromolar range, which requires a sensitive assay. For example, if 5 μM UDP-glucose is to be used in the analysis, the assay must detect products in the 0.1 to 0.5 μM range to insure that initial velocities are being measured. Attempts to use fluorescence to measure the product gave inconclusive results. The method was sensitive enough, but varied from day to day. One set of assay conditions gave the same rate within $\pm$ 5%. If either substrate was held constant and the other varied, a good double reciprocal plot was obtained. However, when both substrates were varied, the variation in the double reciprocal plots increased, and the extrapolated blank values for each assay varied; but the experimentally determined blank remained nearly the same. Whether the

variation was due to contamination of the reagents, interactions of reagent molecules, or variability in the enzyme was not conclusively shown. Instrumental variation was ruled out by using quinine sulfate as a secondary standard. As discussed above, some evidence for multiple forms of the enzyme was found. This is the simplest way to explain the variability of the fluorescence assay, pH stability properties, and the activation phenomenon in crude homogenates (74).

Qualitatively the sequential mechanism was confirmed. Plots with UDP-glucose varied in 10^{-5} M range and $MgPP_i^{2-}$ varied in the 10^{-5} to 10^{-4} M range gave an intersecting pattern. The standard errors were approximately 20% for the Michaelis constants and as high as 100% for the inhibition constants. This is four times as high as the errors normally associated with these constants (113). Although much of the variability is due to the assay, some may be due to the statistical analysis. During these analyses, the variance is assumed to be constant. However, this is not always true when the substrate is varied over a range greater than ten fold. In such instances the variance can be determined experimentally and the proper adjustments made in the computer programs (114).

The fluorescence assay was shown to be proportional to enzyme, and since it is very sensitive, it could be used for estimation of the amount of enzyme in very small or very dilute samples.

SUMMARY

UDP-glucose pyrophosphorylase was purified approximately 500 fold from an acetone powder of bovine mammary tissue. The specific activity of the preparation was 16 I.U. per mg of protein when assayed in the direction of UDP-glucose synthesis. The enzyme has a molecular weight between 400,000 and 500,000 as determined by sucrose density gradient centrifugation and gel filtration. The enzyme is stable from pH 5 to pH 10.8 for at least 3 months and can be heated at 50°C for at least two hours at pH 8.0 without loss in activity. The enzyme is not affected by either mercaptoethanol or dithiothreitol, but is inhibited by 0.2 M sucrose. Kinetic data, pH stability studies, and separations on hydroxylapatite, DEAE-cellulose, and Bio-Gel P-300 all suggested that multiple forms (usually two) could occur, but the data were not conclusive.

Initial velocity and product inhibition studies are consistent with a steady-state ordered mechanism. For this mechanism, product inhibition studies indicate that $MgPP_i^{2-}$ is the first product to be released. The true Michaelis constants were evaluated and found to be very similar to the apparent bovine liver constants. No unusual kinetic properties were observed.

A fluorescence assay was developed which will quantitatively estimate UDP-glucose pyrophosphorylase activity. However, the assay was unsuccessful in accurately determining the kinetic constants in the direction of pyrophosphorolysis.

REFERENCES

1. Caputto, R., Leloir, L. F., Cardini, C. E., and Paladini, A. C., J. Biol. Chem., 184, 833 (1950).

2. Leloir, L. F., and Cardini, C. E., J. Am. Chem. Soc., 79, 6340 (1957).

3. Ginsburg, V., Advances in Enzymology, 26, 35 (1964).

4. Shatton, J. B., Gruenstein, M., Shay, H., and Weinhouse, S., J. Biol. Chem., 240, 22 (1965).

5. Leloir, L. F. Proc. 3rd Intern. Congr. Biochem., Brussels, 1955, Academic Press, New York, 1956, p. 154.

6. Kalckar, H. M. Advances in Enzymology, 20, 111 (1958).

7. Tsuboi, K. K., Fukunaga, K., and Petricciani, J. C., J. Biol. Chem., 244, 1008 (1968).

8. Neufeld, E. F., and Hassid, W. Z., Advances in Carbohydrate Chemistry, 18, 309 (1963).

9. Neufeld, E. F., and Ginsburg, V., Ann. Rev. Biochem., 34, 297 (1965).

10. Kalckar, H.M., and Cutolo, E., 2nd Intern. Congr. Biochem., Paris, 1952, p. 260.

11. Munch-Petersen, A., Kalckar, H. M., Cutolo, E., and Smith, E. E. B., Nature, 172, 1036 (1953).

12. Munch-Petersen, A., Acta Chem. Scand., 9, 1523 (1955).

13. Trucco, R. E., Arch. Biochem. Biophys., 34, 482 (1951).

14. Bernstein, R. L., and Robbins, P. W., J. Biol. Chem., 240, 391 (1965).

15. Nikaido, H., Nakano, K., and Levinthal, M., 7th Intern. Congr. Biochem., Tokyo, Abstract IV, General Sessions A-F, 1967, p. 708.

16. Chojnacki, T., Sawicha, T., and Korzybski, T., Acta Biochem. Pol., 15, 193 (1968).

17. Verachtert, H., Rodriguez, P., Bass, S. T., and Hansen, R. G., J. Biol. Chem., 241, 2007 (1966).

18. Ting, W. K., and Hansen, R. G., Proc. Soc. Exp. Biol. Med., 127, 960 (1968).

19. Axelos, M., Peaud-Lenoel, C., Bull. Soc. Chim. Biol., 51, 261 (1969).

20. Kornberg, A., Advances in Enzymology, 18, 191 (1957).

21. Recondo, E., and Leloir, L. F., Biochem. Biophys. Res. Commun., 6, 85 (1961).

22. Elbein, A. D., Barber, G. A., and Hassid, W. Z., J. Am. Chem. Soc., 86, 309 (1964).

23. Feingold, D. S., Neufeld, E. F., and Hassid, W. Z., J. Biol. Chem., 233, 783 (1958).

24. Cardini, C. E., and Recondo, E., Plant Cell Physiol. (Japan), 3, 313 (1962).

25. Turner, J. F., Aust. J. Biol. Sci., 22, 1145 (1969).

26. Turner, J. F., Aust. J. Biol. Sci., 22, 1321 (1969).

27. Furlong, C. E., and Preiss, J., J. Biol. Chem., 244, 2539 (1969).

28. Glaser, L., J. Biol. Chem., 232, 627 (1958).

29. Abraham, H. D., and Howell, R., J. Biol. Chem., 244, 545 (1969).

30. Knop, J. K., and Hansen, R. G., J. Biol. Chem., 245, 2499 (1970).

31. Albrecht, G. J., Bass, S. T., Seifert, L. L., and Hansen, R. G., J. Biol. Chem., 241, 2968 (1966).

32. Zetsche, K., Z. Naturforsch, 23, 369 (1968).

33. Newell, P. C., and Sussman, M., J. Biol. Chem., 244, 2990 (1969).

34. Ashworth, J. M., and Sussman, M., J. Biol. Chem., 242, 1696 (1967).

35. Zetsche, K., Planta, 89, 244 (1969).

36. Malpress, F. H., Biochem. J., 78, 527 (1961).

37. Baldwin, R. L., J. Dairy Sci., 49, 1533 (1966).

38. Baldwin, R. L., and Milligan, L. P., J. Biol. Chem., 241, 2058 (1966).

39. Kuhn, N. J., and Lowenstein, J. M., Biochem. J., 105, 995 (1967).

40. Fitzgerald, D. K., Ph.D. Thesis, Department of Biochemistry, Oklahoma State University, (1969).

41. Fukasawa, T., Jokura, K., and Kurahashi, K., Biochem. Biophys. Res. Commun., 7, 121 (1962).

42. Fukasawa, T., Jokura, K., and Kurahashi, K., Biochim. Biophys. Acta, 74, 608 (1963).

43. Sundararajun, T. A., Rapin, A., and Kalckar, H. M., Proc. Natl. Acad. Sci., U. S., 48, 2187 (1962).

44. Shapiro, J. A., J. Bacteriol., 92, 518 (1966).

45. Lieberman, M. M., Buchanan, C. E., and Markovitz, A., Proc. Natl. Acad. Sci., U. S., 65, 625 (1970).

46. Taylor, A. L., and Trotter, C. D., Bact. Rev., 31, 332 (1967).

47. Nikaido, H., Levinthal, M., Nikaido, K., and Nakane, K., Proc. Natl. Acad. Sci., U. S., 57, 1825 (1967).

48. Sanderson, K. E., Bact. Rev., 31, 354 (1967).

49. Lowry, O. H., Passonnear, J. V., Hasselberger, F. X., and Schultz, D. W., J. Biol. Chem., 239, 18 (1964).

50. Carlson, D. M., and Hansen, R. G., J. Biol. Chem., 237, 1260 (1962).

51. Berg, P., and Joklik, W. K., J. Biol. Chem., 210, 657 (1954).

52. Verachtert, H., Bass, S. T., Seifert, L. L., and Hansen, R. G., Anal. Biochem., 13, 259 (1965).

53. Oliver, I. T., Biochim. Biophys. Acta, 52, 75 (1961).

54. Steelman, V. S., and Ebner, K. E., Biochim. Biophys. Acta, 128, 92 (1966).

55. Basu, D. K., and Bachhawat, B. K., J. Neurochem., 7, 174 (1961).

56. Fiske, C. H., and Subbarow, Y., J. Biol. Chem., 66, 1375 (1925).

57. Sherman, J. R., Anal. Biochem., 5, 548 (1963).

58. Bertoli, C., and Segal, S., J. Biol. Chem., 241, 4023 (1966).

59. Morrison, J. F., Anal. Biochem., 24, 106 (1968).

60. Kamogawa, A., Kurahashi, K., J. Biochem., 57, 758 (1965).

61. Ginsburg, V., J. Biol. Chem., 232, 55 (1958).

62. Rodriguez, P., Parra, N., Bello, O., and Gaede, K., Biochim. Biophys. Acta, 151, 694 (1968).

63. Emery, R. S., and Baldwin, R. L., Biochim. Biophys. Acta, 136, 223 (1967).

64. Villar-Palasi, C., and Larner, J., Arch. Biochem. Biophys., 86, 61 (1960).

65. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem., 193, 265 (1951).

66. Robinson, H. W., and Hogden, C. G., J. Biol. Chem., 135, 727 (1940).

67. Warburg, O., and Christian, W., Biochem. Z., 310, 384 (1941).

68. Hansen, R. G., Albrecht, G. J., Bass, S. T., and Seifert, L. L., Methods in Enzymology, VIII, 248 (1966).

69. Keppler, D., and Decker, K., Eur. J. Biochem., 10, 219 (1969).

70. Turner, D. H., and Turner, J. F., Biochem. J., 69, 448 (1958).

71. Wright, B. E., in S. H. Hutner and A. Lwoff (Editors), The Biochemistry and Physiology of Protozoa, Vol. 3, Academic Press, New York, 1964, p. 365.

72. Wright, B. E., and Dahlberg, D., J. Bacter., 95, 983 (1968).

73. Levine, S., Gillet, T. A., Hageman, E., and Hansen, R. G., J. Biol. Chem., 244, 5729 (1969).

74. Fitzgerald, D. K., Chen, S., and Ebner, K. E., Biochim. Biophys. Acta, 178, 491 (1969).

75. O'Sullivan, W. J., and Perrin, D. D. Biochemistry, 3, 18 (1964).

76. Walaas, E., Acta Chem. Scand., 12, 528 (1958).

77. Gosselin, R. E., and Coghlon, E. R., Arch. Biochem. Biophys., 45, 301 (1953).

78. Lineweaver, H., and Burk, D., J. Am. Chem. Soc., 56, 658 (1934).

79. Clarke, H. B., Cusworth, D. C., and Datta, S. P., Biochem. J., 58, 146 (1954).

80. Trevelyan, W. E., Mann, P. F. E., and Harrison, J. S., Arch. Biochem. Biophys., 39, 440 (1952).

81. Kornfeld, S., Fed. Proc., 24, 536 (1965).

82. Keppler, D., and Decker, K., Hoppe-Seyler's Z. Physiol. Chem., 350, 8 (1969).

83. Cleland, W. W., Biochim. Biophys. Acta, 67, 104 (1963).

84. Trucco, R. E., Nature, 174 1103 (1954).

85. Neufeld, E. F., Ginsburg, V., Putman, E. W., Franshier, D., and Hassid, W. Z., Arch. Biochem. Biophys., 69, 602 (1957).

86. Gillett, T. A., Levine, S., Bass, S. T., and Hansen, R. G., Fed. Proc., 27, 453 (1968).

87. Johnson, J. C., Shanoff, M., Bass, S. T., Boezi, J. A., and Hansen, R. G., Anal. Biochem., 26, 137 (1968).

88. Ward, C., and Wright, B. E., Biochemistry, 4, 2021 (1965).

89. Wright, B. E., Ward, C., and Dahlberg, D., Biochem. Biophys. Res. Commun., 22, 352 (1966).

90. Roth, R., and Sussman, M., J. Biol. Chem., 243, 5081 (1968).

91. Roth, R., and Sussman, M., Biochim. Biophys. Acta, 122, 225 (1966).

92. Sussman, M., and Osborn, M. J., Proc. Natl. Acad. Sci., U. S. 52, 81 (1964).

93. Isselbacher, K. J., Science, 126, 652 (1957).

94. Gitzelmann, R., Pediat. Res., 3, 279 (1969).

95. Strominger, J. L., Maxwell, E. S., Axelrod, F., and Kalckar, H. M. J. Biol. Chem., 244, 79 (1957).

96. Siegelman, H. W., Wieczorek, G. A., and Turner, B. C., Anal. Biochem., 13, 402 (1965).

97. Whatman Advanced Ion-Exchange Celluloses, Bulletin IE 3, H. Reeve Angel and Co. Ltd., London.

98. Bio-Rad Laboratories, Price List T, Richmond, California.

99. Sephadex-gel Filtration in Theory and Practice, Pharmacia Fine Chemicals, Inc., Upsalla, Sweden.

100. Martin, R. G., and Ames, B. H., J. Biol. Chem., 236, 1372 (1961).

101. Canalco Instruction Manual for the Model 6 System, Canal Industrial Corporation, Rockville, Maryland.

102. Sephadex Technical Data Sheet No. 1, Pharmacia Fine Chemicals Inc., Upsalla, Sweden.

103. Worthington Catalogue, Worthington Biochemical Corporation, Freehold, New Jersey.

104. Tsai, C. M., Ph.D. Thesis, Department of Biochemistry, Oklahoma State University (1969).

105. Andrews, P., Biochem. J., 96, 595 (1965).

106. Steelman, V. S., and Ebner, K. E., unpublished observation, Department of Biochemistry, Oklahoma State University.

107. Jakoby, W. B., Anal. Biochem., 26, 295 (1968).

108. Teorell, T., and Stenhagen, E., Biochem. Z., 299, 416 (1938).

109. Smith, M. E., and Smith, L. B., Biol. Bull., 96, 233 (1949).

110. Samejima, T., Kamato, M., and Shibata, K., J. Biochem., 51, 181 (1962).

111. Morrison, J. F., O'Sullivan, W. J., and Ogston, A. G., Biochim. Biophys. Acta, 52, 82 (1961).

112. Lowry, O. H., and Lopez, J. A., J. Biol. Chem., 162, 421 (1946).

113. Cleland, W. W., Nature, 198, 463 (1963).

114. Cleland, W. W., Advances in Enzymology, 29, 1 (1967).

115. Morrison, J. F., and James, E., Biochem. J., 97, 39 (1965).

116. Najjar, V. A., The Enzymes, 6, 161 (1962).

117. Paladini, A. C., and Leloir, L. F., Biochem. J., 51, 426 (1952).

118. Roth, M., Methods of Biochemical Analysis, 17, 189 (1969).

119. Cuatrecasas, P., J. Biol. Chem., 245, 3059 (1970).

120. Bernardi, G., and Kawasaki, T., Biochim. Biophys. Acta, 160, 301 (1968).

121. Berlin, R. D., Arch. Biochem. Biophys., 134, 120 (1969).

122. Powanda, M. C., Muniz, O., and Dietrich, L. S., Biochemistry, 8, 1869 (1969).

VITA

Robert Roscoe Aksamit

Candidate for the Degree of

Doctor of Philosophy

Thesis: PURIFICATION, PROPERTIES, AND KINETIC ANALYSIS OF UDP-GLUCOSE PYROPHOSPHORYLASE FROM BOVINE MAMMARY TISSUE

Major Field: Chemistry

Biographical:

Personal Data: Born in Fairbury, Nebraska, February 17, 1944, the son of Roscoe W. and Leatha L. Aksamit.

Education: Attended public schools in Rawlins, Wyoming; graduated from Rawlins High School, Rawlins, Wyoming, in May, 1962; received the Bachelor of Science degree from the University of Wyoming, Laramie, Wyoming, with a major in Chemistry in August, 1965; completed requirements for the Doctor of Philosophy degree in May, 1971.

Professional Experience: Served as a research assistant at Oklahoma State University from September, 1965 to October, 1970.

Honorary Societies: Phi Lambda Upsiolon, Associate member Sigma Xi.