THE ISOLATION AND SYNTHESIS OF GUANOSINE DIPHOSPHATE GLUCOSE

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY

Don Marvin Carlson

1961

This is to certify that the

thesis entitled

THE ISOLATION AND SYNTHESIS OF

GUANOSINE DIPHOSPHATE GLUCOSE

presented by

Don Marvin Carlson

has been accepted towards fulfillment
of the requirements for

Ph. D. degree in Biochemistry

R. G. Hansen

Major professor

Date 10/6/61

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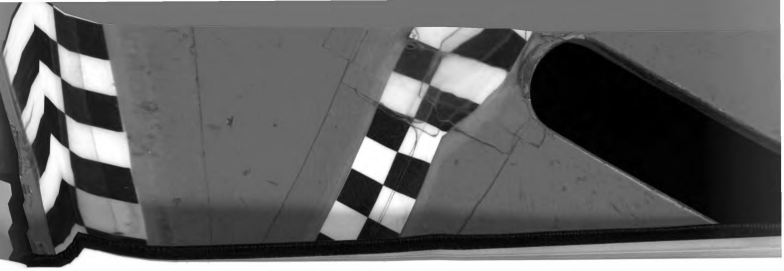


THE ISOLATION AND SYNTHESIS OF GUANOSINE DIPHOSPHATE GLUCOSE

By

Don Marvin Carlson

A THESIS

Submitted to the School for Advanced Graduate Studies
of Michigan State University in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

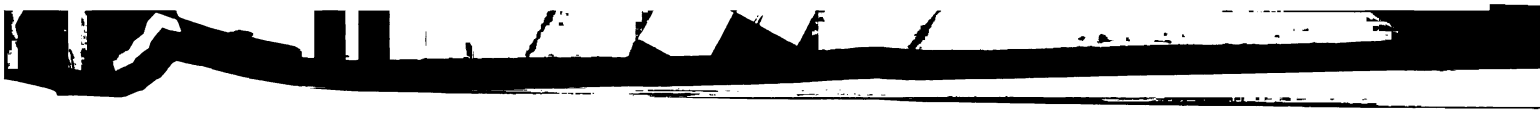
Department of Biochemistry

1961

ACKNOWLEDGMENTS

The author wishes to express his appreciation to Dr. R. G. Hansen for his helpful advice and continuous interest in this project.

The author is especially grateful to his wife Eunice, and his sons, Tim and Tom, for their interest, understanding, and love during the course of this study.



To Eunice

VITA

Don M. Carlson was born on March 11, 1931, at Walhalla, North Dakota. He graduated from Cavalier High School, Cavalier, North Dakota in 1949 and attended North Dakota Agricultural College at Fargo for two quarters before entering the U. S. Army in 1950. After his discharge from the service, Mr. Carlson resumed his studies at North Dakota where he received his B.S. in Agriculture in 1956. The requirements for the Master of Science degree were completed in January of 1958 after two years in residence at the University of Illinois. Graduate studies were continued with the aid of a research assistantship in the Department of Agricultural Chemistry at Michigan State University. In January, 1960, a National Institutes of Health Predoctoral Research Fellowship was awarded to support the completion of his graduate education. A postdoctoral position at the Rackham Arthritis Research Unit, University of Michigan, was recently accepted.

THE ISOLATION AND SYNTHESIS OF GUANOSINE DIPHOSPHATE GLUCOSE

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AN ABSTRACT

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ABSTRACT

The presence of guanosine diphosphate mannose and guanosine diphosphate glucose has been demonstrated in nucleotide extracts of lactating bovine mammary gland. Tentative evidence is presented for the presence of guanosine diphosphate fucose and guanosine diphosphate fructose. An enzyme from mammary tissue has been partially purified which is capable of forming guanosine diphosphate glucose from guanosine triphosphate and glucose-1-phosphate. Of seven different enzyme sources used, all of which contained uridine diphosphate glucose pyrophosphorylase, only mammary tissue was capable of synthesizing guanosine diphosphate glucose. Guanosine diphosphate glucose was chemically synthesized by reacting bis-(tri-n-octylammonium)-D-glucose-α-1-phosphate with 4-morpholine N,N'-dicyclohexylcarboxamidinium guanosine-5' phosphoromorpholidate in anhydrous pyridine. When subjected to both chemical and enzymic assays, the enzymically and chemically synthesized compounds were identical. The reversibility of the enzymic reaction was demonstrated by (1) the incorporation of labeled pyrophosphate into guanosine triphosphate and (2) the recovery of glucose-1-phosphate from guanosine diphosphate glucose and pyrophosphate. The enzyme was shown to be distinct from uridine diphosphate glucose pyrophosphorylase. Because of the characteristic type of reaction which it catalyzes, this enzyme has been named guanosine diphosphate glucose pyrophosphorylase.

TABLE OF CONTENTS

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. . . . . .

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. . . . . .
. . . . . .
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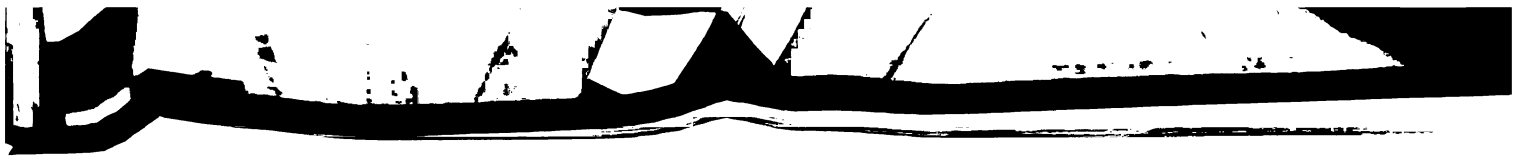
TABLES

FIGURES



INTRODUCTION

INTRODUCTION

Nucleotide derivatives of hexoses and pentoses play a major role in carbohydrate metabolism. Since the classical demonstration of the mediation of UDP-glucose[1] in the interconversion of galactose-1-P and glucose-1-P, the reports on the isolation and synthesis of nucleotide derivatives have become voluminous. Of the sugar nucleotides the uridine diphosphate derivatives have been studied most thoroughly, but it is now evident that combinations occur which involve other purine and pyrimidine bases.

Sugar nucleotides appear to be widespread in nature and are found to participate in a large number of biochemical reactions, such as: 1/-oxidation-reduction, 2/polysaccharide synthesis, 3/pyrophosphorolysis, 4/carbohydrate transfers, 5/epimerization, 6/epimerization and reduction and 7/decarboxylation; for example,

1. UDP-glucose + 2DPN + H_2O $\longrightarrow$ UDP-glucuronic acid + 2 DPNH + $2H^+$
2. UDP-glucose + $(glucosyl)_n$ $\longrightarrow$ UDP + $(glucosyl)_{n+1}$
3. UDP-glucose + PPi $\rightleftharpoons$ UTP + glucose-1-P
4. UDP-glucose + galactose-1-P $\rightleftharpoons$ UDP-galactose + glucose-1-P
5. UDP-glucose $\rightleftharpoons$ UDP-galactose
6. GDP-mannose + TPNH + H^+ $\longrightarrow$ GDP-L-fucose + TPN + H_2O
7. UDP-glucuronic acid $\longrightarrow$ UDP-xylose + CO_2

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[1]The following abbreviations are used: AMP, ADP, and ATP, adenosine mono-, di-, and triphosphate; ADPR, adenosine diphosphoribose; DCC, dicyclohexylcarbodiimide; DPN, oxidized diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; EDTA, ethylenediaminetetracetate; GMP, GDP and GTP, guanosine mono-, di-, and triphosphate; IMP, inosine monophosphate; Pi, inorganic phosphate; PPi, inorganic pyrophosphate; TDP, thymidine diphosphate; TPN, oxidized triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; Tris, tris-(hydroxymethyl)-aminomethane; UMP, UDP, and UTP, uridine mono-, di-, and triphosphate.

These reactions may be independent of each other or they may be interrelated, e.g., the oxidation of UDP-glucose to form UDP-glucuronic acid followed by the incorporation of the uronic acid moiety into mucopolysaccharides.

While reports of lactose synthesis in cell free systems have appeared, they have not received confirmation. The most recent report hypothesized that UDP-galactose combines with glucose-1-P to yield lactose-1-P which is subsequently hydrolyzed to lactose and inorganic phosphate. The inability of various laboratories to confirm this report coupled with experiments with whole tissue which indicated that free glucose is the galactosyl acceptor[1], prompted the investigation of other nucleotide components of lactating mammary gland with the thought that they may be implicated in lactose or oligosaccharide biosynthesis.

The object of this thesis is to establish the presence of GDP-glucose in mammary gland preparations and to report for the first time the chemical synthesis and biosynthesis of GDP-glucose. GDP-glucose is synthesized from GTP and glucose-1-P by a partially purified enzyme from mammary gland. GDP-mannose is synthesized by the same preparation, but evidence is presented which suggests that these two syntheses are accomplished by two distinct enzymes. Of seven different preparations studied only mammary gland was found to be a source of the enzyme capable of forming GDP-glucose. A comparison of the chemically synthesized GDP-glucose with the enzymically synthesized compound established the

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[1]A communication from Dr. W. Z. Hassid, University of California, reports that lactose has been synthesized with cell-free preparations of guinea pig mammary gland from UDP-galactose and free glucose.

structure of the isolated and synthesized nucleotide as guanosine 5'-diphosphate-α-D-glucose.

LITERATURE REVIEW

LITERATURE REVIEW

While the physical and chemical properties and biochemical functions of the adenine and uridine nucleotides have been studied intensively, the guanosine compounds have only recently received attention. Although ATP was shown to be a naturally occurring substance in 1929 (1,2), it was not until 1953 that Bergkvist and Deutsch (3) discovered GTP as a contaminant of ATP isolated from rabbit muscle. One year later Cabib and Leloir (4) isolated GDP-mannose from baker's yeast. The ultraviolet absorption spectrum, the identification of the sugar moiety after mild acid hydrolysis, the reaction with snake venom 5'-nucleotidase, and the analytical data for total and acid-labile phosphate, guanosine, and reducing value after hydrolysis suggested that GDP-mannose had a structure similar to UDP-glucose (5). It was postulated by Cabib and Leloir that GDP-mannose may serve as a donor of mannose residues for the synthesis of polymers which are present in the yeast cell wall. As yet, however, there is no direct evidence to support this assumption.

GDP-mannose has since been reported to be present in hen's oviduct (6), the molds Penicillium chrysogenum (7) and Eremothecium ashbyii (8), the red alga Porphyra perforata (9), guinea pig mammary gland (10), sheep mammary gland (11) and the milk of the sheep, goat and sow (12). The biosynthesis of GDP-mannose was first reported by Munch-Petersen (13). The reaction was shown to be catalyzed by a pyrophosphorylase extracted from brewer's yeast according to reaction (1). Reversibility

of this reaction was demonstrated by the incorporation of P^{32} into GTP

(1) GTP + mannose-1-P $\rightleftharpoons$ GDP-mannose + PPi

when radioactive pyrophosphate was added. The equilibrium constant of the reaction was determined to be about 1, which is similar to that reported for UDP-glucose pyrophosphorylase (14). The mannose enzyme has been separated from UDP-glucose pyrophosphorylase but not from UDP-acetylglucosamine pyrophosphorylase (15).

Ginsburg (16,17) has demonstrated the TPNH dependent conversion of GDP-mannose into GDP-fucose by dialyzed crude extracts of Aerobacter aerogenes. The overall multi-step reaction may be written as follows:

GDP-D-mannose + TPNH + H^+ $\longrightarrow$ GDP-L-fucose + TPN + H_2O

The product of this reaction was identified as guanosine 5'-diphosphate-L-fucose by chemical and enzymic analyses. A pathway for the biosynthesis of GDP-L-fucose has been suggested (18): (1) GDP-D-mannose loses water and rearranges to form GDP-4-keto-6-deoxy-D-mannose, and (2) by a double enolization, GDP-4-keto-6-deoxy-D-mannose is converted to GDP-4-keto-6-deoxy-L-galactose, which is then stereospecifically reduced by TPNH to GDP-L-fucose. This hypothetical pathway is based on the presence of a keto-function in intermediates formed during the reaction. Chemical reduction of these intermediates gave predominantly 6-deoxytalose and 6-deoxymannose.

Another example of a nucleotide derivative implicated in the synthesis of a deoxy sugar is the formation of TDP-rhamnose from TDP-glucose (19). This reaction also requires a source of TPNH. Kornfeld and Glaser (20) have reported that DPN is required in the overall reac-

tion in addition to TPNH. In the absence of TPNH, incubation of TDP-glucose, enzyme and DPN leads to the formation of a sugar which appeared to be a 4-keto-6-deoxyhexose. It is probable that the formation of GDP-L-fucose and TDP-rhamnose proceed via similar mechanisms.

Pontis, et al. (21) have reported the presence of small quantities of GDP-glucose and GDP-fructose in association with GDP-mannose isolated from a mold. The sugars were identified by chromatography and ionophoresis after acid hydrolysis. The resorcinol-HCl absorption spectrum and the indole-H_2SO_4 colorimetric test were used to specifically identify fructose. From quantitative estimations of the sugars it was reported that glucose and fructose together could account for approximately 6% of the total hexose present in the GDP-mannose fraction.

MATERIALS AND METHODS

MATERIALS AND METHODS

Preparation of substrates. Nucleotides were purchased from Sigma Chemical Company and Pabst Brewing Company. Glucose-1-P-C^{14} was prepared by Dr. R. G. Hansen from C^{14}-labeled starch by the action of phosphorylase. Galactose-1-P was prepared by the method of Hansen, et al. (22) and mannose-1-P was prepared by the procedure of Colowick[1] (23). The lithium salt of GDP-mannose was provided by Dr. S. Roseman, University of Michigan, and the GDP-mannose isolated from yeast was a gift from R. K. Bretthauer. The chemical preparation of GDP-glucose is included in Experimental Procedures and Results.

Preparation of enzymes. UDP-glucose dehydrogenase was purified from calf liver by the procedure of Strominger, et al. (25). Crystalline phosphoglucomutase was prepared from rabbit muscle (26) and glucose-6-P dehydrogenase from dried brewer's yeast (27). Crotalus atrox venom was obtained from the Ross Allen Reptile Institute, Silver Springs, Florida, and was used as a source of nucleotide pyrophosphatase. This preparation gave quantitative cleavage of UDP-glucose to glucose-1-P and presumably UMP as measured by the formation of glucose-1-P and by the disappearance of UDP-glucose.

Qualitative measurements. Nucleotides were separated on ion exchange columns (28). Charcoal was prepared for nucleotide adsorbtion by the method of Threlfall (29). Paper chromatography on Whatman No. 1 filter

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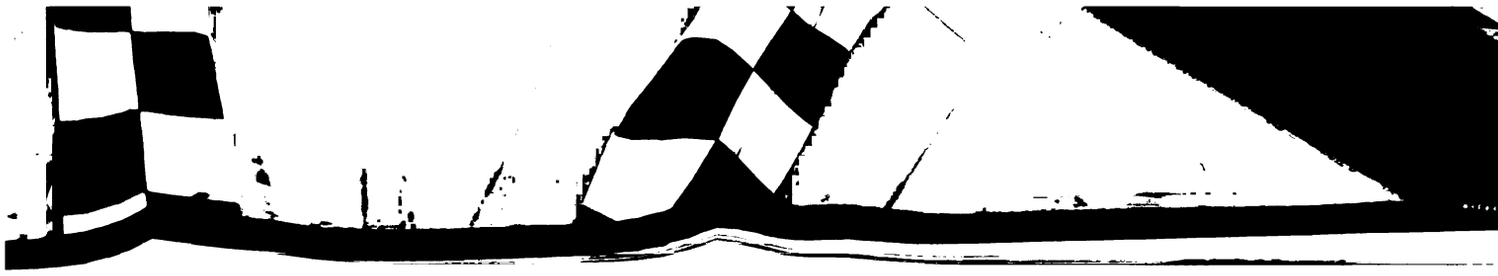
[1]The α-D-mannose-1-P was prepared by R. K. Bretthauer by a slight modification of Colowick's procedure. Penta-acetyl-α-D-mannose was prepared as a syrup by the method of Hudson and Dale (24). This syrup was then treated according to Colowick's procedure without crystallization.



paper was employed for identifying nucleotides by using the following solvent systems: ethanol-ammonium acetate, pH 7.5 (30), ethanol-ammonium acetate, pH 3.8 (30), and isobutyric acid-ammonium hydroxide-water (31). An ultraviolet lamp (Mineralight SL 2537) was used for the detection of nucleotides on paper. Photographs of ultraviolet absorbing spots on paper chromatograms were made by contact prints prepared by slight modification of the procedure of Markham and Smith (32) as outlined by Wilken (33). The paper electrophoretic conditions of Wade and Morgan (34), employing 0.05 M citrate buffer, pH 4.4, were used. Solvents used for the identification of sugars were butanol-pyridine-water (35), phenol-water (36) and ethylacetate-pyridine-water (37). Sugars were detected on paper by the ammonical-silver nitrate method (38).

Quantitative measurements. Total phosphorus determinations were performed by the method of King (39) and acid-labile and inorganic phosphorus by the method of Fiske and SubbaRow (40). Ribose was assayed by the Mejbaum pentose determination (41) and reducing sugars by the method of Park and Johnson (42). Protein was measured by the Lowry method (43). A liquid scintillation counter was used for the determination of radioactivity. The sample was dissolved in 0.01 ml water and 1 ml absolute ethanol to which was added 14 ml of a solution made from 4.0 g 2,5-diphenyloxazole and 50 mg 1,4-bis-2-(5-phenyloxazole)-benzene made up to one liter with toluene (44). Radioactive spots were detected by exposing paper chromatograms to No-Screen X-ray film. The spots were quantitated with a gas-flow counter equipped with a thin Mylar

window Geiger-Mueller tube. Absorption spectra were obtained with a Beckman DK-2 Recording Spectrophotometer. Enzyme assays involving the oxidation or reduction of pyridine nucleotides were performed on a Beckman DU Spectrophotometer equipped with a Gilford cuvette changer and recording attachment (45). UDP-glucose was measured with UDP-glucose dehydrogenase by following the formation of DPNH. Glucose-1-P was measured by following the formation of TPNH, as catalyzed by phosphoglucomutase and glucose-6-P dehydrogenase.

EXPERIMENTAL PROCEDURES AND RESULTS

EXPERIMENTAL PROCEDURES AND RESULTS

Isolation of GDP-hexoses. Lactating bovine mammary gland obtained from a local abattoir was excised and frozen in dry ice for transport to the laboratory. The frozen tissue was then pulverized and extracted with 0.6 N perchloric acid in a Waring Blender. After centrifugation the supernatant was brought to pH 6 with 5 N KOH and the precipitate removed by filtration. The filtrate was placed directly on a Dowex 1-formate column for separation of the nucleotides by the chromatographic procedure of Hurlbert, et al. (28). GDP-hexoses were eluted immediately after ADP and were contaminated by this fraction (Figure 1). To remove electrolytes the principle GDP-hexose peak was adsorbed on norite and the nucleotides eluted with 50 percent ethanol containing 1 percent ammonia. This nucleotide peak was further purified by paper chromatography in pH 7.5 ammonium acetate-ethanol[1]. Characterization of some of the components of this peak is included with the characterization of the enzymically prepared GDP-glucose.

Biosynthesis of GDP-glucose. After glucose was tentatively identified as a component of the GDP-hexose fraction from mammary gland, it was possible to demonstrate the biosynthesis of GDP-glucose from GTP and glucose-1-P by mammary gland extracts. The enzyme was detected by

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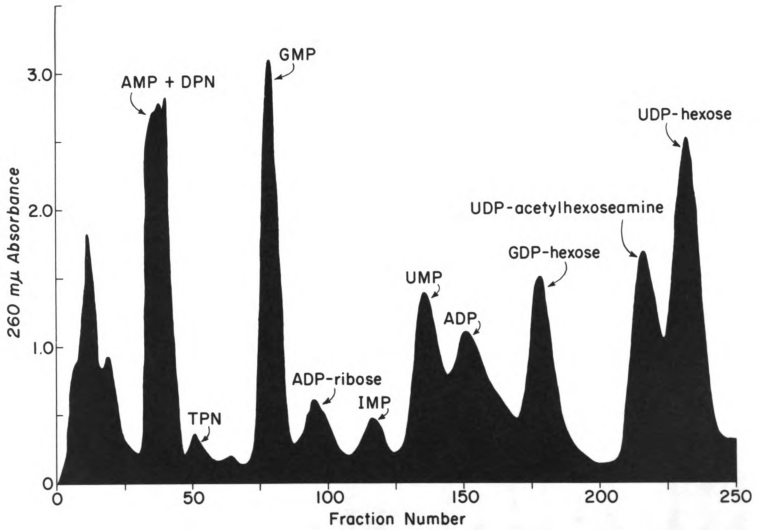
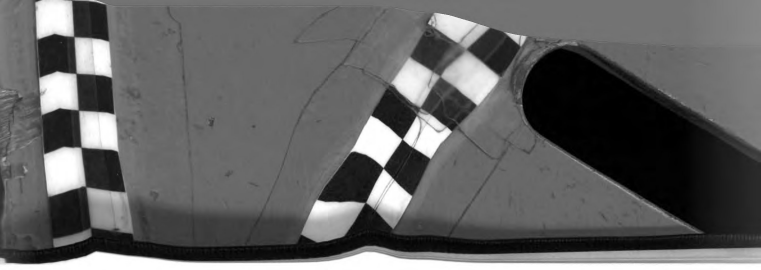
[1]During purification by paper chromatography a band was detected which had both U.V. adsorption and ninhydrin-positive material. After elution and lyophilization Doctor Okuhara hydrolyzed the material for 20 hours in 6 N HCl and identified glutamic acid, cysteic acid, glycine and taurine by using two-dimensional paper chromatography with water-saturated phenol in the first direction and butanol-propionic acid and water (46) in the second direction. The amino acids were detected and identified using the polychromatic ninhydrin spray of Moffat and Lytle (47).

Figure 1. Elution sequence of nucleotides from lactating bovine mammary gland.


AMP + DPN
GMP
UDP-hexose
UDP-acetylhexoseamine
GDP-hexose
UMP
ADP
ADP-ribose
IMP
TPN
260 mμ Absorbance
3.0
2.0
1.0
0
0
50
100
150
200
250
Fraction Number



adding about 20 mg of protein to 1 μmole GTP, 2 μmoles of glucose-1-P and 2 μmoles of ATP made to a total volume of 1 ml with mixed buffer.[1] After incubation for one hour the reaction was stopped by heating at 100° for 1 minute and a portion of the supernatant chromatographed in pH 7.5 ammonium acetate-ethanol.

Bovine mammary gland used as a source of the enzyme was the same as that used in the isolation of GDP-hexose. The frozen tissue was pulverized before the acetone powder was prepared. This latter procedure included two extractions with 10 volumes of cold acetone (-10°) followed by extraction with 5 volumes of cold ether before drying. Rat mammary gland acetone-powder was prepared from the posterior glands of animals that had been lactating for 7 to 10 days.

The acetone-powder was extracted with 10 volumes of mixed buffer in a Servall Omni-mixer for 10 minutes and the resulting homogenate centrifuged at 10,000 x g. The precipitate was discarded and 0.05 volume of 1 M $MnCl_2$ was added to the supernatant with stirring. A heavy precipitate usually formed. As the addition of the $MnCl_2$ lowered the pH to approximately 6, proteins were removed at this point as well as nucleic acids. Stirring was continued for about 5 minutes and the material was centrifuged at 10,000 x g. The precipitate was discarded and the supernatant brought to 0.60 ammonium sulfate saturation (37 g/100 ml supernatant), stirred for 20 minutes and centrifuged. The supernatant was discarded and the precipitate was dissolved in 4 ml

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[1]The mixed buffer used consisted of the following: 0.1 M tris, 0.01 M $MgCl_2$, 0.001 M EDTA and 0.001 M mercaptoethanol, brought to pH 7.5 with 5 N acetic acid.

of mixed buffer per gram of acetone powder. This preparation was used as the source of the enzyme.

Effect of ATP on GDP-glucose synthesis. Phosphatases present in mammary tissue, e.g., apyrase (48), could readily cause the destruction of nucleotide triphosphates. The effect of ATP on GDP-glucose synthesis was therefore considered. The following incubation conditions were employed: 0.75 ml of enzyme (prepared as described on p. 13) was added to 5 μmoles of GTP, 20 μmoles glucose-1-P, 6 μmoles ATP (where indicated) and 20 μmoles of NaF in a total volume of 1 ml. Four identical tubes each with and without ATP were incubated for 1 hour at room temperature and the reaction stopped by heating at 100° for 1 minute. After centrifugation the appropriate supernatants were combined and the nucleotides separated on a column of Dowex 1-formate as previously described. As shown in Table 1, a 51 percent increase in GDP-glucose formation resulted from the presence of ATP. This increase in GDP-glucose and the concurrent decrease in GDP may be due to (1) the inhibition of a nucleotide pyrophosphatase, or (2) the regeneration of GTP from GDP or GMP by a nucleotide kinase reaction.

Chemical synthesis of GDP-glucose.[1] GDP-glucose was chemically prepared by following the procedure outlined for the synthesis of GDP-mannose (49).

1. Preparation of 4-morpholine N,N'-dicyclohexylcarboxamidinium guanosine-5' phosphoromorpholidate (GMP-morpholidate) (50). A column of Dowex 50-hydrogen form was converted to the morpholine form by

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[1]The author wishes to thank Dr. Saul Roseman for his assistance in the preparation of the GDP-glucose.

Table 1. The effect of ATP on the enzymic formation of GDP-glucose.

Nucleotide peak	μmoles -ATP	μmoles +ATP	Percent change
GMP	0.36	0.40	+14.0
GDP-glucose	3.07	6.06	+51.4
GDP	7.16	1.56	- 76
GTP	3.54	5.52	-38.5
Total	14.13	13.54	

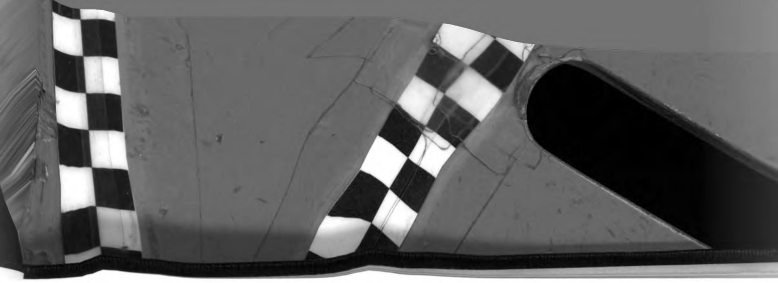
washing with a 10 percent aqueous solution of distilled morpholine until the eluate was strongly basic. The column was then washed with water until the eluent was neutral. An aqueous solution of 1.434 g of $Na_2GMP \cdot H_2O$ (3.23 mmoles) was placed on the resin and washed with 130 ml of water. This eluted 3.09 mmoles of GMP for a recovery of 97 percent. After removal of the water in vacuo, the morpholine-GMP was dissolved in 30.9 ml water, 30.9 ml t-butanol and 1.05 ml distilled morpholine, and connected to a condensor and a dropping funnel which contained 2.54 g DCC in 46.4 ml t-butanol. DCC was added at a rate of about 10 ml/hour with refluxing for five hours. An aliquot was then removed for electrophoresis in 0.05 M phosphate buffer, pH 7.5. A light ultraviolet absorbing spot of GMP was present together with a heavy spot which had moved about one-half the distance of the GMP. This was the GMP-morpholidate. Extended refluxing and the addition of more DCC and morpholine did not convert the remaining GMP into the morpholidate. Upon cooling of the mixture to room temperature a precipitate of substituted urea formed which was removed by filtration and washed free of the morpholidate with 5-butanol. After removal of most of the alcohol in vacuo a precipitate formed which was transferred with the butanol to a separatory funnel and extracted 3 times with two volumes of ether. The aqueous layer was dried in vacuo and the remaining traces of water removed with an oil pump. Methanol was added to dissolve the dry, glassy material and the solution transferred to 2-50 ml centrifuge tubes. Each tube was filled to capacity with cold ether and the precipitate was triturated,

centrifuged and the ether decanted. Ether was added twice more with trituration and centrifugation and, after decanting, the remaining ether was removed *in vacuo*. The sample was dried at 80° *in vacuo* for 4 hours and the yield was 1.97 g of GMP-morpholidate. Molecular weight, determined by ultraviolet absorption, was 728 g/m for a recovery of 87.6 percent.

2. Preparation of bis-(tri-*n*-octylammonium)-D-glucose-α-1-phosphate. DiK-glucose-1-P·$2H_2O$ (7.5 mmoles) was converted to the free acid by passing it through a column of Dowex 50-hydrogen form. Pyridine was added to make the solution alkaline when it was evaporated to about 5 ml, then 7.7 ml of tri-*n*-octylamine was added in 5 ml of pyridine. After removal of the pyridine *in vacuo*, the material was left overnight in the cold room.

3. Preparation of GDP-glucose. The tri-*n*-octylammonium salt of glucose-1-P was dissolved in 150 ml of pyridine (10 ml/0.05 mmole of nucleotide for reacting guanosine compounds as they are less soluble than uridine derivatives) and azeotropically evaporated by continuous addition of anhydrous pyridine.[1] GMP-morpholidate was added (1.5 mmoles) and again the preparation was azeotropically evaporated. The final volume was adjusted with pyridine to the point of solution of the morpholidate. To minimize the effects of light and moisture, the flask was removed, quickly stoppered, wrapped with aluminum foil and stored in a desiccator with $CaCl_2$. After 6 days a portion was removed

[1]Anhydrous pyridine was prepared by refluxing pyridine over calcium hydride for 10 hours and then distilling the pyridine onto calcium hydride. The metal hydride used to store the pyridine should have the fine material removed to prevent cloudiness.



and assayed by electrophoresis. Only one major compound was detectable and this corresponded to GDP-glucose. After evaporation of the pyridine, 30 ml of water containing 2 g of sodium acetate were added to the flask. Following this 20 ml of ether were added and the contents transferred to a separatory funnel. After four extractions of the aqueous phase with equal volumes of ether, the combined ether extracts were backwashed with water (3 times with 10 ml each time). Traces of ether were removed in vacuo and the aqueous portion was placed on a column of Dowex 1-chloride (2 x 35 cm). The column was washed with water and then eluted successively with 0.05 M, 0.1 M, 0.3 M and 0.6 M lithium chloride. Ultraviolet absorbing material eluted with 0.3 M lithium chloride was pooled and evaporated to dryness. Methanol (75 ml) was added, the solution distributed evenly in 4-250 ml centrifuge bottles, and the bottles were filled to capacity with cold acetone. The precipitate which formed was washed twice with cold acetone-methanol (95:5), then with acetone and finally with ether. The GDP-glucose was dried for 10 hours in a desiccator connected to a vacuum pump and left overnight at 0°. The yield was 730 mg and assuming the hexahydrate, the molecular weight was 709 g/m. A yield of 1.03 mmoles or 69.4 percent based on the amount of GMP-morpholidate was obtained.

Characterization of guanosine diphosphate glucose. The components of the purified GDP-hexose fraction, the product resulting from incubating GTP and glucose-1-P with mammary gland preparations, and the chemically synthesized GDP-glucose were characterized using the

following criteria:

1. Absorption spectra. The enzymically and chemically synthesized GDP-glucose and the isolated GDP-hexose all exhibited typical guanosine spectra (31) (Figures 2, 3, and 4) with the characteristic shift in absorption maxima and minima at pH 11.

2. Chemical analyses. Chemical analyses for phosphorus, ribose and hexose agreed with guanosine values obtained by quantitative ultraviolet absorption as measured at 260 mμ. The three GDP-hexose preparations compared favorably with the theoretical values; e.g., one mole of each of guanosine, acid-labile phosphorus, ribose and reducing sugar per mole of sugar nucleotide (Table 2).

3. Hydrolysis with acid. Figure 5 illustrates the effect of hydrolysis in 0.01 N HCl on the nucleotide portion of GDP-glucose. Hydrolysis for 15 minutes and 3 hours gave rise to GDP and GMP, respectively. The quantitative appearance of reducing sugar when the compound is hydrolyzed at pH 2 is shown in Figure 6. The chemically and enzymically prepared GDP-glucoses are identical. These hydrolysis curves are also similar to that obtained by Cabib and Leloir for GDP-mannose (5).

4. Chromatographic and electrophoretic characterization of the hexose nucleotides and their components. Chromatographic mobilities of various hexose nucleotides with respect to GMP are shown in Table 3. The isolated GDP-hexose and the chemically and enzymically formed GDP-glucose have the same mobility in three solvent systems and are

Figure 2. Absorption spectra of isolated GDP-hexoses.

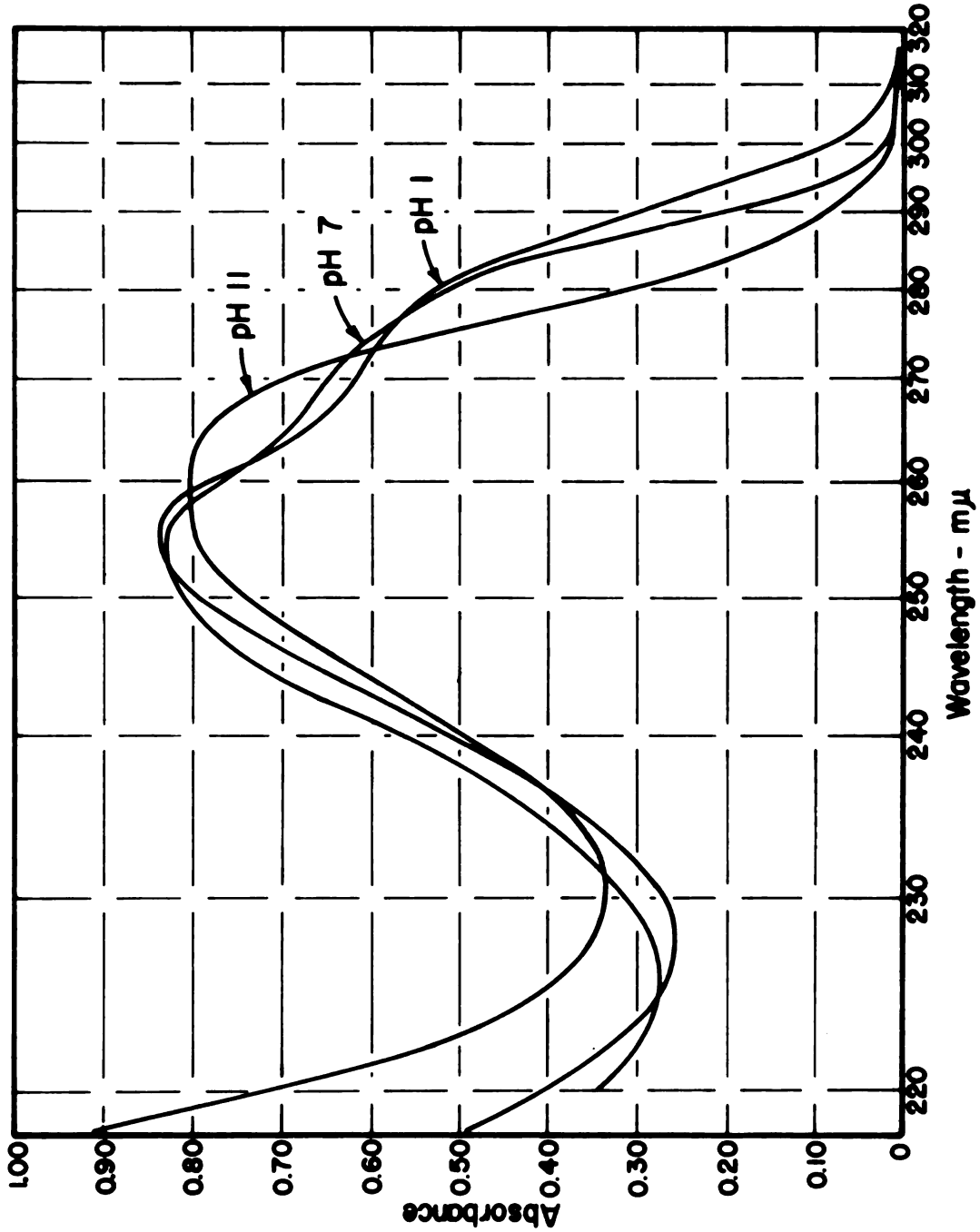

pH 11
pH 7
pH 1
Absorbance
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0
220
230
240
250
260
270
280
290
300
310
320
Wavelength - mμ

Figure 3. Absorption spectra of chemically synthesized GDP-glucose.

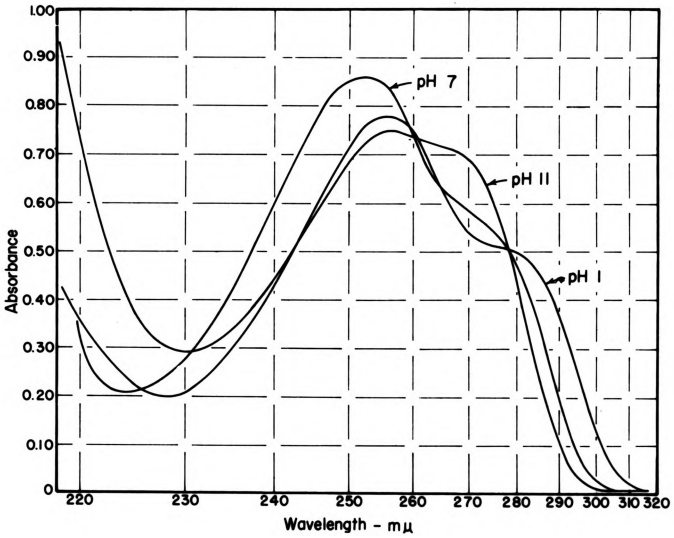

1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0
Absorbance
pH 7
pH 11
pH 1
220
230
240
250
260
270
280
290
300
310
320
Wavelength - mμ

Figure 4. Absorption spectra of enzymically synthesized GDP-glucose.

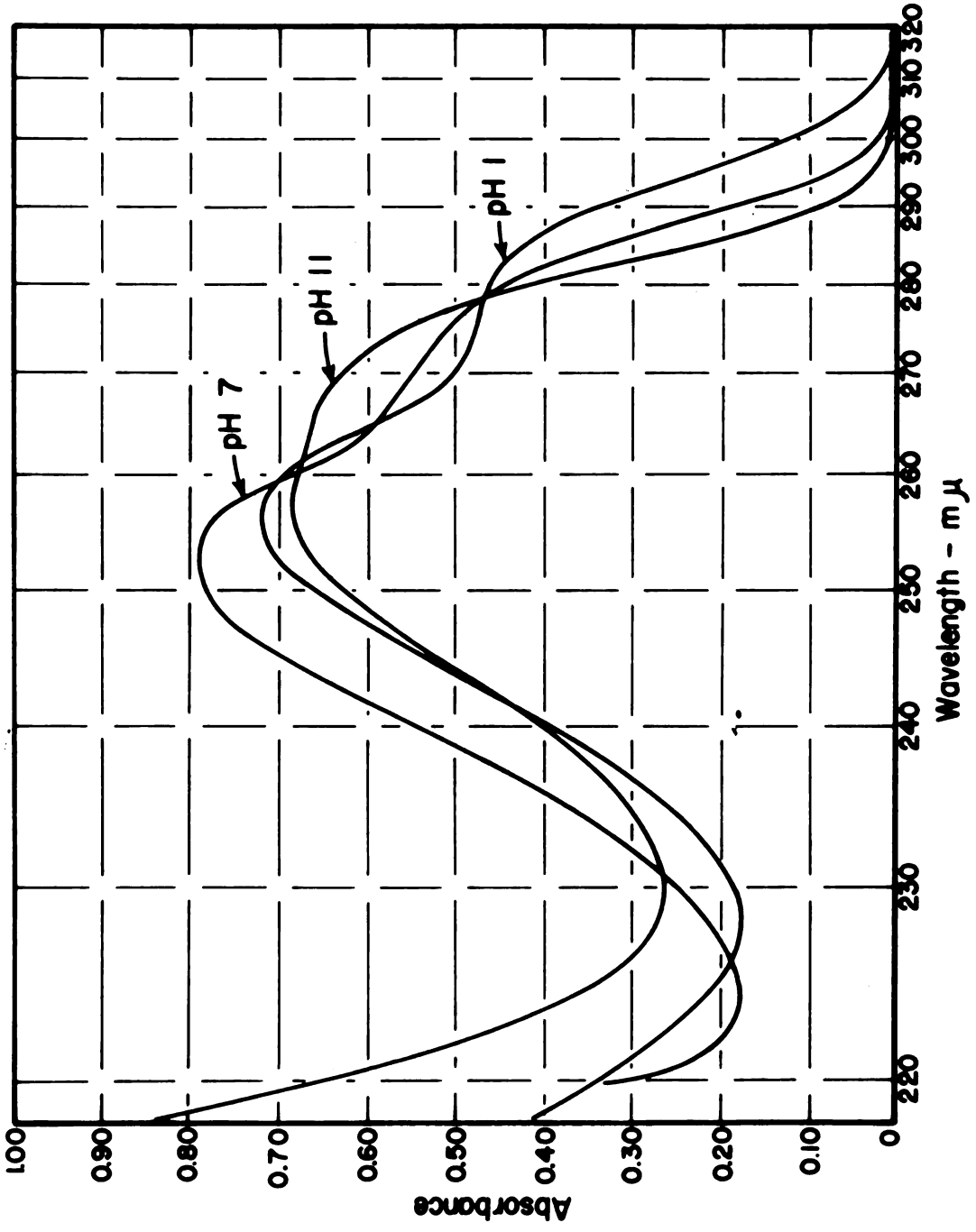

pH 1
pH 11
pH 7
Absorbance
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0
220
230
240
250
260
270
280
290
300
310
320
Wavelength - mμ

Table 2. Chemical analyses of GDP-hexoses.

	GDP-glucose		GDP-hexose	
	Chemically Synthesized	Enzymically[1] Synthesized	Isolated[1]	Theoretical
Guanosine	1.03	1.09	1.10	1.00
Total Phosphorus[2]	2.00	2.00	2.00	2.00
Acid-Labile Phosphorus	0.96	1.06	1.04	1.00
Ribose	1.00	1.14	1.12	1.00
Reducing Value	0.98	1.03	1.08	1.00

[1]Guanosine values corrected for interfering ultraviolet absorbing materials according to the following equation (5):

$$\text{Guanosine concentration } (\mu\text{moles}) = \frac{(A_{250} - A_{\lambda})}{\alpha_{m,250} - \alpha_{m,\lambda}} \times 100$$

where A_{250} is the absorbancy at 250 mμ and A_{λ} is the absorbancy at wavelength λ ; $\alpha_{m,250}$ is the extinction coefficient at 250 mμ and $\alpha_{m,\lambda}$ is the extinction coefficient at wavelength λ . The same results were obtained with λ = 230 mμ or λ = 280 mμ.

[2]Results based on total phosphorus value as 2.

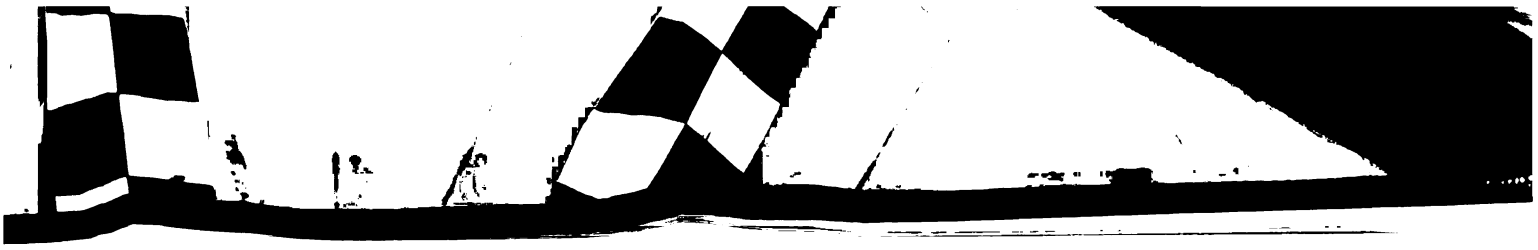
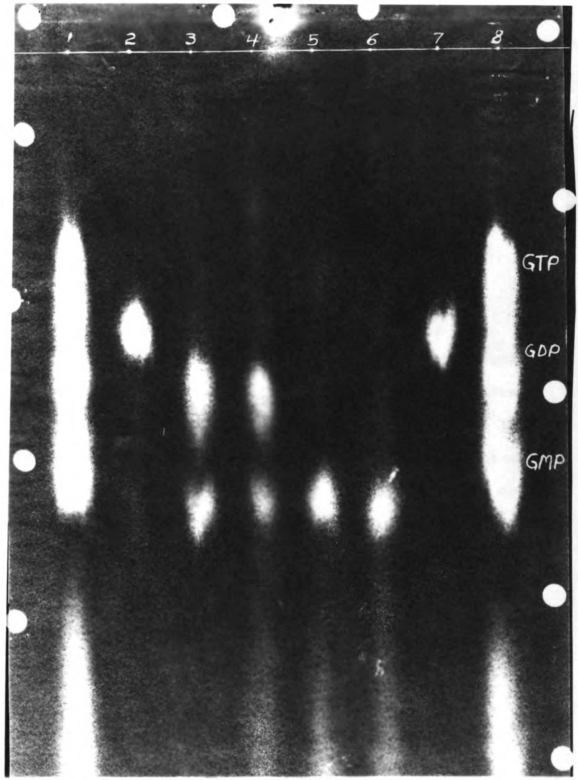


Figure 5. Effect of acid hydrolysis on GDP-glucose.

Chromatograms were developed with isobutyric acid-NH_4OH-H_2O for 24 hours and photographed to reveal the ultraviolet absorbing spots. The spots at the origin are as follows: 1 and 8 are standards of GMP, GDP, and GTP; 2, 3, and 5 are enzymically prepared GDP-glucose, before hydrolysis, and after hydrolysis for 15 minutes and 3 hours, respectively, at pH 2; 7, 4, and 6 are chemically synthesized GDP-glucose before hydrolysis, after hydrolysis for 15 minutes and 3 hours, respectively, at pH 2.


1
2
3
4
5
6
7
8
GTP
GDP
GMP

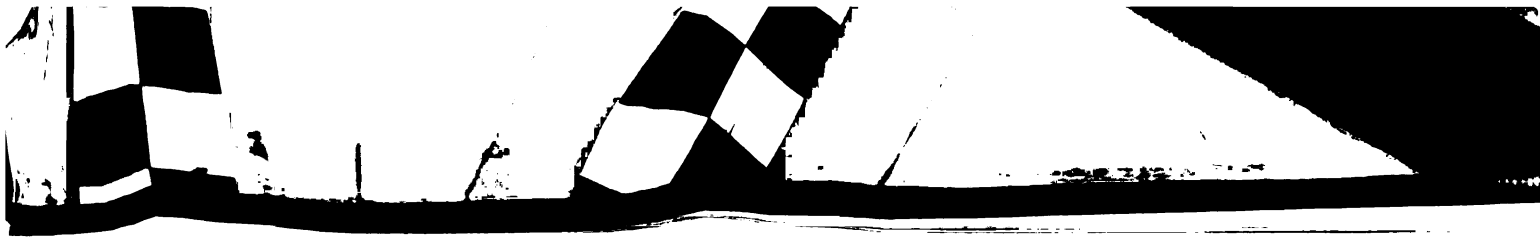


Figure 6. Release of reducing sugar from GDP-glucose.

Tubes containing either enzymically synthesized GDP-glucose or chemically synthesized GDP-glucose in 0.01 N HCl were heated at 100° and aliquots removed at the designated time intervals. After neutralization, reducing sugar was determined. Each aliquot was checked for guanosine concentration by 260 mμ absorption to minimize errors introduced by evaporation.

$$\frac{\mu\text{moles guanosine}}{\mu\text{moles glucose}} \times 100 = \text{percent hydrolysis.}$$

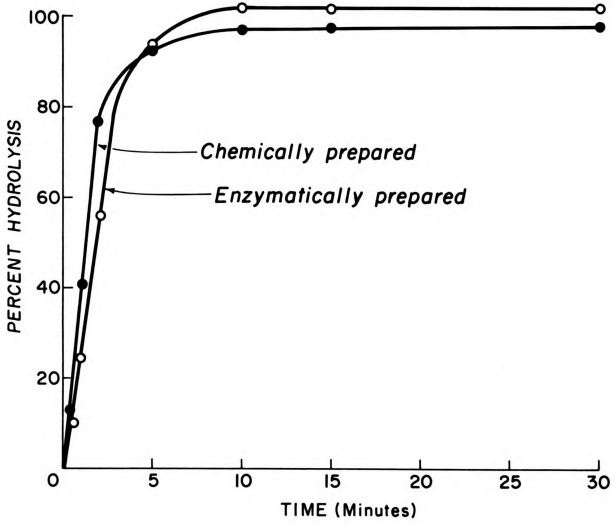

100
80
60
40
20
0
5
10
15
20
25
30
PERCENT HYDROLYSIS
TIME (Minutes)
Chemically prepared
Enzymatically prepared

Table 3. Chromatographic and electrophoretic mobilities of hexose nucleotides.[1]

	Source	Ethanol-ammonium acetate pH 7.5	Ethanol-ammonium acetate pH 3.8	Isobutyric acid-ammonium hydroxide-water	Electrophoresis in citrate buffer
GDP-glucose	Chemical	1.34	0.28	0.65	1.24
GDP-glucose	Enzymatic	1.35	0.28	0.65	1.23
GDP-mannose	Chemical	1.34	0.27	0.67	1.24
GDP-hexose	Isolated	1.36	0.27	0.66	1.22
UDP-glucose		2.74	0.84	0.64	1.34

[1]Mobility relative to GMP.

electrophoretically identical in 0.05 M citrate buffer, pH 4.4. Table 4 shows the relative chromatographic mobilities of the sugars released from the GDP-hexose preparations by acid-hydrolysis. Chemically and enzymically formed GDP-glucoses gave only glucose upon hydrolysis. Isolated GDP-hexoses gave compounds corresponding to mannose and glucose with faint spots with mobilities similar to fructose and fucose. Fructose and mannose have the same mobility in butanol-pyridine and water and these are shown to be separated with phenol and water. The faint fucose spot was not detectable when phenol-water was used because of the darker background encountered with ammonical-silver nitrate as the developing reagent.

5. Hydrolysis with snake venom nucleotide pyrophosphatase. Treatment of both the chemically and enzymically formed GDP-glucose with snake venom gave quantitative liberation of glucose-1-P as determined by the reduction of TPN in the presence of phosphoglucomutase and glucose-6-phosphate dehydrogenase. As phosphoglucomutase is specific for the α-form of glucose-1-P, the compounds exist in the α-configuration as does UDP-glucose or are rapidly converted to the α-form by the snake venom preparation.

6. Incorporation of glucose-1-P-C^{14}. Incubation of glucose-1-P-C^{14} and GTP with the mammary gland enzyme yielded a radioactive nucleotide which was chromatographically identical to GDP-glucose. Acid hydrolysis of the isolated nucleotide and subsequent co-chromatography of the hexose portion in three solvents yielded only glucose.

Table 4. Paper chromatography of sugars released by acid hydrolysis of nucleotides.[1]

	Source	Butanol-pyridine-water	Ethyl acetate-pyridine-water	Phenol-water
Mannose		0.83		0.68
Fucose		0.93		0.94
Fructose		0.83		0.79
Galactose		0.66		0.64
Glucose		0.73	0.70	0.60
UDP-glucose		0.76	0.70	0.60
GDP-glucose	Chemical	0.76	0.69	0.60
GDP-glucose	Enzymatic	0.76	0.69	0.60
GDP-hexose	Isolated	0.75		0.58
		0.84		0.67
		0.94		0.79

[1]Mobility relative to ribose.

Formation of hexose nucleotides catalyzed by pyrophosphorylases from different sources.

Calf liver acetone powder was the same as that used for the purification of UDP-glucose dehydrogenase. Dried brewer's yeast was purchased from the Anheuser-Busch Brewing Company. The Eremothecium ashbyii culture was purchased from the American Type Culture Collection (6747) and was cultivated according to MacLaren (51). Laying hens were sacrificed and the oviducts were removed and placed in ice. An acetone powder was prepared as before. Hansenula holstii was provided by R. K. Bretthauer. In all cases extracts were made as previously described for mammary gland preparations.

Pyrophosphorylase activity of the seven preparations with respect to the synthesis of UDP-glucose, GDP-mannose and GDP-glucose is recorded in Table 5. With the assay conditions described, only mammary gland was a source for an enzyme that forms detectable amounts of GDP-glucose whereas UDP-glucose pyrophosphorylase is found in all seven preparations. GDP-mannose pyrophosphorylase activity was not found in calf liver, hen oviduct or Eremothecium ashbyii. Hen oviduct contains a very active phosphatase which liberates over 90 percent of the acid-labile phosphorus from GTP in about 20 minutes confounding the measurement of pyrophosphorylase in this preparation. Quantitatively, GDP-glucose synthesis exceeded GDP-mannose synthesis by 10-fold in bovine mammary gland extracts. Although the GDP-glucose pyrophosphorylase was not separated from the GDP-mannose pyrophosphorylase in mammary gland, preparations from other sources which synthesize

Table 5. Catalysis of the hexose nucleotides by pyrophosphorylases.

Enzyme source	UDP-glucose	GDP-mannose	GDP-glucose
Bovine mammary gland	+	+	+
Rat mammary gland	+	+	+
Saccharomyces cerevisiae	+	+	-
Hansenula holstii	+	+	-
Calf liver	+	-	-
Hen oviduct	+	-	-
Eremothecium ashbyii	+	-	-

GDP-mannose were not able to synthesize GDP-glucose. On the basis of present data, it is most likely that GDP-glucose synthesis is due to a specific pyrophosphorylase.

UDP-glucose dehydrogenase specificity. It is interesting to note that the incubation of GDP-glucose with UDP-glucose dehydrogenase gave no reduction of DPN (Figure 7), substantiating the specificity of this dehydrogenase.

Partial purification of GDP-glucose pyrophosphorylase and demonstration of reversibility. By using the chromatographic assay procedure described (p. 10), it was possible to show that the enzyme capable of forming GDP-glucose was precipitated by 0.35 saturation of ammonium sulfate (19.9 g/100 ml of supernatant) as illustrated in Figure 8. Chromatographic procedures are too laborious and time consuming for quantitating the enzyme assay to serve as the basis for purification. Therefore, an assay was developed which involved the reduction of TPN via the following reactions:

$$\text{GDP-glucose} + \text{PPi} \rightleftharpoons \text{glucose-1-P} + \text{GTP}$$

$$\text{Glucose-1-P} \rightleftharpoons \text{glucose-6-P}$$

$$\text{Glucose-6-P} + \text{TPN} \longrightarrow \text{6-phosphogluconate} + \text{TPN} + H^+$$

1. Assay conditions. This assay could not be successfully adapted to the crude enzyme from bovine mammary gland because of the presence of a very active nucleotide pyrophosphatase which catalyzed the reaction:

$$\text{GDP-glucose} \longrightarrow \text{Glucose-1-P} + \text{GMP}$$

Figure 7. UDP-glucose dehydrogenase specificity.

The protocol for the assay was as follows:

	1	2	3
	ml added		
GDP-glucose, 10 μmoles/ml.....	-	.02	-
UDP-glucose, 10 μmoles/ml.....	-	-	.02
DPN, 25 μmoles/ml.....	.02	.02	.02
Glycine buffer, 0.1 M, pH 8.7.....	.46	.44	.44
UDP-glucose dehydrogenase, 80,000 units/ml.....	.02	.02	.02

After 60 minutes, UDP-glucose was added to cuvette number 2.

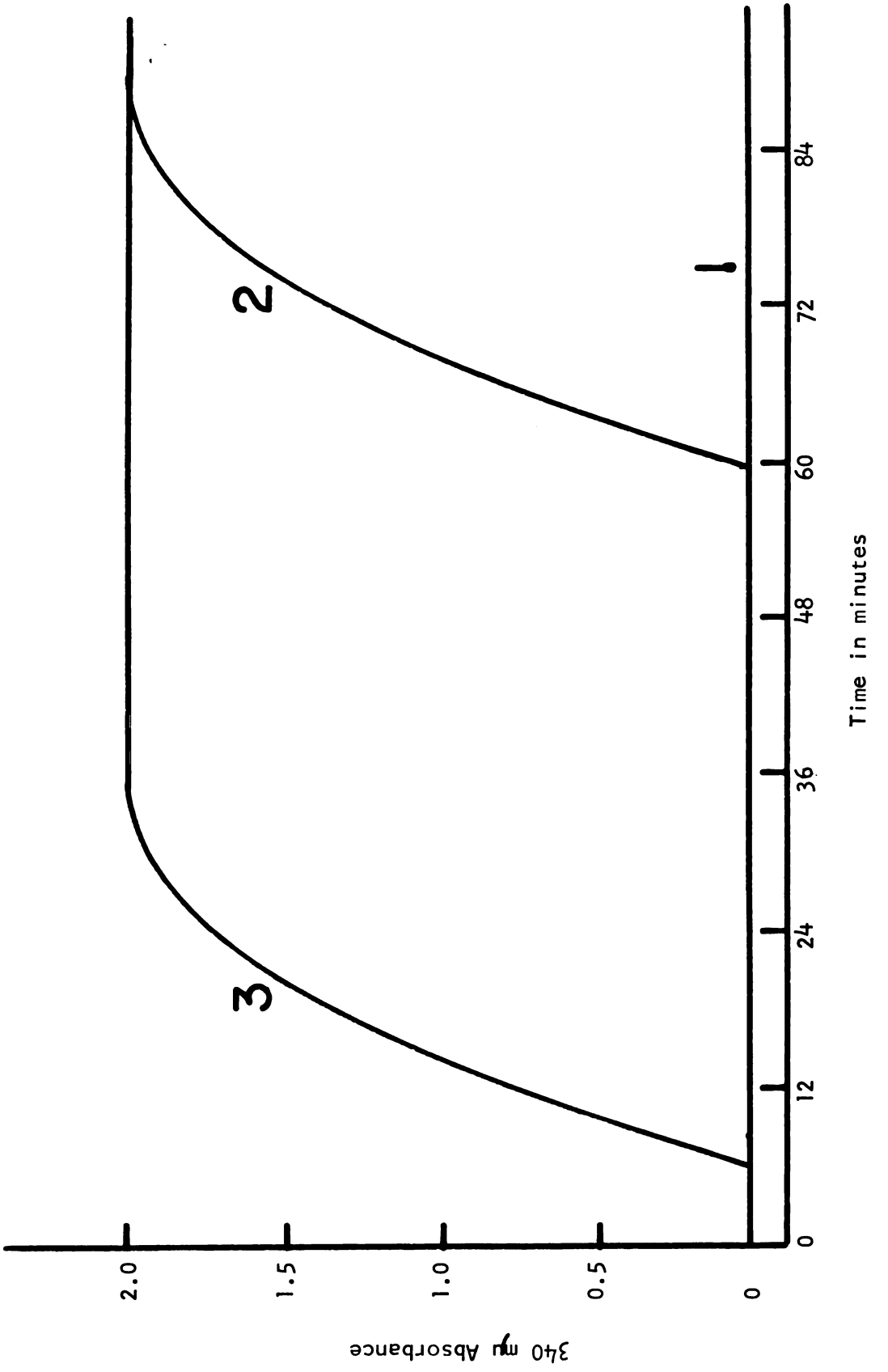
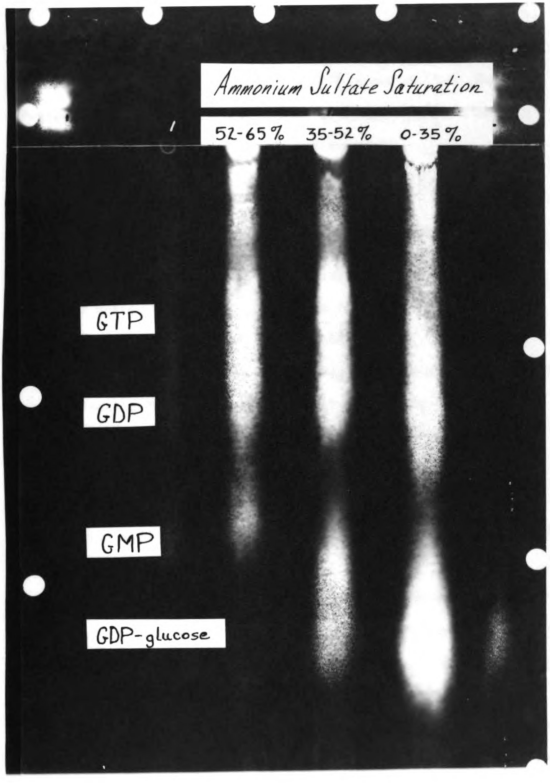
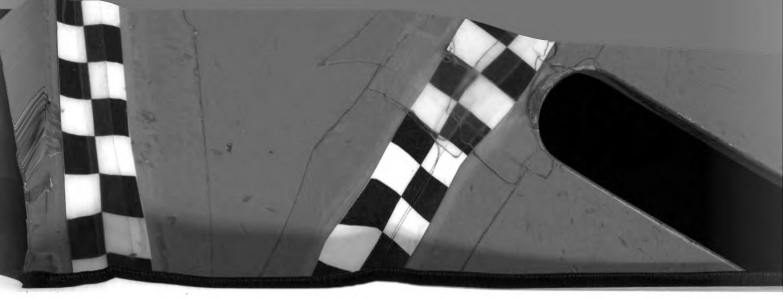

340 mμ Absorbance
2.0
1.5
1.0
0.5
0
0
12
24
36
48
60
72
84
Time in minutes
1
2
3

Figure 8. Fractionation of GDP-glucose pyrophosphorylase with ammonium sulfate.

The chromatographic assay used is described in the text.


Ammonium Sulfate Saturation
52-65%
35-52%
0-35%
GTP
GDP
GMP
GDP-glucose

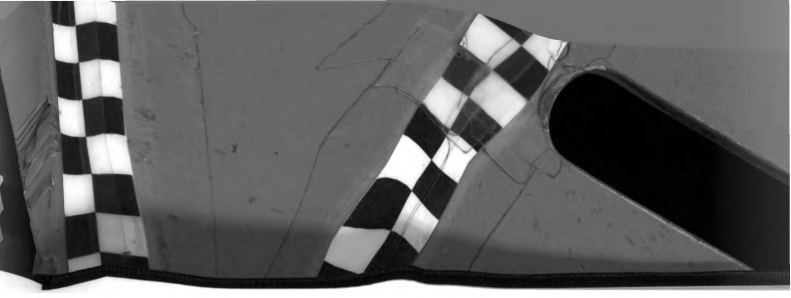


With rat mammary gland acetone powder as the enzyme source it was possible to utilize the spectrophotometric assay outlined above for GDP-glucose pyrophosphorylase. The assay protocol was as follows:

GDP-glucose	0.2 μmoles
PPi	1.5 μmoles
TPN	0.5 μmoles
Phosphoglucomutase	0.02 ml
Glucose-6-P dehydrogenase	0.02 ml
Mixed buffer	To final volume of 0.5 ml
Enzyme preparation	0.05 ml

Phosphoglucomutase and glucose-6-P dehydrogenase were added in excess. Controls lacking PPi and TPN were run simultaneously and all assays were corrected for TPNH formation due to nucleotide pyrophosphatase activity. There was no reduction of TPN until rat mammary gland enzyme was added. A unit of enzyme is defined as the amount of activity that will form one μmole of glucose-1-P per minute under the conditions of the assay.

2. Fractionation of rat mammary gland with ammonium sulfate. One gram of rat mammary gland acetone powder was homogenized with 15 ml of mixed buffer in an Omnimixer. The homogenate was centrifuged at 10,000 x g and the precipitate discarded. Saturated ammonium sulfate, pH 8.3, was added dropwise to the supernatant (4.7 ml/10 ml supernatant) and the precipitate was removed by centrifugation. The precipitate was dissolved in 0.5 ml of mixed buffer and is referred to as A.S.1. The next fraction was precipitated by the addition of 3.2



ml of saturated ammonium sulfate to the first supernatant. The resulting precipitate was dissolved in 1 ml of buffer and referred to as A.S. II. A.S. III was prepared by the addition of 4.8 ml of ammonium sulfate to the supernatant of A.S. II and the precipitate dissolved in 1 ml of buffer. The approximate ammonium sulfate saturation is given with the summary of purification in Table 6. The change in the ratio of UDP-glucose pyrophosphorylase to GDP-glucose pyrophosphorylase is evidence that the formation of GDP-glucose is due to a separate enzyme.

The suitability of the assay for further purification studies is demonstrated in Figure 9. The reaction is linear over at least a five-fold change in enzyme concentration. Figure 10 demonstrates the requirement for PPi and shows the effect of different levels in the reaction mixture on the rate of reaction. An increase in the reaction rate is noted up to 1.5 μmoles of PPi. Increasing the concentration to 2.5 μmoles has an inhibitory effect while higher concentrations caused turbidity in the cuvette. There was no loss of activity after centrifugation of the crude homogenate for 1 hour at 100,000 x g, demonstrating that the enzyme is located in the soluble fraction.

3. Incorporation of P^{32} into GTP. Attempts to demonstrate the incorporation of labeling into GTP from PPi^{32}[1] were not successful with the crude enzyme extracts. However, when the enzyme preparation A.S. II was used, incorporation of labeling from PPi into GTP was

[1]The PPi^{32} was prepared by pyrolysis of Pi^{32} obtained from the Oak Ridge National Laboratories, Oak Ridge, Tennessee.

Table 6. Purification of GDP-glucose pyrophosphorylase.

Fraction	Ammonium sulfate saturation	ml	Total units	Total Protein (mg)	Specific Activity	Ratio of Activity[1]
Crude homogenate	0	10	0.515	129.5	.004	4.85
A.S. I	.23	0.5	0.05	7.78	.006	1.30
A.S. II	.40	1.0	0.384	24.72	.016	2.73
A.S. III	.60	1.0	.008	18.23	<.001	8.20

[1]Ratio of UDP-glucose pyrophosphorylase activity to GDP-glucose pyrophosphorylase activity.

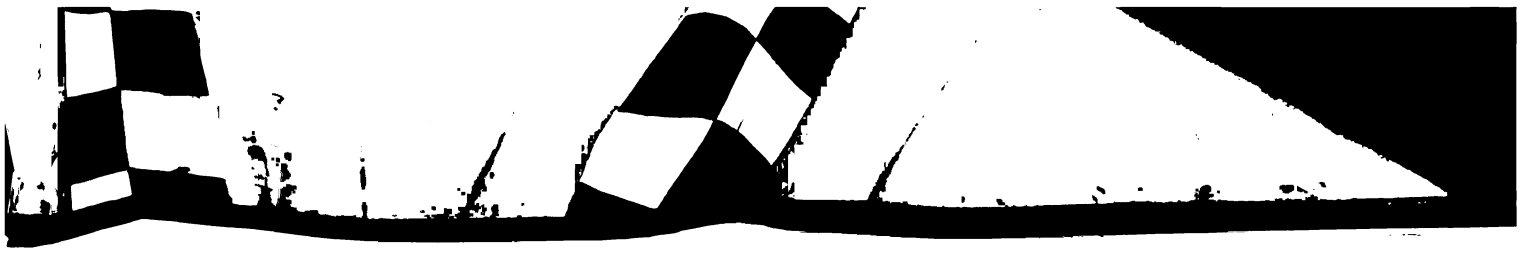


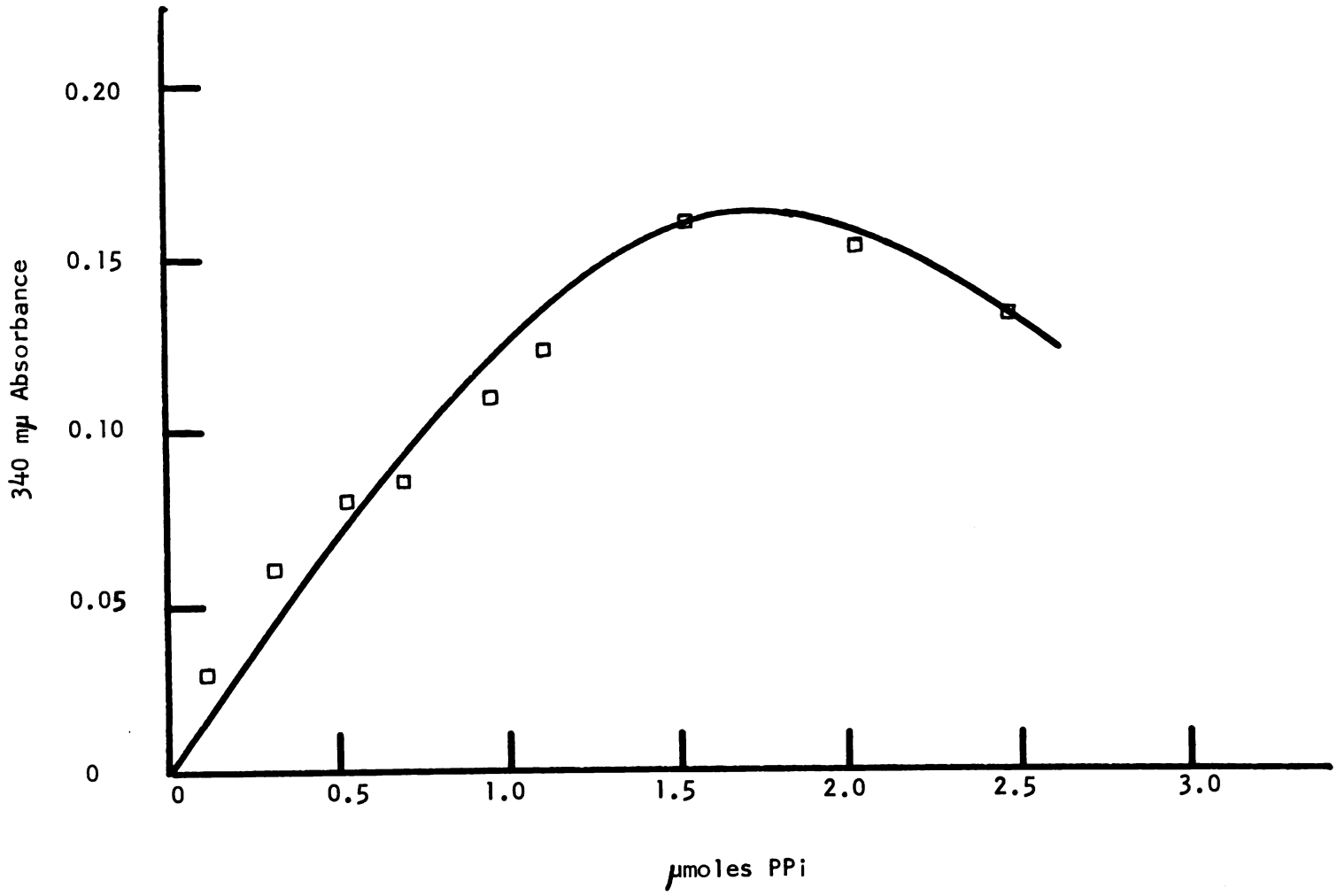
Figure 9. Effect of enzyme concentration on reaction rate.

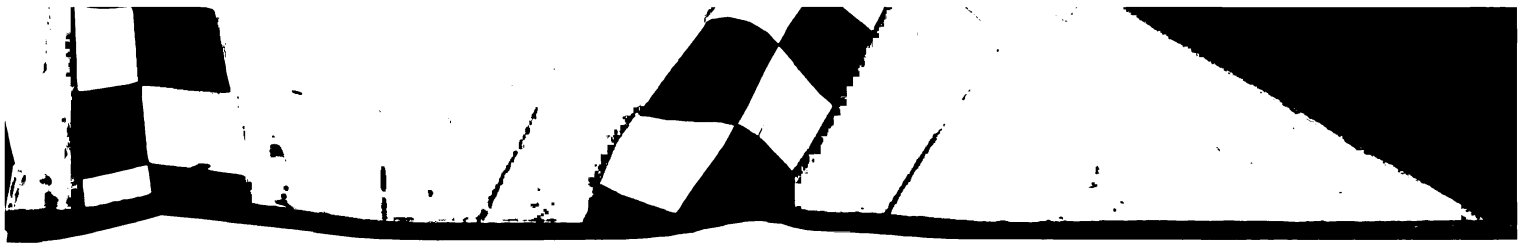
For details on the assay, see the text.

340 mμ Absorbance
0
0.1
0.2
0.3
0.4
0.5
0
0.1
0.2
0.3
0.4
0.5
0.6
M1 enzyme

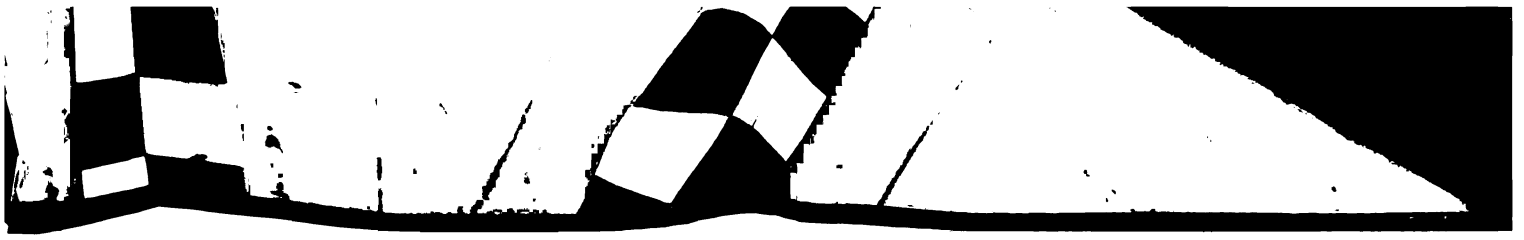
Figure 10. Effect of pyrophosphate concentration on reaction rate.

The details are given in the text.

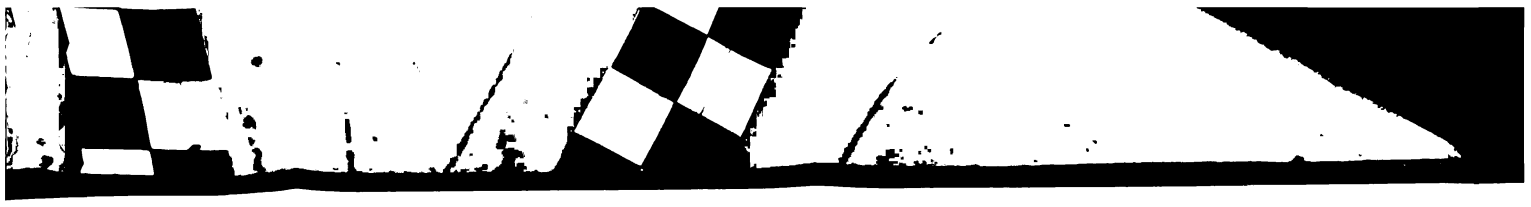

0.20
0.15
0.10
0.05
0
340 mμ Absorbance
0
0.5
1.0
1.5
2.0
2.5
3.0
μmoles PPi



readily demonstrated. Two incubations were prepared as shown for the enzyme assay on p. 45, except the amount of GDP-glucose was doubled, with no PPi in the control and 2.15 μmoles (150,000 cpm/μmole) in the second tube. These were incubated for 30 minutes at room temperature and the reaction terminated by heating at 100° for 1 minute. Nucleotides were adsorbed on norite and the norite was washed exhaustively with water. The nucleotides were then eluted with 15 percent aqueous pyridine and portions of the water wash and the pyridine eluent were counted with a gas flow counter. The water wash contained a total of 276,000 cpm and the pyridine eluent contained 31,200 cpm. The nucleotide fraction accounts for about one-tenth of the P^{32} recovered. The labeled GTP co-chromatographed with authentic GTP in ethanol-ammonium acetate, pH 7.5 and was identical by electrophoresis in 0.05 M, citrate buffer, pH 4.4



DISCUSSION

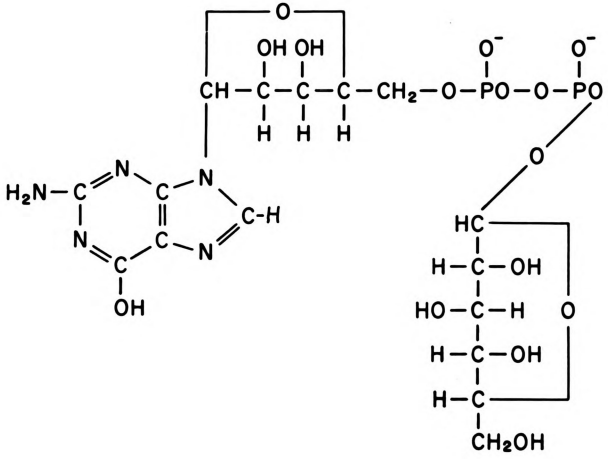


DISCUSSION

The natural occurrence and biosynthesis of GDP-glucose has now been adequately demonstrated. By using chemical and enzymic analyses, the structure of GDP-glucose is consistent with guanosine 5'-diphosphate α-D-glucose as shown in Figure 11. With the previously published occurrence of GDP-hexoses, it is becoming increasingly evident that the guanosine nucleotides probably have a significant function in carbohydrate metabolism, but this largely remains to be demonstrated. The participation of these sugar nucleotides in polymer formation is still speculative. As yet the only direct metabolic role of GDP-mannose is as a precursor of GDP-fucose, the sugar portion of which may be incorporated into mucopolysaccharides.

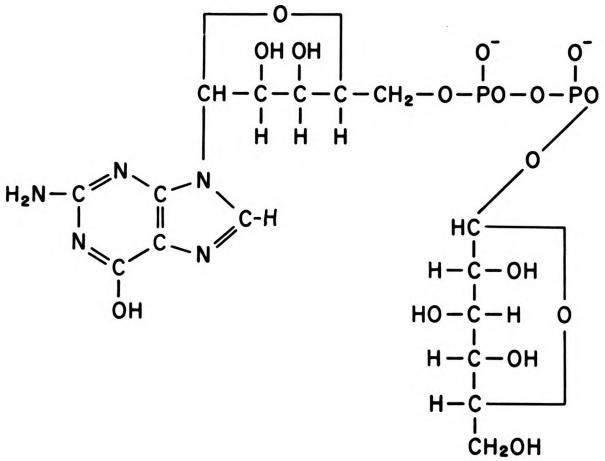
Mammary tissue was the only preparation studied which contained GDP-glucose pyrophosphorylase. The reason for this is not obvious. As there were no transformations of GDP-glucose into other carbohydrate derivatives during the incubations with mammary tissue, GDP-glucose likely serves as a glucosyl donor for some polymer, e.g., glycoprotein or glycolipid. Experiments designed to test this hypothesis are now in progress. The possibility that sugar nucleotides could serve as a storage form of nucleoside triphosphates should not be overlooked. In mammary tissue GDP-hexose, in the presence of PPi, could serve as a source of GTP, which has been shown to be required for protein synthesis.

Robbins and Uchida (52) have demonstrated the presence of glucose in type-specific polysaccharides of phage-infected Salmonella. This



Guanosine diphosphate glucose

Figure 11. Structure of guanosine diphosphate glucose.



Guanosine diphosphate glucose

seems to be the only difference in the polysaccharide produced by phage-infected and non-infected cells, as glucose is absent in the latter. Attempts to demonstrate UDP-glucose as the glucosyl donor were unsuccessful, but some evidence was obtained which suggested that GDP-glucose may be the glucosyl donor.[1] However, the presence of an enzyme, e.g. GDP-glucose pyrophosphorylase, that could catalyze the formation of GDP-glucose has not been demonstrated.

Two nucleotide oligosaccharides have recently been isolated from goat colostrum by Jourdian, et al. (53). While the ability of uridine sugar nucleotides to transfer their carbohydrate moiety to primer polymers has been well established, this is the first report of the isolation of a nucleotide oligosaccharide. The oligosaccharide portion of both compounds contained N-acetylglucosamine, galactose, and sialic acid. The distinguishing feature between the two compounds isolated was the sialic acid portion, one contained N-acetylneuraminic acid and the other contained N-glycolylneuraminic acid. This new type of nucleotide may be involved in the biosynthesis of mixed polymers like glycoproteins and glycolipids. Attempts to synthesize nucleotide oligosaccharides are now in progress[2].

Mendicino has suggested[3] that the following reaction may be present in mammary gland and that it could be responsible for the GDP-mannose formation:

GDP-glucose + mannose-1-P $\rightleftharpoons$ GDP-mannose + glucose-1-P

As the formation of GDP-glucose is required for the above reaction,

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[1,2,3]Personal communications.

the mannose-1-P used would have to be contaminated with glucose-1-P. This, in the author's opinion, is not the case. Also, by replacing PPi with mannose-1-P in the spectrophotometric assay glucose-1-P should be formed. This then could be measured by the reduction of TPN as discussed previously. There was no reduction of TPN which suggests that GDP-mannose was not formed by a transferase of this type in the preparations studied.

SUMMARY

SUMMARY

The biosynthesis and chemical synthesis of guanosine diphosphate glucose has been reported for the first time. Isolation and characterization of guanosine diphosphate hexoses from lactating bovine mammary gland has demonstrated the presence of guanosine diphosphate mannose and guanosine diphosphate glucose. Tentative evidence for the presence of guanosine diphosphate fucose and guanosine diphosphate fructose is presented. The enzyme responsible for catalyzing the synthesis of guanosine diphosphate glucose has been shown to be separate from uridine diphosphate glucose pyrophosphorylase. The product of the reaction has been identified as guanosine 5'-diphosphate- α -D-glucose.

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