ABSTRACT

CARBOHYDRATE METABOLISM

I. ACCESSING HETEROZYGOSITY FOR GALACTOSEMIA BY ENZYMATIC MEANS

II. METABOLISM OF MANNOSE BY Hansenula holstii

by Roger Keith Bretthauer

Part I

Galactosemia is a metabolic disorder characterized by a deficiency of the enzyme galactose-1-phosphate uridyl transferase. A quantitative assay for this enzyme in erythrocytes has been developed and applied to 14 families of known galactosemic children and to a group of 106 normal individuals. Three distinct groups of enzyme activities are apparent: affected children having little or none; a group including all parents of affected children having an intermediate level; and normal controls having a full level. These results confirm that this disorder is hereditary and imply that it is transmitted as a simple, autosomal recessive trait. Preliminary studies on comparison of the incidence of galactosemic heterozygotes in a normal and mentally retarded population have resulted in the detection of at least two heterozygotes from the 138 normal individuals examined.

Part II

The metabolism of mannose by Hansenula holstii NRRL Y-2448 has been investigated in relation to the

extracellular phosphorylated mannan produced by this organism. Mannose is phosphorylated and isomerized to fructose-6-phosphate, as is glucose. The presence of mutases was demonstrated by the conversion of glucose-1-phosphate and mannose-1-phosphate to the corresponding hexose-6-phosphates. Oxidation of glucose-6-phosphate and 6-phosphogluconic acid occurred in the presence of triphosphopyridine nucleotide. The presence of pyrophosphorylases capable of catalyzing the formation of guanosine diphosphate mannose, guanosine diphosphate glucose, and uridine diphosphate glucose from the respective nucleoside triphosphates and hexose-1-phosphates was established. No interconversion of guanosine diphosphate hexoses or oxidation of uridine diphosphate glucose was detected.

Acid soluble nucleotides, including guanosine diphosphate hexoses and uridine diphosphate hexoses, were isolated and identified. The guanosine diphosphate hexoses contained mannose and minor amounts of glucose and fructose.

The synthesis of phosphomannan by washed, resting cells from glucose and inorganic phosphate and by whole cultures from C^{14}-mannose is described. No incorporation of radioactivity from C^{14}-guanosine diphosphate mannose or C^{14}-mannose-1-phosphate into phosphomannan was achieved.

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II. METABOLISM OF MANNOSE BY *Hansenula holstii*

by

Roger Keith Bretthauer

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To Donna

VITA

Roger K. Bretthauer was born on January 4, 1935, at Morris, Illinois. He graduated from the Yorkville High School in 1952 and attended Blackburn College for two years. After transferring to the University of Illinois, he received the B.S. degree in Agriculture in 1956 and the M.S. degree from the Department of Dairy Science in 1958. Remaining formal academic training and research requirements for the Ph.D. degree were completed in the Department of Biochemistry at Michigan State University. A postdoctoral position at the Department of Bacteriology, University of Wisconsin, has been accepted.

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CARBOHYDRATE METABOLISM

I. ACCESSING HETEROZYGOSITY FOR GALACTOSEMIA BY ENZYMATIC MEANS

INTRODUCTION

Recent advances in biochemical studies of hereditary metabolic disorders have revealed in many cases that the disorder is characterized by an enzymic deficiency. Heterozygous carriers of these disorders have been detected by various techniques, in some instances by direct measurement of the deficient enzyme. Many of these disorders are thought to be transmitted by autosomal recessive genes, as both parents of an affected individual appear to be heterozygous with respect to the trait.

One such metabolic disorder which has been shown to be characterized by a single enzymic deficiency is galactosemia. The lack of Gal-1-P uridyl transferase[1] results in an inability to convert galactose, a normal constituent of milk, to the common metabolite glucose. The affect is clearly illustrated by the *in vitro* accumulation of Gal-1-P in erythrocytes when incubated with galactose. If death does not occur, the secondary abnormalities which result, if all dietary galactose is not removed from the affected person, are cataracts, mental retardation and enlargement of the liver.

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[1]The following abbreviations are used: Gal-1-P, galactose-1-phosphate; G-1-P, glucose-1-phosphate; ATP and ADP, adenosine tri- and diphosphate; UTP and UDP, uridine tri- and diphosphate; UDPG, uridine diphosphate glucose; UDPGal, uridine diphosphate galactose; PP, inorganic pyrophosphate; DPN, oxidized diphosphopyridine nucleotide.

The discovery of the biochemical defect of galactosemia presents an unique opportunity to study the enzymic expression of a heterozygous state and to elucidate the mode of inheritance of this disorder. Previous methods have not been sufficiently sensitive to rigorously define this problem.

The purpose of this thesis is to present a method which defines the heterozygote, and which, when applied to family studies, has revealed an autosomal recessive mode of inheritance. Preliminary studies on the incidence of heterozygosity in a normal and mentally retarded population are also presented.

LITERATURE

The first report of galactosemia was in 1908 by Von Reuss (1). Since that time, many other cases with similar clinical features have appeared in the literature. In general the symptoms of the fully manifested disease are jaundice, hepatomegaly, cirrhosis, cataracts, galactosuria, amino-aciduria, and failure to grow. Normally these symptoms occur soon after birth and if not detected, may result in severe cataracts, mental retardation, and often death. Early exclusion of all dietary galactose was found to relieve this condition, and thus the eventual finding of an enzymic deficiency in galactose metabolism as the biochemical defect (2,3).

Schwarz et al. (4) noted an accumulation of Gal-1-P in erythrocytes of galactosemic persons when incubated in the presence of galactose. The significance of this discovery was realized when Kalckar and associates (5-7) demonstrated a deficiency of Gal-1-P uridyl transferase (reaction 2 below) in both erythrocytes and liver of affected individuals. Other enzymes involved in galactose metabolism (reactions 1, 3, and 4 below) were present in normal amounts.

The discovery of such an enzymatic deficiency was possible only after much detailed experimentation with various biological sources revealed the following pathway of galactose metabolism:

1) Galactose + ATP $\xrightarrow{\text{Galactokinase}}$ Gal-1-P + ADP

2) Gal-1-P + UDPG $\xrightleftharpoons[\text{transferase}]{\text{Gal-1-P uridyl}}$ G-1-P + UDPGal

3) UDPGal $\xrightleftharpoons{\text{UDPGal-4-epimerase}}$ UDPG

4) G-1-P + UTP $\xrightleftharpoons{\text{UDPG pyrophosphorylase}}$ UDPG + PP

5) Gal-1-P + UTP $\xrightleftharpoons{\text{UDPGal pyrophosphorylase}}$ UDPGal + PP

The irreversible phosphorylation of galactose resulting in Gal-1-P is followed by its conversion to G-1-P (reaction 2) which involves UDPG. UDPGal is formed which supplies the galactose essential for galactolipids, mucopolysaccharides and other galactose containing compounds. In the absence of this reversible reaction, Gal-1-P accumulates and thus the utilization of galactose is affected. In reaction 3 UDPGal is converted to UDPG by the enzyme UDPGal-4-epimerase. As this reaction is reversible, UDPGal can therefore be formed from UDPG, which in turn can be synthesized from G-1-P via reaction 4. These reactions offer an explanation of why the body can satisfy its needs for galactose even on a galactose free diet. Reaction 5, catalyzed by UDPGal-pyrophosphorylase, offers an additional alternative pathway by which Gal-1-P can be incorporated into the nucleotide UDPGal.

Whether Gal-1-P uridyl transferase is completely absent in galactosemic persons is not clear. Simon and Topper (2) found that erythrocytes of galactosemic persons oxidized

galactose at about 0.5% of normal. Eisenberg et al. (8) administered C^{14}-galactose and menthol to a galactosemic adult and found about 3% of the radioactivity in the urine in 24 hours as the glucuronide of menthol. Thus it appears that the block at reaction 2 may not be complete, or that galactose is being metabolized via alternate routes. One such possible by-pass, UDPGal-pyrophosphorylase (reaction 5), has been demonstrated in mammalian tissues (9).

Earlier reports of this disorder mentioned that siblings of both sexes were affected and that parents were phenotypically normal. Such reports suggest an autosomal, recessive mode of inheritance. Holzel and Komrower (10), employing an oral galactose loading test, demonstrated abnormal galactose tolerance in one parent in each of four families. In one instance of consanquinity, both parents exhibited abnormal tolerance. While suggesting a hereditary etiology, the data did not prove a recessive mode of inheritance, in which both parents should be heterozygous. In a more extensive application of this method, Donnell et al. (11) found that a group of mothers and fathers of affected children could be distinguished from a group of normals on a statistical basis. However, the overlap of individual values was so considerable that the technique has to be regarded as unsuitable for defining the heterozygous condition. Similar limitations apply to the UDPG consumption test, originally designed by Kalckar and coworkers (5-7) to detect the

homozygous condition. The kinetic limitations of this test were pointed out by Anderson et al. (12), who were unable to detect decreased activity of Gal-1-P uridyl transferase in parents of affected children. Hsia et al. (13) have employed this previous method in attempting to identify heterozygotes, but examination of their data reveals extensive overlap between normals and assumed heterozygotes.

Concurrent with the studies to be reported in this thesis, Kirkman and Bynum (14) developed a manometric assay based on the oxidation of Gal-1-P by hemolyzates under conditions of limiting transferase levels. Application of this method to 18 parents of galactosemic children resulted in approximately 64% of the activity as in normal controls. However, significant overlapping of groups was still apparent. This technique is also quite complex and costly, thus limiting its practical usefulness.

In an attempt to preserve the relative simplicity of the original UDPG consumption test as described by Kalckar and coworkers (5-7), and unequivocally define the mode of inheritance of this disorder, the assay was quantitated by altering substrate concentrations, hemolyzate concentration, and time of incubation. The description of this assay and results of its application to 14 families each having at least one member affected by galactosemia are presented in this report.

The numerous case reports of galactosemic persons

have revealed that frequently mental development is impaired. It was, therefore, of interest to apply the quantitative transferase assay to a mentally retarded population and see if a significant number of these individuals have abnormal galactose metabolism. Concurrent with these studies, examination of a normal population as controls might reveal the incidence of heterozygosity and homozygous recessives. Preliminary results of these studies are also presented in this report.

MATERIALS AND METHODS

UDPG and DPN were purchased from Sigma Chemical Company. The α-D-Gal-1-P was synthesized according to the procedure described by Hansen *et al.* (15). UDPG dehydrogenase was isolated from calves liver and purified through Step 5 of the procedure of Strominger *et al.* (16). This preparation contained 37,500 units/ml. UDPGal-4-epimerase was purified from *Saccharomyces fragilis* and kindly furnished by Dr. R. J. Forrest (unpublished procedure). This enzyme was also purified by the method of Maxwell and Szulmajster (17) and used successfully. Both preparations contained about 50,000 units/ml. Spectrophotometric determinations were carried out in a Beckman DU spectrophotometer with a photomultiplier attachment and pinhole filter.

EXPERIMENTAL PROCEDURES AND RESULTS

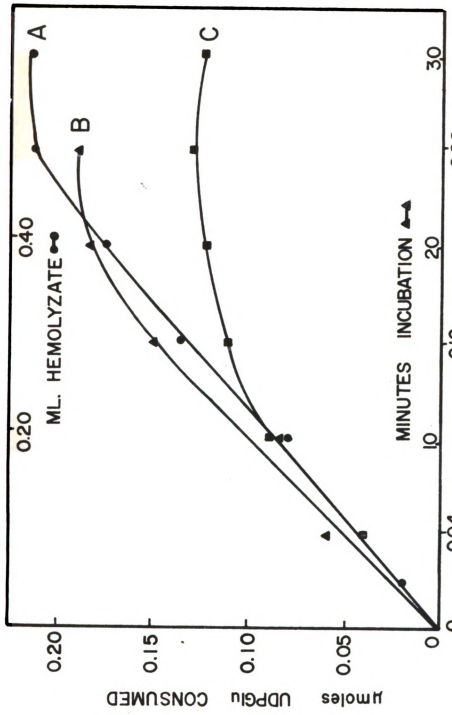
Development of quantitative assay

Blood samples were drawn by venipuncture into balanced oxalate tubes. They were iced and centrifuged in the cold within 30 to 60 minutes. The erythrocytes were washed three times with cold 0.9% NaCl. The last centrifugation was done under standard conditions to provide uniform packing. The erythrocytes were stored frozen until use. Prior to measurement of the enzyme, a hemolyzate was prepared by thawing the frozen erythrocytes and adding an equal volume of cold distilled water.

The principle of the assay is similar to the most recent enzymic assays published by Anderson *et al.* (12) and Maxwell *et al.* (18) with the following major exceptions: the present procedure employs a higher concentration of UDPG and a higher ratio of Gal-1-P to UDPG, less hemolyzate, and a shorter incubation period. A four-fold excess of Gal-1-P over that of UDPG was necessary for optimum transferase activity. While using these assay conditions, namely 0.20 μmole UDPG and 0.80 μmole Gal-1-P, the amount of hemolyzate was varied in a 15 minute incubation period to determine the optimum enzyme concentration. In similar experiments, the substrate concentration and the incubation time were varied while the amount of hemolyzate was held constant at 0.20 ml (Fig. 1). Reasonably good linearity was achieved

Figure 1. Gal-1-P uridyl transferase activity as a function of hemolyzate concentration, time of incubation, and substrate concentration.

Unless otherwise indicated, the reaction mixture contained 100.0 μmoles of glycine, pH 8.7, 0.2 μmole UDPG and 0.8 μmole Gal-1-P (always added in a 1:4 ratio), and 0.2 ml hemolyzate in a total volume of 0.45 ml except for those incubations with varying hemolyzate concentration where the final volume was 0.7 ml. Incubation time was 15 minutes at 37° C. Remaining details of assay are described in the text. A, variable hemolyzate concentration; B, variable time of incubation; C, variable substrate concentration.


A
B
C
0.20
0.40
ML. HEMOLYZATE
0.20
0.15
0.10
0.05
0
μmoles UDPGlu CONSUMED
0
0.04
0.12
0.20
10
20
30
MINUTES INCUBATION
μmoles UDPGlu ADDED

until the UDPG became limiting at approximately 0.50 ml hemolyzate (curve A) or at 20 minutes incubation time (curve B). In the presence of excess substrate (more than 0.12 μmole UDPG), incubation time and/or hemolyzate became limiting (curve C). The difference in slopes of these three curves resulted from using different blood samples for each of the three experiments. For routine determinations the following conditions were selected which afforded a wide range of measurable values with the same assay conditions: 0.20 ml hemolyzate, 0.20 μmole UDPG, 0.80 μmole Gal-1-P, 100.0 μmoles glycine, pH 8.7, in a total volume of 0.45 ml incubated 10 minutes at 37° C. A control incubation lacking the Gal-1-P was run with every sample under identical conditions. All incubations were run in cellulose nitrate tubes. The reagents were all added to the cellulose nitrate tubes in an ice bucket, the hemolyzate being added last. At the moment of placing the tubes in a 37° C water bath, timing was started. At 10 minutes the tubes were placed in a boiling water bath for one minute with shaking, cooled, and the precipitate centrifuged off. The clear supernatant was used for the spectrophotometric UDPG determination.

The microcuvettes contained 0.10 μmole DPN, 45.0 μmoles glycine, pH 8.7, and 0.01 ml of incubation supernatant in a total volume of 0.48 ml. Having the control cuvette (lacking incubation supernatant) set at zero absorption, the absorbancy at 340 mμ of the two test cuvettes was recorded.

UDPG dehydrogenase (0.01 ml) was then added to the control cell and reset to zero absorption before a similar addition to each test cell. After the increase in optical density had ceased, 0.01 ml UDPGal-4-epimerase was added to each cell in the same manner as described for the dehydrogenase and further reduction of DPN recorded. The difference in optical density increases of the two test cells before addition of the epimerase was calculated as μmoles UDPG consumed in the transferase catalyzed reaction. The difference in increases of optical density of the two test cells after addition of epimerase was calculated as μmoles UDPGal formed. The UDPGal recovered was 85 to 105% of the UDPG consumed.

In the application of the method, three families were available for examination to determine if the modified assay would clearly distinguish the heterozygotes from the clinical patient and normal controls. The results of such studies are presented in Table I. Enzyme levels are expressed as μmoles UDPG consumed per hour of incubation per ml of erythrocytes and per gram of hemoglobin (determined according to Anderson et al. (12)).

Normal controls ranged from 4.8 to 8.1 μmoles UDPG/hr/ml erythrocytes. Of special significance is that in every family, both parents exhibited a lower value of transferase, ranging from 2.9 to 3.7 μmoles UDPG/hr/ml erythrocytes. As expected, no activity was exhibited by the galactosemics.

Table I. Gal-1-P Uridyl Transferase Levels in Erythrocytes

	Individual (sex)	μmole UDPG/hr	
		per ml erythrocytes	per gm Hb
Family			
1	Affected child	0	0
	Mother	2.9	8.7
	Father	3.5	10.3
	Sibling (F)	3.0	9.0
	Sibling (M)	7.0	21.2
	Sibling (F)	6.0	--
2	Affected child	0	0
	Mother	3.7	10.8
	Father	3.6	10.7
3	Affected child	0	0
	Mother	3.0	9.4
	Father	3.6	10.7
Controls			
9	Child - male	7.6	23.0
10	Child - male	6.9	21.5
11	Adult - male	8.1	25.4
12	Adult - female	5.9	18.8
25	Adult - male	6.0	17.7
51	Adult - female	6.0	18.3
52	Adult - female	5.0	14.9
53	Child - female	6.5	21.7
58	Child - male	5.0	15.8
60	Adult - male	4.8	14.7
61	Adult - female	5.0	14.7
Mean			
	Galactosemics	0	0
	Carriers	3.33	9.94
	Normals	6.14	19.0
Range			
	Galactosemics	0	0
	Carriers	2.9-3.7	8.7-10.8
	Normals	4.8-8.1	14.7-25.4

Other members of the family resulted in either normal or intermediate levels of enzyme. This data clearly demonstrates that the assay is quantitative and capable of detecting the heterozygous condition with no overlap of values. As both parents exhibited an intermediate level of transferase, a recessive mode of inheritance is implicated.

Studies on families with galactosemic children

A more detailed genetic study of several more known galactosemic families was undertaken to more clearly define the expected range of transferase levels for galactosemics, heterozygotes, and normals. Fourteen families, including the three previous families, were available in which a known case of galactosemia had occurred. Blood samples were obtained from affected children, their siblings, their mothers, and from all but three of the fathers. Also, blood samples were obtained from a great number of other relatives.

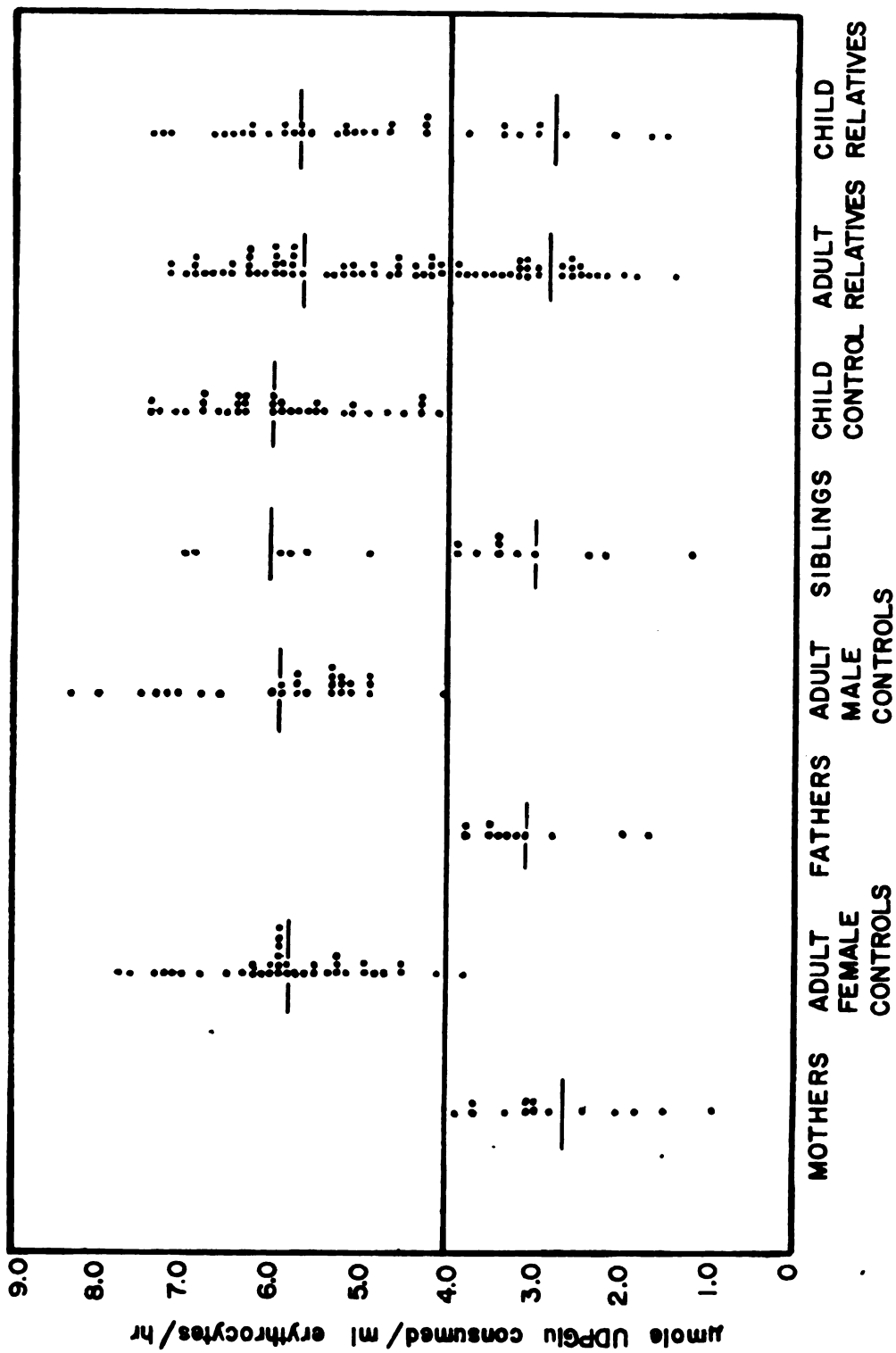
Normal controls were chosen from resident physicians, nurses, and other adults and children in which no family history of galactosemia was evident or in which no history of mental retardation, liver dysfunction, or metabolic disease was evident.

All of the previous samples were taken at the Childrens Hospital of Los Angeles, packed in dry ice, and shipped by air to East Lansing.

A total of 278 individuals were examined. Fig. 2 presents a scattergram of all the data obtained divided

Figure 2. Scattergram of values for Gal-1-P uridyl transferase activity from normals and fourteen families with galactosemic children.

The lines in each column represent the mean value.


μmole UDPGlu consumed/ ml erythrocytes/ hr
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
MOTHERS
ADULT FEMALE CONTROLS
FATHERS
ADULT MALE CONTROLS
SIBLINGS
CHILD CONTROL
ADULT RELATIVES
CHILD RELATIVES

into the various groups considered. The distribution into normal and heterozygous categories is evident, including the expected division among relatives and siblings of affected children.

Table II summarizes the mean values for Gal-1-P uridyl transferase from erythrocytes of 106 normal controls, subdivided by age and sex. The mean values of the groups and their standard deviation approximate each other. The mean value for normal controls has been taken as 5.9 ± 1.0 μmole UDPG consumed/ml erythrocytes/hr.

Data from members of affected families are presented in Table III. Some transferase activity was found in erythrocytes from 3 of the 14 affected children. Two of these had previously received blood transfusions.

Values of transferase in the erythrocytes of the parents studied averaged 2.9 ± 0.83, and all values were less than 3.9 μmoles UDPG consumed/ml erythrocytes/hr. All controls had values above 4.0, with one exception at 3.8. A value of 4.0 was arbitrarily selected as the value which differentiated heterozygotes from normals. Values of transferase in erythrocytes from unaffected siblings of affected children ranged from 1.1 to 7.0. Application of the arbitrary dividing value of 4.0 yielded two groups with values for means and standard deviations of 3.0 ± 0.87 and 6.0 ± 0.78, respectively. The similarity of these means and standard deviations to those obtained with known heterozygotes

Table II. Activity of Gal-1-P Uridyl Transferase in Erythrocytes from Normal Controls

	Gal-1-P Uridyl Transferase (μmole UDPG/ml erythrocytes/hr)			
	No.	Mean	Range	Standard Deviation
Adult males	28	5.9	4.0-8.3	1.06
Adult females	39	5.8	3.8-7.8	0.95
Child males	21	6.0	4.3-7.4	0.98
Child females	18	6.0	4.1-8.0	1.14
All adults	67	6.0	3.8-8.3	0.99
All children	39	6.0	4.1-8.0	1.05
All males	49	5.9	4.0-8.3	1.02
All females	57	5.9	3.8-8.0	1.01
Total control group	106	5.9	3.8-8.3	1.01

Table III. Activity of Gal-1-P Uridyl Transferase in Erythrocytes from Affected Families

Family No.	Individual (sex)	Gal-1-P Uridyl Transferase (μmole UDPG/ml erythrocytes/hr)
1	Affected child	0
	Mother	3.7
	Sibling (F)	4.9
	Sibling (M)	3.4
2	Affected child	0
	Father	3.4
	Mother	2.8
	Sibling (F)	2.9
	Sibling (M)	6.9
	Sibling (F)	5.9
3	Affected child	0
	Father	3.5
	Mother	3.7
4	Affected child	0
	Father	3.5
	Mother	2.9
5	Affected child	0.6
	Father	3.8
	Mother	1.8
	Sibling (F)	3.4
6	Affected child	0
	Father	3.2
	Mother	3.3
	Sibling (M)	5.8
	Sibling (F)	2.1
	Sibling (F)	2.3
7	Affected child	0
	Father	3.0
	Mother	0.9
	Sibling (M)	1.1
8	Affected child	0.3
	Mother	3.0

Table III. (cont.)

Family No.	Individual (sex)	Gal-1-P Uridyl Transferase (μmole UDPG/ml erythrocytes/hr)
9	Mother	3.9
	Sibling (M)	3.9
	Sibling (M)	3.9
	Sibling (M)	3.1
	Sibling (M)	3.7
	Sibling (F)	7.0
10	Affected child (M)	0
	Affected child (F)	0
	Father	3.3
	Mother	2.9
11	Father	1.6
	Mother	2.0
	Sibling (M)	5.6
12	Affected child	0
	Father	3.8
	Mother	2.4
	Sibling (M)	3.4
13	Affected child	0
	Father	2.8
	Mother	1.5
14	Affected child	0.64
	Father	1.9
	Mother	3.0

Summary

	Mean	Range	S.D.
Affected children (13)	0.1		
Fathers and mothers (25)	2.9	0.9-3.9	0.83
Fathers (11)	3.1	1.6-3.8	0.72
Mothers (14)	2.7	0.9-3.9	0.89
Siblings (17)	4.1	1.1-7.0	1.7
Assumed heterozygote (11)	3.0	1.1-3.9	0.87
Assumed normal (6)	6.0	4.9-7.0	0.78

(2.9 ± 0.83) and normal individuals (5.9 ± 1.0) is apparent. On a theoretical basis, two-thirds of the unaffected siblings would be expected to be heterozygotes. In the division into groups as made, 11 siblings fall into the category of heterozygotes out of a total of 17.

The results from the 117 relatives studied are given in Table IV. When the data are divided into the same two groups, close correspondence with mean values for heterozygotes and normals is again evident.

Studies on a normal and mentally retarded population

Samples of blood were obtained from inmates at the State Prison of Southern Michigan for a control group of physically and mentally normal individuals. As a random population was desired, volunteer inmates were sampled with no attempt at selection. Samples were drawn, washed, and assayed for transferase activity under conditions identical to those previously described.

A total of 132 individuals were studied. The transferase activities of these subjects are presented in Fig. 3 as a scattergram.

Pertinent data of these studies are presented in Table V. As the previous studies revealed an arbitrary dividing line of 4.0 μmoles UDPG consumed/ml erythrocytes/hr for the normal and heterozygous groups, this group was therefore divided into a subgroup of values 4.0 and above and a subgroup of 3.9 and below. In addition the group was analyzed

Table IV. Activity of Gal-1-P Uridyl Transferase in Erythrocytes from Relatives of Affected Families

	Gal-1-P Uridyl Transferase (μmole UDPG/ml erythrocytes/hr			
	No.	Mean	Range	Standard Deviation
Individuals assumed as heterozygotes*				
Adult males	10	2.7	2.4-3.7	0.71
Adult females	19	2.9	1.8-3.9	0.66
Child males	6	2.8	1.6-3.8	0.81
Child females	4	2.6	1.4-3.3	0.86
Total group	39	2.8	1.4-3.9	0.70
Individuals assumed as normals*				
Adult males	12	5.6	4.1-7.8	1.14
Adult females	38	5.7	4.1-7.2	0.91
Child males	12	6.1	4.3-8.5	1.17
Child females	16	5.6	4.3-7.4	0.90
Total group	78	5.7	4.1-8.5	0.99

*Arbitrary division at 4.0 on basis of results from parents of known affected children.

Figure 3. Scattergram of values for Gal-1-P uridyl transferase activity from normal prison population and parents of assumed heterozygotes.

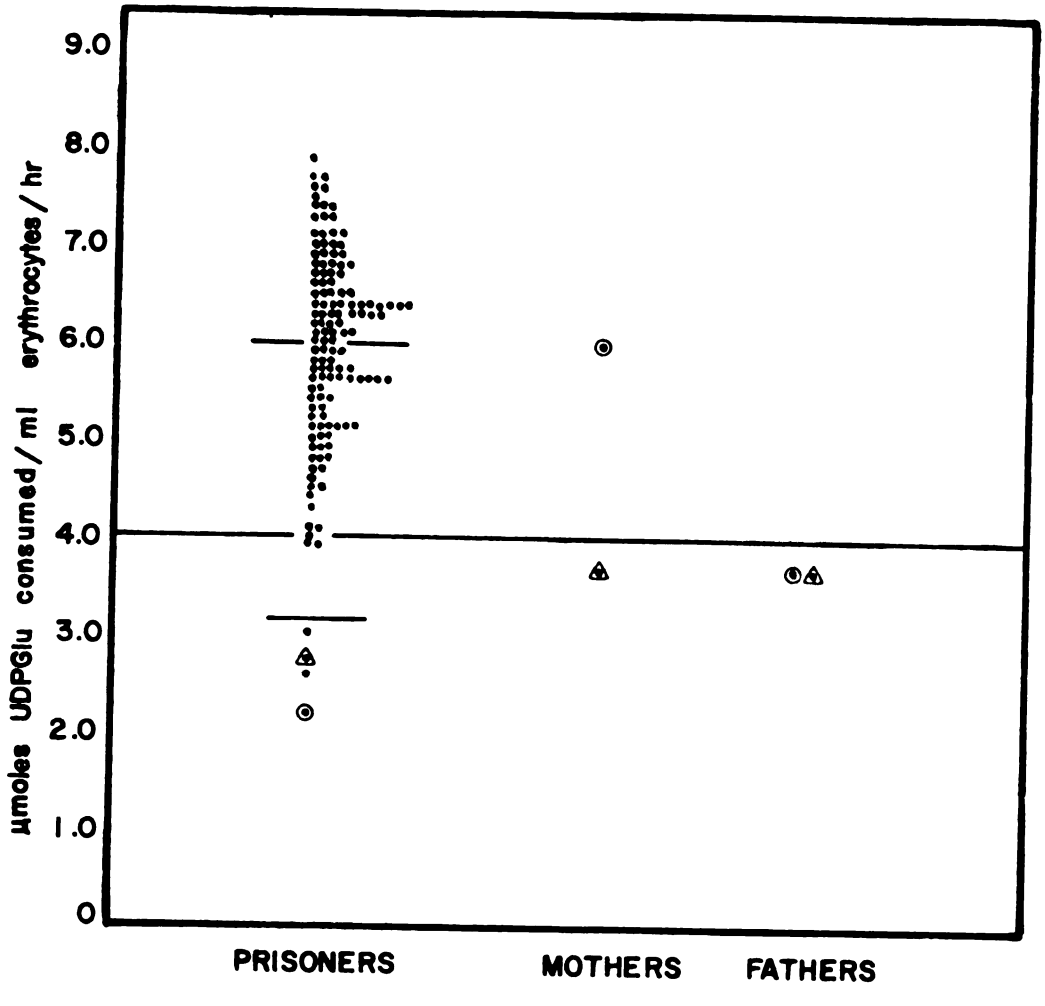

9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
µmoles UDPGlu consumed / ml erythrocytes / hr
PRISONERS
MOTHERS
FATHERS

Table V. Activity of Gal-1-P Uridyl Transferase in Erythrocytes from Normal Controls

Group	μmoles UDPG/ml erythrocytes/hr			
	No.	Mean	Range	Standard Deviation
4.0 and above	126	6.0	4.0-7.9	0.60
3.9 and below	6	3.1	3.3-3.9	0.58
Complete	132	5.9	2.2-7.9	0.60

as a whole. The group above 4.0 has a mean value of 6.0, which corresponds very well to the normal mean value found previously of 5.9. Values below 3.9 have a mean of 3.1, corresponding closely to 2.9 which was found for fathers and mothers of galactosemic persons.

To ascertain that the values below 4.0 were real and reproducible, the three lowest values were twice redetermined, each time on a fresh sample of blood. Similar values were obtained.

Parents of two of these suspected heterozygotes were sampled, along with the wife and two children of one. The data obtained are presented in Table VI. In Family 1, the father exhibited a transferase value characteristic of the heterozygous range, while the mother was normal. In Family 2, both parents exhibited heterozygous levels of transferase while the wife and children both showed normal values. The heterozygous condition of the two inmates are thus confirmed in that at least one of the parents in each case carries the recessive trait.

Preliminary studies on a mentally retarded population have revealed that the overall distribution of transferase activities is lower, averaging around 5.0. Many values below 4.0 were observed with apparently no sharp dividing line. Whether this lower distribution is significant must await further study with a larger population. Also, repeat determinations of the low values and family studies are

Table VI. Activity of Gal-1-P Uridyl Transferase in Erythrocytes from Suspected Heterozygous Families

Family No.	Individual	μmoles UDPG/ml erythrocytes/hr
1	Suspected heterozygote	2.2
	Mother	6.0
	Father	3.7
2	Suspected heterozygote	2.7
	Mother	3.7
	Father	3.7
	Wife	6.2
	Daughter	4.6
	Son	5.1

required to ascertain whether or not these are truly heterozygotes. It is possible that in such a population, the normal range of transferase activities is signficantly lowered so that the heterozygous range would have to be reevaluated. Further studies on this problem are in progress.

DISCUSSION

The present procedure for the assay of Gal-1-P uridyl transferase has proven practical and sufficiently sensitive to permit identification of the heterozygote. Application of the method in studies of families was successful in making this identification before knowledge of the family relationships. It is apparent from the results presented that three different groups are defined. The first consists of the homozygotes with essentially no transferase activity. The second consists of the normal controls and some family members with normal values for transferase activity. A third group, heterozygotes, with intermediate levels of transferase activity was derived entirely from members of affected families.

No significant statistical difference exists between the means of the various groups into which the 106 normal controls were divided ($t = .99$, $p \geq .05$). Therefore, the overall mean value and standard deviation of 5.9 ± 1.0 have been taken as the mean and standard deviation of all normal individuals (Table II).

When fathers and mothers of affected children are considered together as one group, the mean value for transferase and the standard deviation are 2.9 ± 0.83 (Table III). No statistically significant difference could be shown to exist between fathers and mothers ($t = 1.3$,

$p < .10$)

A highly significant difference was found when mean values from fathers and mothers of known homozygotes are compared with the mean of the entire group of 106 normal controls ($t = 13.9$, $p < 10^{-6}$).

In Table III it is noted that transferase values of some parents are below 2.0 (Families 5, 7, 11, 13, 14). The possibility exists that these low values are in the upper end of the range for homozygotes, in fact that the parents are homozygotes with a very mild form of the disease. However, every well-documented galactosemic studied has essentially no transferase activity in erythrocytes. Further, patients with a mild form of galactosemia should show milk intolerance or an abnormal response to galactose loading, and none of the parents in question showed any such indications (11). All of the parents in this study were without clinical symptoms and physical characteristics of galactosemia.

The most convincing evidence for the heterozygosity of the parents lies in the findings on grandparents which were available for study in Families 5, 6, 8, 11, 12, and 14. In Families 5, 6, 11, and 14, one parent of each heterozygous parent of the affected child was found to have an intermediate transferase level. In Families 8 and 12, grandparents on both sides of the affected childs family were not available, but those which were examined again

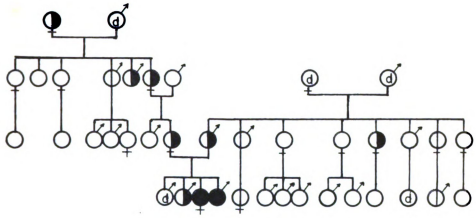
revealed one heterozygous grandparent. In Families 12 and 14 (Fig. 4), the heterozygous condition was found in one great-grandparent. Consequently, the data on the grandparents indicate heterozygosity in the parents with low values.

Only one normal case overlaps values assigned as heterozygous (Table II) but some values in both normal and heterozygous groups are close to the arbitrary dividing line. Normal distribution curves constructed from the data revealed a theoretical overlap of only 5% of the total area of both curves combined. This is insignificant as a population with an equal number of heterozygotes and normal individuals is entirely hypothetical. In a random population, the incidence of galactosemia must be so low that the possibility of heterozygosity is minimal in an individual whose erythrocytes yield a transferase value near 4.0. In families of children with galactosemia, it is probable that an individual near the 4.0 region is heterozygous. In both cases, family studies are required to clarify the point.

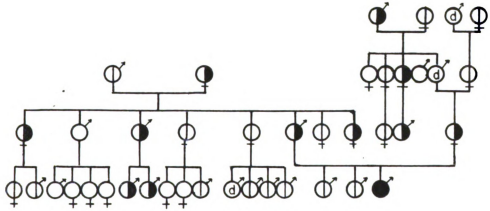
The studies on prisoners, which were assumed to be a normal population, have revealed that the heterozygote appears with an incidence of at least 2 per 132 persons. This is a minimum figure, as only 2 of the 4 persons revealing a low transferase level in Fig. 3 were further clarified by showing at least one parent in each case to also have

Figure 4. Pedigrees of Families 12 and 14 of children with galactosemia.

●, galactosemia; ◑, heterozygous range;
⦶, normal range; ○, not tested; d, deceased.



FAMILY 12



FAMILY 14

a transferase level characteristic of the heterozygous range. Assuming two heterozygotes (aA) from a total of 132 individuals, the frequency (q) of the recessive gene (a) would be one in 132. Therefore, the frequency (q^2) of the homozygous recessive (aa) would be approximately one in 17,400. For comparison, the homozygous recessive disorder phenylketonuria has been reported (19) to occur with an incidence of one in 25,000 (U.S.A.).

Comparison of the incidence of the heterozygous state of galactosemia in a mentally retarded population might reveal a significant difference from normal. Indeed, Lippman (19) has proposed that mental deficiency may be correlated with heterozygosity for many different metabolic errors occurring simultaneously. Preliminary studies have revealed a large number of mentally retarded persons having transferase levels in the heterozygous range (below 4.0). However, these must be clarified by family studies and evaluated with respect to the overall distribution obtained from such a population.

As opposed to the possible complications of the galactose tolerance test, the direct measurement of the defective enzyme in erythrocytes is a practical approach to detection of the heterozygote for galactosemia. A simplified colorimetric method, based on the quantitative procedure described in this thesis, has recently been developed by Nordin *et al.* (20). Thionicotinamide

diphosphopyridine nucleotide is used in place of DPN in the UDPG dehydrogenase catalyzed oxidation of UDPG, and as the reduced form of this DPN analog has an absorption maximum at 400 mμ, a standard visual spectrophotometer will serve for the assay. This method has been demonstrated to be as equally reliable in differentiating the heterozygote as the method described in this thesis, both being useful tools in confirming a previous diagnosis, in clinical study, and in genetic counseling.

SUMMARY

A quantitative assay for Gal-1-P uridyl transferase in erythrocytes has been developed and applied to 14 families in which a known case of galactosemia has occurred. The method is sensitive enough to categorize the individuals examined into three groups: the galactosemic children having little or no enzyme (homozygous recessives); a group, including all parents, having an intermediate level of enzyme (heterozygotes); and a group with a full level of enzyme. These results substantiate the conclusion that galactosemia is a hereditary metabolic disorder and imply that it is transmitted as a simple autosomal recessive trait. Preliminary data on the incidence of heterozygosity in a normal and mentally retarded population are presented.

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CARBOHYDRATE METABOLISM

II. METABOLISM OF MANNOSE BY *Hansenula holstii*

INTRODUCTION

Recent investigations have revealed that sugar nucleotides serve as glycosyl donors in the synthesis of polysaccharides. Primer molecules are usually required which serve as the glycosyl acceptors, resulting in the release of nucleoside diphosphate. From thermodynamic considerations, synthesis from sugar nucleotides is a much more favorable route than the classical phosphorylase type of reaction, suggesting the possibility that the former reaction is the physiological synthetic route and the latter the route for breakdown.

Demonstrated thus far has been the biological synthesis of glycogen, starch, cellulose, and a β-1,3-glucan from UDPG[1] hyaluronic acid from UDPGA and UDPAG, Type III pneumoccal polysaccharide from UDPGA and UDPG, and chitin from UDPAG. Evidence for the participation of sugar

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[1]Abbreviations used are: UMP, UDP, and UTP, uridine mono-, di-, and triphosphate; AMP, ADP, and ATP, adenosine mono-, di-, and triphosphate; GMP, GDP, and GTP, guanosine mono-, di-, and triphosphate; DPN and DPNH, oxidized and reduced diphosphopyridine nucleotide; TPN and TPNH, oxidized and reduced triphosphopyridine nucleotide; UDPG, UDPGA, UDPGalA, and UDPAG, uridine diphosphate glucose, glucuronic acid, galacturonic acid, and N-acetylglucosamine; GDPM and GDPG, guanosine diphosphate mannose and glucose; M-1-P, mannose-1-phosphate; M-6-P, mannose-6-phosphate; G-1-P, glucose-1-phosphate; G-6-P, glucose-6-phosphate;, G-1,6-diP, glucose-1,6-diphosphate; 6-PGA, 6-phosphogluconic acid; F-6-P, fructose-6-phosphate; Pi, inorganic phosphate; PP, inorganic pyrophosphate; EDTA, ethylenediaminetetra-acetic acid; Tris, tris(hydroxymethyl) aminomethane.

nucleotides in polysaccharide synthesis received further support upon demonstration of the presence of acid soluble nucleotides containing the glycosyl monomer of the polysaccharide and enzymes required for the synthesis of these sugar nucleotides from free hexoses.

The monomeric units of an exocellular phosphorylated mannan produced by various strains of Hansenula was shown by D. R. Wilken (unpublished data) to result from incorporation of glucose units intact. From known enzymic reactions, incorporation of glucose into this polysaccharide might occur by several pathways such as: 1) phosphorylation of glucose and subsequent isomerization and mutation to M-1-P which might serve as a glycosyl donor in a classical phosphorylase type reaction; 2) pyrophosphorolysis of the M-1-P with GTP to form GDPM which could serve as a glycosyl donor; 3) pyrophosphorolysis of G-1-P with GTP to form GDPG followed by epimerization to GDPM (not a known enzymic reaction). The present report is concerned with the biosynthesis of this phosphomannan, including identification of nucleotides and enzymes which might be assumed to function in the conversion of hexose to polymer, and attempts to synthesize polymer directly from GDPM.

LITERATURE

Glycogen

The pioneering work in glycosyl transfer from sugar nucleotides to polysaccharides, reported by Leloir and Cardini (1) in 1957, was the discovery of the catalysis by a rat liver enzyme preparation of glucose transfer from UDPG to primer glycogen. More detailed studies of this reaction carried out by Leloir et al. (2) with a rat muscle enzyme revealed the requirement of G-6-P for maximal activity. Among many other compounds tested, glucosamine-6-P, F-6-P, and galactose-6-P were found to be active. The function of the activator remains obscure, however, as radioactive G-6-P was not incorporated into the synthesized glycogen in the presence of unlabeled UDPG. Primer polysaccharide was needed to attain maximal activity. Native glycogen was a better primer than partially degraded products while soluble starch was approximately 20% as active as glycogen.

The polysaccharide formed in this system was carefully characterized. In addition to being stable in hot alkali and insoluble in 50% ethanol as anticipated, the product was degraded to its expected components. Glycogen formed from C^{14}-UDPG yielded radioactive maltose and a trace of glucose upon hydrolysis with β-amylase. Hydrolysis of this maltose with maltase produced only radioactive glucose. Also, degradation of radioactive glycogen with

phosphorylase followed by acid hydrolysis of the phosphate ester formed resulted in only radioactive glucose.

Robbins et al. (3) found a similar enzyme in pigeon breast muscle which was concentrated in microsomal particles. Since this enzyme contained a high concentration of glycogen, it was suggested that the enzyme is actually adsorbed to the polysaccharide.

Leloir and Goldenberg (4) extended the studies with the liver enzyme and found it also to be associated with polysaccharide in the particulate fraction. Washing this fraction with soluble starch resulted in a 300-fold purification of the enzyme, which still retained requirements for G-6-P and primer. Reversal of the reaction was attempted but not achieved. Similar glycogen-synthesizing enzymes have been described from human skeletal and heart muscle (5), and from brain tissue (6).

Some insight into the possibility of oligosaccharides being an intermediate glycosyl acceptor of glucose from the sugar nucleotide for glycogen synthesis was furnished by Olavarria (7). Having detected radioactive oligosaccharides when radioactive UDPG and the rat muscle enzyme were incubated, the problem was further investigated with the rat liver particulate enzyme. Incubation of radioactive oligosaccharides and unlabeled UDPG with the enzyme did not result in radioactive glycogen. Alternatively, incubation of the enzyme with radioactive glycogen and unlabeled

UDPG did result in the formation of radioactive oligosaccharides. Furthermore, in short time incubations with labeled UDPG, glycogen formed had a higher specific activity than did the oligosaccharides. The differences were still greater if only the exterior chains of the glycogen were measured for radioactivity. These data indicate that the oligosaccharides are not intermediates in the synthesis of glycogen, but rather are degradation products.

Starch

The biosynthesis of starch and oligosaccharides from UDPG was reported by Leloir et al. (8). The active enzyme, in association with starch granules, was isolated from the embryos and cotyledons of Phaseolus vulgaris. All attempts to separate the enzyme from the starch granules were negative. Starch biosynthesized from C^{14}-UDPG was separated into its components, amylose and amylopectin. As both fractions contained C^{14}-glucose, there was no indication of one fraction being a precursor of the other. That the label was indeed incorporated into starch was shown by β-amylase degradation to labeled maltose.

This enzyme preparation catalyzed the transfer of glucose from UDPG to malto-oligosaccharides. This was clearly demonstrated by the synthesis of higher oligosaccharides from UDPG and lower oligosaccharides. Maltose but not glucose was active in this system. The oligosaccharides were not intermediates in the transfer

of glucose from UDPG to starch as shown by the lack of incorporation of label from labeled maltotriose into starch when incubated with or without UDPG and the enzyme. Alternatively, the oligosaccharides were not formed from starch as there was no incorporation of label into oligosaccharides when radioactive starch was incubated with the enzyme. It appears then that the malto-oligosaccharides compete with starch for accepting glucose from UDPG.

The addition of the glucosyl group to the nonreducing end of the acceptor was demonstrated by the formation of labeled gluconic acid and unlabeled glucose after bromine oxidation and hydrolysis of maltotetraose enzymically synthesized from unlabeled UDPG and maltotriose labeled in the reducing end group. Similarly, borohydride reduction and hydrolysis of the same maltotetraose resulted in the formation of labeled sorbitol and unlabeled glucose. Alternatively, borohydride reduction of maltotetraose synthesized from C^{14}-UDPG and unlabeled maltotriose resulted in the formation of labeled glucose and unlabeled sorbitol.

Cellulose

The biosynthesis of cellulose has been studied by numerous investigators using different approaches to the problem. Minor, Greathouse, and coworkers in a series of papers (9-12) established that Acetobacter xylinum, when grown on specifically labeled glucose, produced

cellulose of which the monomer units retained in general the position of the label.

Greathouse (13) and Colvin (14) reported the first cell-free synthesis of cellulose. As with growing cells, cell-free extracts incorporated labeled glucose intact into the cellulose but ATP was required to obtain good yields.

The role of hexose-phosphates in cellulose synthesis in A. xylinum was investigated by Schramm et al. (15). Both extracts and cell suspensions were used to demonstrate individual enzymes which catalyzed phosphorylation of hexoses and their subsequent conversion to CO_2, acetate, ethanol, and cellulose. Distribution of label in glucose units of cellulose produced from specifically labeled substrates suggested that hexose-phosphates are involved in the overall process of cellulose synthesis, probably without cleavage of the hexose residue. However, resting cells were not capable of metabolizing α- or β-G-1-P, G-6-P, or UDPG. As cellulose is found extracellularly, it was suggested that the role of the hexose-phosphates is to furnish a diffusible intermediate which contains the carbon skeleton but in which the phosphate group is modified or replaced.

The transfer of glucose residues from UDPG to cellulose in the presence of a particulate enzyme system from A. xylinum and soluble cellodextrins was demonstrated by

Glaser (16). Although incorporation of label from UDPG was low, partial acid hydrolysis of the synthesized polysaccharide revealed radioactive glucose, cellobiose, cellotriose, cellotetraose, and larger oligosaccharides. Further digestion of the partial acid digest with Aspergillus niger cellulase produced only radioactive glucose. Digestion with Myrothecium verrucaria cellulase produced radioactive glucose and cellobiose, as this enzyme splits cellobiose at a very slow rate. These characteristics imply that the structure of the polysaccharide produced is a β-1,4-linked glucose polymer (cellulose). Primer dependence was established by pretreatment of the particulate enzyme with M. verrucaria cellulase. Without added primer, this enzyme had essentially no activity. Cellodextrins of higher molecular weight exhibited more activity as primer than did those of low molecular weight. Net synthesis and not simply exchange of glucose residues was demonstrated by the lack of isotope dilution in the cellulose upon incubation of enzyme labeled cellulose, and unlabeled UDPG.

Some doubt as to whether UDPG is the immediate precursor of cellulose has been cast by recent work of Colvin (17). Ultra-filtered 80% ethanol extracts of active A. xylinum cells were found to contain a compound which was rapidly converted to cellulose microfibrils when slowly evaporated to dryness at 40° C. This conversion was accelerated by a heat-labile, extracellular material in

the medium of the active cells, presumably an enzyme. When C^{14}-glucose was utilized by the active cells, no labeled cellobiose, cellotriose, cellotetraose, or UDPG could be recovered. Further investigations on the nature of this compound revealed that after adsorption of the ethanol extract on a trisilicate-celite column, the active compound could be eluted off (18). The compound, having chemical characteristics of a glyco-lipid, was radioactive when extracted from cells grown on C^{14}-glucose. The radioactivity was transferred to cellulose microfibrils when the compound was incubated with ultra-filtered medium. As no lipid was found to accumulate in the medium, a cyclic transport system was suggested whereby the lipid alternately leaves and enters the cell wall, transporting an activated glucose molecule to the extracellular acceptor.

Chitin

Glaser and Brown (19) demonstrated the synthesis of chitin from C^{14}-UDPAG by a particulate fraction of Neurospora crassa. The product of the enzymic reaction was characterized by chitinase degradation of partial acid digests to C^{14}-N-acetylglucosamine. Carbon^{14}-N'-N-diacetylchitobiose was also identified in the partial acid digest. For maximal activity, the particulate enzyme required soluble chitodextrins as primer and N-acetylglucosamine. Labeled soluble primer was incorporated into insoluble chitin only in the presence of UDPAG. Net synthesis of chitin was demonstrated

by a lack of isotope dilution when C^{14}-chitin was incubated with unlabeled UDPAG and also by direct chemical measurement of the hexosamine content. Reversibility of the reaction was attained by incorporation of label into UDPAG upon incubation of C^{14}-chitin and UDP with the enzyme. A soluble enzyme was obtained by butanol treatment which had an absolute primer requirement, but was not activated by N-acetylglucosamine.

Hyaluronic Acid

Investigations concerned with the biosynthesis of hyaluronic acid prior to 1955 are thoroughly reviewed by Dorfman (20). Briefly, it has been shown that in Group A streptococci the glucosamine and glucuronic acid residues of hyaluronic acid are derived from glucose without cleavage of the glucose molecule. Cifonelli and Dorfman (21) in further investigations on the biosynthesis of this polymer examined the nucleotides present in streptococcus capable of synthesizing hyaluronic acid. Boiling water extracts of washed cells were chromatographed on Dowex-1 (Cl) columns. Sugar nucleotides found were UDPG, UDPAG, and large amounts of UDPGA. The authors suggested that these compounds may be intermediates in hyaluronic acid synthesis.

The first cell-free synthesis of hyaluronate chains was reported in 1955 by Glaser and Brown (22). Cell-free homogenates of Rous chicken sarcoma were capable of catalyzing the incorporation of label from

C^{14}-N-acetylglucosamine-6-P and UTP or from C^{14}-UDPAG, along with UDPGA into hyaluronic acid chains. However, much of the label was lost on dialysis or reprecipitation of the carrier hyaluronic acid, indicating that the actual products formed were oligosaccharide chains.

Markowitz et al. (23) were able to confirm the work of Glaser and Brown (22) and conclusively show the net synthesis of hyaluronic acid from UDPGA and UDPAG. A particulate fraction from Group A streptococcus catalyzed the incorporation of radioactivity of N-acetylglucosamine from tritiated UDPAG into the acetylglucosamine moiety of hyaluronic acid in the presence of nonlabeled UDPGA. The incorporation of the radioactivity of glucuronic acid from UDPGA into the glucuronic acid moiety of hyaluronic acid in the presence of nonlabeled UDPAG was also shown.

The labeled product was characterized first by adsorption and elution from Dowex-1 (Cl) resin without change in radioactivity and secondly, hydrolysis of the polymer yielded labeled glucosamine only from polymer synthesized from labeled UDPAG, and labeled glucuronic acid only from polymer synthesized from labeled UDPGA. Finally, degradation of the synthesized hyaluronate by Clostridium perfringens hyaluronidase to the unsaturated disaccharide 3-O-(β-D-Δ, 4,5-glucoseenpyranosyluronic acid) 2-deoxy-2-acetamido-D-glucose resulted in recovery of 80% of the label in this disaccharide. This enzyme system had an absolute $MgCl_2$

requirement and was slightly activated by ATP and N-acetyl-glucosamine-1-P. The presence of UDPAG-pyrophosphorylase in the enzyme preparation was shown to be the reason for the N-acetylglucosamine-1-P activation, as incorporation of this phosphate ester proceeded only if UTP were present. No absolute primer requirement was demonstrated, although the washed particulate enzyme contained traces of nondialyzable uronic acid.

Synthesis of hyaluronic acid from the respective sugar nucleotides has more recently been reported by Schiller et al. (24) with enzymes from rat fetus skin.

Type III Pneumococcal Polysaccharide

Bernheimer (25) in 1953 showed that resting suspensions of Type III pneumococci could synthesize capsular polysaccharide from glucose, but little or none was formed from glucuronic acid or cellobiuronic acid. The importance of these observations was realized after later investigations of the nucleotides and enzymes present in various types of pneumococci. Smith et al. (26) demonstrated in a non-capsulated strain derived from a Type II organism and a capsulated Type III organism the presence of UDPAG, UDPGA, and UDPG. UDPG pyrophosphorylase was present, along with enzymes necessary to transform glucose to G-1-P. Further investigations revealed the presence of UDPG dehydrogenase in a noncapsulated strain (27). UDPGalA was isolated from a strain of pneumococcus Type I (28), and later,

the epimerase which interconverted UDPGA and UDPGalA with the mediation of DPN (29). As Type I capsular polysaccharide contains galacturonic acid, the finding of UDPGalA and enzymes necessary for its formation in these bacteria strongly suggested their participation in capsule formation. This was further substantiated by Austrian et al. (30) who showed by transformation and genetic studies that synthesis of capsular polysaccharide by certain strains of pneumococci is dependent on an intact pathway of uridine diphosphate glycosyl metabolism. The lack of only one enzyme resulted in a block of polysaccharide synthesis.

Smith et al. (31,32) reported the incorporation of radioactivity from C^{14}-UDPG and C^{14}-UDPGA into glucose and glucuronic acid residues, respectively, of Type III pneumococcal capsular polysaccharide (β-1,4-glucuronosido-glucose), resulting in net synthesis of polymer. A partially fractionated, cell-free enzyme preparation was used which subsequently was shown to contain all activity in a sedimentable fraction. The labeled polysaccharide was characterized in several ways. It was precipitated with the specific rabbit anti-Type III capsular serum. Upon acid hydrolysis of the polysaccharide, C^{14}-glucose and/or C^{14}-glucuronic acid were obtained, depending on which labeled sugar nucleotide was used as precursor. Also, the labeled polysaccharide was depolymerized by the specific Type III capsular depolymerase, rendering 90%

of the label dialyzable.

Primer dependence was not rigorously established, as addition of primer polysaccharide did not stimulate synthesis. However, various enzyme preparations were found to contain different amounts of polysaccharide, and the rate of synthesis was proportional to this polysaccharide concentration. It appears, therefore, that in this case primer molecules are associated with the enzyme.

β-1,3-Glucan

Feingold et al. (33) reported the synthesis of a β-1,3-linked glucan from UDPG, the reaction being catalyzed by a particulate enzyme found in various plants. The polysaccharide formed was immobile in chromatographic solvents in which oligosaccharides migrated. Upon partial acid hydrolysis and subsequent chromatography of the polymer synthesized from C^{14}-UDPG, seven radioactive oligosaccharides were observed. These migrated identically to oligosaccharides prepared from laminaran, a β-1,3-linked glucan. The 1,3 linkage was further established by lead tetraacetate oxidation of the disaccharide obtained from a partial acid hydrolyzate. As previously shown (34), such oxidation of a reducing sugar having a 1,3 linkage with glucose as the reducing moiety produces arabinose. Arabinose was obtained, indicating a 1,3 linkage. In addition, the arabinose was radioactive, proving the incorporation of label from C^{14}-UDPG into the polymer. The β-type linkage

of the radioactive oligosaccharides obtained by partial acid hydrolysis of the polymer was proven by degradation to glucose with β-glucosidase.

The particulate enzyme was capable of catalyzing polymer formation from UDPG without added primer. The digitonin solubilized enzyme exhibited an absolute primer or activator requirement, glucose or oligosaccharides of cellulose and laminarin being active. Primer glucose was not, however, incorporated into the polymer.

Xylo-Oligosaccharides

Feingold, et al. (35) reported the ability of an asparagus extract, prepared similarly to the β-1,3-glucan synthetase from mung bean seedlings (33), to catalyze the transfer of xylose from UDPxylose to β-1,4-linked xylo-oligosaccharide acceptors. It was shown by cochromatography and coelectrophoresis that radioactive xylo-oligosaccharides were formed which contained one more pentose unit (3 to 6 units) than the initial xylo-oligosaccharide acceptor (2 to 5 units). Also, total acid hydrolysis yielded xylose as the sole radioactive product. A β-1,4-linked xylo-oligosaccharide primer was required in very high concentration for maximal transfer to occur (molar ratio $\frac{\text{acceptor}}{\text{UDPxylose}} > 100$). Similar concentrations of di-, tri-, tetra-, and penta-xylo-oligosaccharides were necessary as primer. This apparent identical affinity of the enzyme for each of the acceptors accounts for the

observed one-step reaction, without formation of xylan. The possibility of xylose transfer by this enzyme to a higher molecular weight primer was not investigated.

Synthesis of polysaccharides by glycosyl transfer from sugar nucleotides have in most cases required primer. Lack of primer dependence in hyaluronic acid and Type III pneumococcal polysaccharide synthesis has not been rigorously established, as traces of polysaccharide constituents were found associated with the enzyme. These findings lead to the very interesting question of how does a primer molecule arise. A possible explanation was furnished by Illingworth et al. (36) who demonstrated the de novo synthesis of amylose chains from G-1-P by phosphorylase in the absence of primer. Crystalline muscle phosphorylase, free of amylo-1,4 → 1,6-transglucosidase (branching enzyme), α-amylase, amylo-1,6-glucosidase, and essentially free of any carbohydrate (0.3 glucose monomer per monomeric unit of phosphorylase), when incubated in large quantities with G-1-P after an initial lag period catalyzed the release of Pi and the formation of amylose chains. This lag period could be abolished by addition of minute quantities of glycogen. When catalytic quantities of phosphorylase were incubated with G-1-P, no release of Pi occurred. However, addition of catalytic quantities of branching enzyme resulted in release of Pi, the lag period and running rate of the reaction being dependent

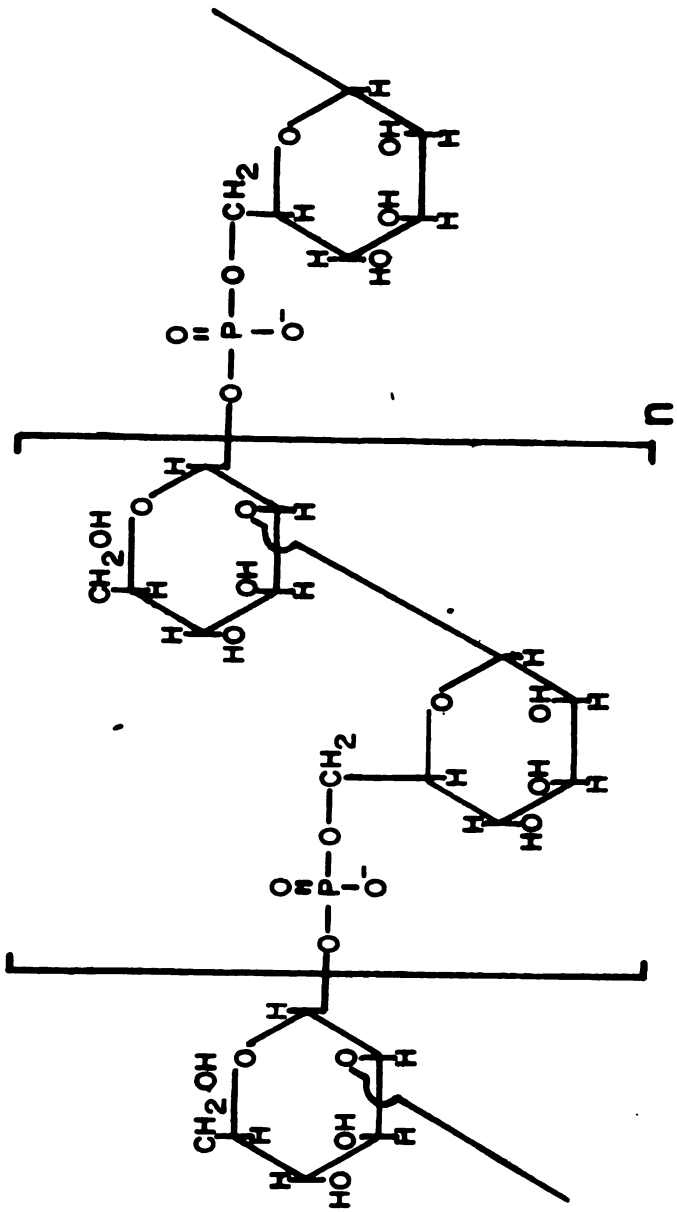
on the branching enzyme concentration. The stimulation of synthesis by branching enzyme and glycogen are both apparently due to the creation of multiple end groups. It is suggested that such a catalytic system (phosphorylase and branching enzyme) could conceivably account for the initial synthesis of glycogen in embryonic tissues.

Phosphomannan

The production of extracellular phosphorylated mannans by various strains of Hansenula has recently been reported (37,38). Laboratory production (39), characterization, and properties (40) of the phosphomannan produced by Hansenula holstii NRRL Y-2448 have been described in more detail. This particular polymer consists of mannose and phosphate in a ratio of 5:1. Investigations on the structure of the polymer (private communication) produced by Hansenula capsulata NRRL Y-1842 (mannose to phosphate ratio of 2.5:1) are consistent with that shown in Fig. 5.

The biosynthesis of Hansenula holstii NRRL Y-2448 polymer was investigated by Wilken (unpublished data). Cells grown on glucose-1-C^{14} produced polymer which retained essentially all the label (84.3%) in carbon 1 of the mannose residues. The remaining label in carbon 6 was ascribed to a slight equilibration of the hexose-phosphates with triose phosphates and subsequent incorporation of hexose into phosphomannan. The suggestion by Wilken that GDPM may be the glycosyl donor was enhanced by a preliminary

Figure 5. Structure of phosphomannan.



report (41) that sugar nucleotides are present in these organisms.

MATERIALS AND METHODS

Quantitative Measurements

Radioactivity was measured in a Packard Instrument Company Tri-Carb liquid scintillation counter. The counting solution consisted of 4.0 g 2,5-diphenyloxazole and 50 mg 1,4-bis-2-(5-phenyloxazole)-benzene made up to 1 liter with toluene (42). Usually 0.1 ml of aqueous solution to be counted was added to 5 ml of absolute ethanol and 10 ml of counting solution. Polysaccharide samples were counted by adding 1 to 5 mg of dry sample to 10 ml of a thixotropic gel suspension (2.5% thixotropic gel in the previously described solution). The counting vial was shaken vigorously to evenly disperse the sample in the gel. This technique resulted in proportionality of counts to sample added over a range of 1 to 10 mg with an efficiency of 56%. Radioactive spots were detected and counted on paper chromatograms with a gas flow counter equipped with a thin Mylar window Geiger-Mueller tube.

Pyridine nucleotide reduction and oxidation were measured on a Beckman model DU spectrophotometer equipped with a Gilford cuvette changer and recording attachment (43). Phosphorus was determined by the Fiske-SubbaRow procedure as described by Leloir and Cardini (44). Ketoses were detected by the resorcinol method of Roe as described by Ashwell (45) and reducing sugars were

measured by the procedure of Park and Johnson (46). Phosphomannan was estimated by the phenol-sulfuric acid method of Dubois et al. (47). Phosphomannan gave identical color development as equimolar amounts of mannose (assuming that the polymer contained 14% moisture (40)). Protein was measured spectrophotometrically (48).

Qualitative Measurements

Nucleotides were separated on ion exchange resin columns by the method of Hurlbert et al. (49) as described by Forrest and Hansen (50). Norite A (Pfanstiehl Laboratories, Incorporated) was prepared by washing with 2 N HCl, 0.05 EDTA, 95% ethanol, and water, followed by drying. Nucleotides were chromatographed on Whatman No. 1 and/or No. 3 filter paper in a descending direction with the following solvents (51): isobutyric acid-ammonium hydroxide-water, pH 3.7 (66:1:33 v/v), 95% ethanol-1.0 M ammonium acetate, pH 7.5 (7:3 v/v), or isobutyric acid-ammonium hydroxide-water, pH 4.3 (57:4:39 v/v). Nucleotides were detected on paper with an ultraviolet lamp (Mineralight SL-2537). Photographs of ultraviolet absorbing spots on paper chromatograms were made by contact prints prepared by a slight modification of the procedure of Markham and Smith (52) as described by Wilken and Hansen (53). Carbohydrates were chromatographed on Whatman No. 1 filter paper and developed with water-saturated phenol (54) or butanol-pyridine-water (6:4:3 v/v) (55). Sugars were detected

on paper with alkaline-silver nitrate (56). Radioactive spots were detected by exposing paper chromatograms to No-Screen X-ray film.

Chemicals

The tricyclohexylammonium salt of 6-PGA was obtained from the California Corporation for Biochemical Research. K_2-G-1-P was purchased from Sigma Chemical Company. Ba-F-6-P and Ba-M-6-P were purchased from Nutritional Biochemicals Corporation. Ba-G-6-P was made by the procedure of Wood and Horecker (57). Ba-M-1-P was synthesized according to Colowick (58) with the following modifications: penta-acetyl mannose was prepared by the method of Hudson and Dale (59) from D (+) mannose (Pfanstiehl Laboratories, Incorporated) and acetobromomannose by the method of Levene and Sobotka (60), neither product being crystallized. All barium salts were converted to the sodium salts by precipitation of the barium as $BaSO_4$ by addition of Na_2SO_4. Tricyclohexylammonium salts were treated with Dowex-50 (H) and neutralized with NaOH. GDPM was synthesized by the method of Roseman et al. (61) and used as the lithium salt. All other nucleotides were purchased from Sigma Chemical Company and Pabst Brewing Company.

The barium salt of C^{14}-M-1-P and the lithium salt of C^{14}-GDPM were synthesized as previously described from uniformly labeled C^{14}-D-mannose (Schwarz Bioresearch, Incorporated) and had specific activities of 8×10^4

dpm/μmole. The lithium salt of C^{14}-M-1-P was recovered from the Dowex-1 (Cl) resin column used in purification of the C^{14}-GDPM by precipitation from methanol with acetone in the same manner as for the sugar nucleotide (61). C^{14}-G-1-P was prepared as the crystalline dipotassium salt from uniformly labeled leaf starch (62) by phosphorolysis with crystalline muscle phosphorylase (63) and had a specific activity of 5×10^5 dpm/μmole.

EXPERIMENTAL PROCEDURES AND RESULTS

Nucleotide Components

Hansenula holstii NRRL Y-2448 was grown on 6% glucose for 36 hours and harvested by centrifugation at 12,000 x g for 15 minutes. Approximately 215 gm of wet packed cells were obtained from 3 liters of growth medium. The wet cells were extracted in an Omni-mixer at 50,000 rpm for 15 minutes with 2 volumes of cold 0.6 N perchloric acid. After centrifugation, the supernatant was immediately neutralized to pH 6.8 with 5 N KOH. The pellet was re-extracted as outlined above. The neutralized extracts were allowed to stand at 3° C for 12 hours before filtering through Whatman No. 50 filter paper to remove potassium perchlorate. Based on an approximate molar extinction coefficient at 260 mμ of 10,000, 570 μmoles of nucleotides were obtained.

The following procedures including elution of the column were carried out at 3° C. The total extract was placed on a 1.8 x 40 cm Dowex-1-10 x 200-400 mesh formate resin column which had been filled in 2 cm layers with no external pressure applied. After washing with 500 ml water, about 340 μmoles were calculated to have been retained by the resin. The column was developed as described by Forrest and Hansen (50) using a 500 ml mixing

flask and collecting 12.5 ml fractions at about 1 ml per minute. The various peak tubes (Fig. 6) were combined, lyophilized, and taken up in 20 ml water. (For quantitative purposes, all tubes from 121 through 199 were collected in fractions as: K, 121-139; L, 140-155; M, 156-167; N, 168-181; and N_1, 182-199). Norite (0.5) gm was added, collected on a Buchner funnel, and washed with 10 ml water. The nucleotides were eluted by stirring the Norite with 50 ml of 15% aqueous pyridine. Following filtration, pyridine was removed by extracting the filtrate twice with 50 ml of $CHCl_3$ and the aqueous layer was lyophilized.

Further purification of peaks K, L, M, N, and N_1 was achieved by applying the freeze dried material to Whatman No. 3 filter paper and chromatographing in ethanol-ammonium acetate, pH 7.5. Bands migrating with authentic GDPM were found in peaks K, L, M, N, and N_1 and UDPG in peaks N and N_1. These were eluted with water, lyophilized, and rechromatographed on Whatman No. 1 filter paper (previously washed with 1 N acetic acid, 95% ethanol, and water) in isobutyric acid-ammonium hydroxide-water, pH 4.3. The bands corresponding to GDPM and UDPG were eluted and analyzed.

Spectra were identical for the GDPM and UDPG isolated from the various peaks of the column with authentic nucleotides. Typical absorption spectra of GDPM and UDPG from peak N_1 are shown in Fig. 7. Quantitative analysis by ultraviolet absorption and reducing values are presented

Figure 6. Elution pattern of acid soluble nucleotides from Dowex-1 (formate) column.

The column was developed with: 1) 2 N formic acid; 2) 4 N formic acid; 3) 4 N formic acid and 0.2 N ammonium formate; 4) 4 N formic acid and 0.4 N ammonium formate; 5) 4 N formic acid and 0.8 N ammonium formate. Principal components of each peak are: C and D, DPN and AMP; E, TPN; F, GMP; J, ADP; K and L, GDPHexose; M, UDP-N-acetylhexosamine; N, UDPHexose; N_1, mixutre of K, L, M, and N; P, ATP; P_2, UDP; R, UTP and GTP.

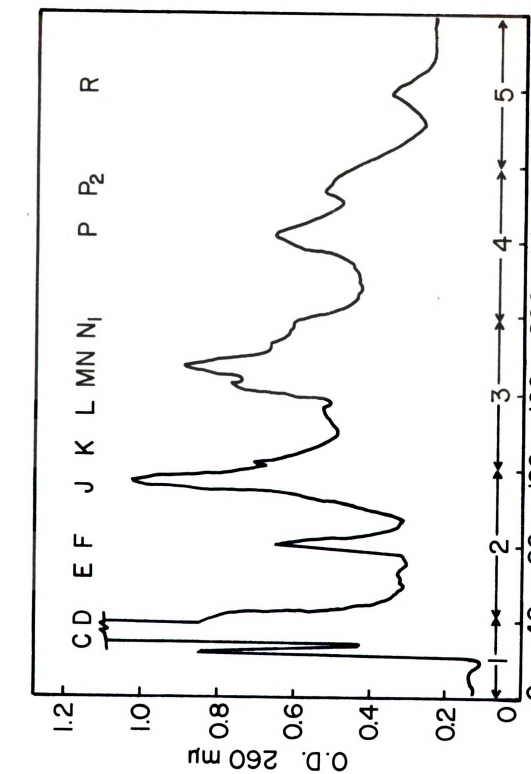

C D E F J K L M N N1 P P2 R
O.D. 260 mμ
1.2
1.0
0.8
0.6
0.4
0.2
0
0 40 80 120 160 200 240 280 320 360
1 2 3 4 5
FRACTION NUMBER

Figure 6. Elution pattern of acid soluble nucleotides from Dowex-1 (formate) column.

The column was developed with: 1) 2 N formic acid; 2) 4 N formic acid; 3) 4 N formic acid and 0.2 N ammonium formate; 4) 4 N formic acid and 0.4 N ammonium formate; 5) 4 N formic acid and 0.8 N ammonium formate. Principal components of each peak are: C and D, DPN and AMP; E, TPN; F, GMP; J, ADP; K and L, GDPHexose; M, UDP-N-acetylhexosamine; N, UDPHexose; N_1, mixutre of K, L, M, and N; P, ATP; P_2, UDP; R, UTP and GTP.

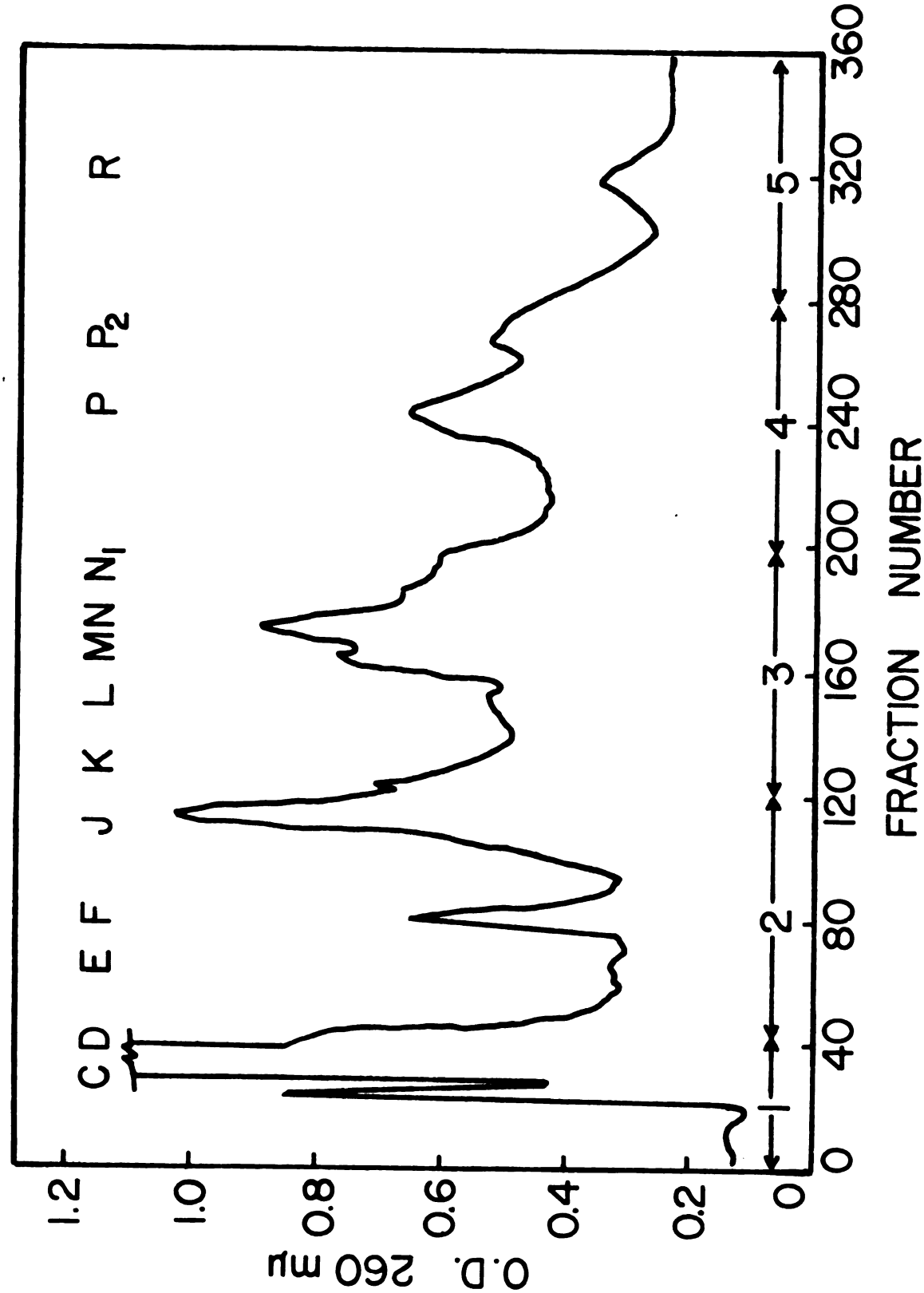
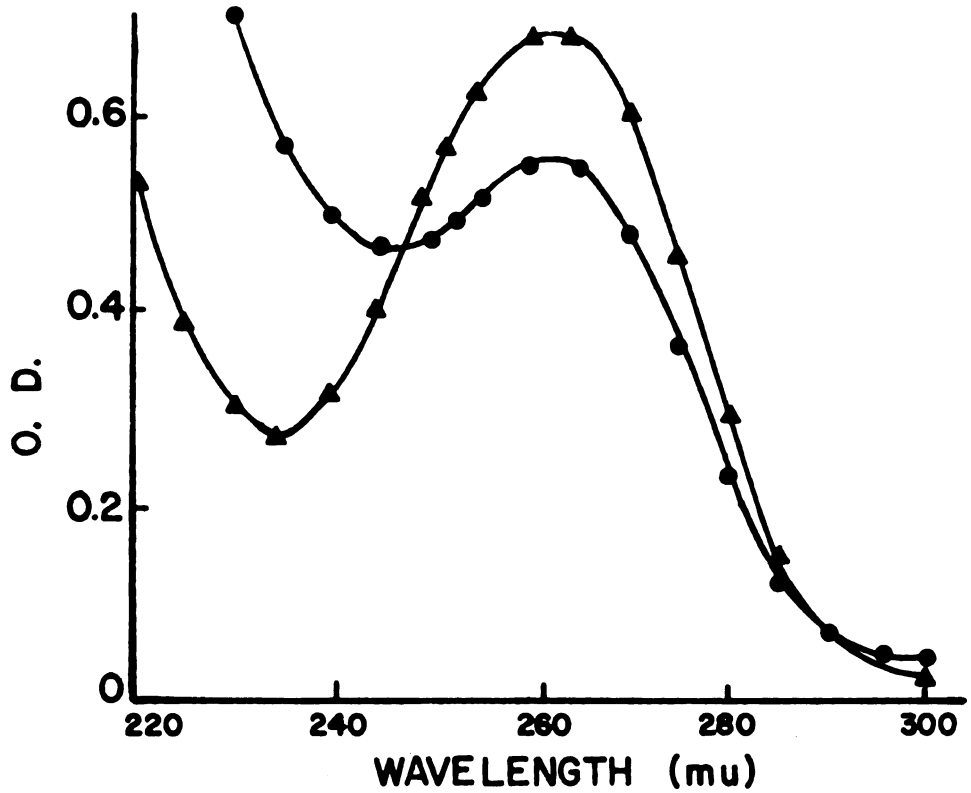

O.D. 260 mμ
1.2
1.0
0.8
0.6
0.4
0.2
0
0
40
80
120
160
200
240
280
320
360
1
2
3
4
5
FRACTION NUMBER
CD
E
F
J
K
L
MN
N1
P
P2
R

Figure 7. Ultraviolet absorption spectra for isolated sugar nucleotides.

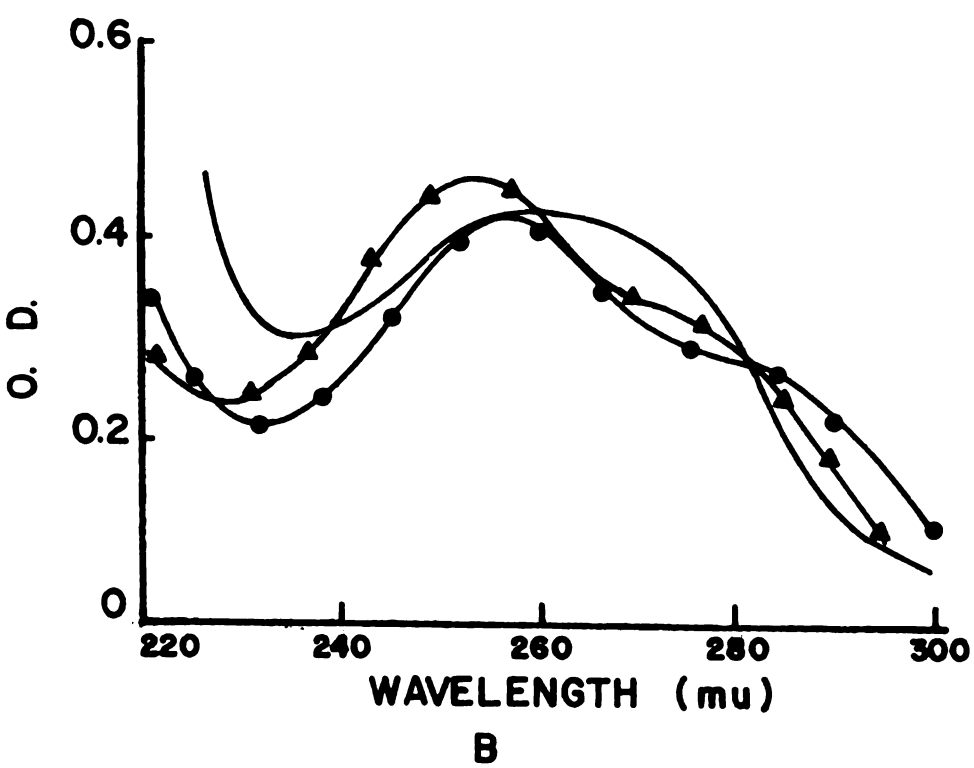
The pH was adjusted to the desired value with 5 N HCl and 5 N NaOH.

A. Spectra of UDPHexoses. ▲—▲ , pH 7;
●—● , pH 11.

B. Spectra of GDPHexoses. ●—● , pH 1;
▲—▲ , pH 7;
—— , pH 11.


0.6
0.4
0.2
0
O. D.
220
240
260
280
300
WAVELENGTH (mu)

A


0.6
0.4
0.2
0
O. D.
220
240
260
280
300
WAVELENGTH (mu)

B

in Table VII. The high reducing value for the GDPM samples before hydrolysis may be due to impurities, or due to partial hydrolysis of the mannose residue during the determination. Chemically synthesized GDPM also gave, before hydrolysis, about 25% of the total reducing value obtained after hydrolysis. Nevertheless, reducing equivalents approach one mole per mole of base as determined by spectral analysis. A total of 1.28 μmoles GDPM and 2.45 μmoles UDPG were isolated, a ratio of GDPM to UDPG of 0.52.

The sugar moieties of these two nucleotides were released by hydrolysis in 0.1 N HCl for 15 minutes at 100° C. The samples were deionized by passing through a 5.0 x 0.6 cm resin bed composed of equal amounts of Amberlite IR-120 (H) and IR-45 (OH). Following washing of the resin with 3 ml of water, the samples were concentrated and chromatographed in phenol-water and butanol-pyridine-water. Development with alkaline $AgNO_3$ revealed a heavy glucose and light galactose spot from the UDPG samples. From the GDPM samples, mannose was the principal component but in addition a detectable amount of fructose and possibly glucose were also present.

A portion of the GDPM sample was hydrolyzed in 0.01 N HCl for 15 minutes at 100° C. The acid was removed by repeated evaporation and the sample chromatographed in ethanol-ammonium acetate, pH 7.5. Ultraviolet absorbancy spots were observed which migrated exactly with authentic

Table VII. Analysis of Guanosine Diphosphate Hexose and Uridine Diphosphate Hexose Fractions

Fraction	Nucleotide	Base	Reducing Sugar++		
			Before Hydrolysis	After Hydrolysis	Difference
		μmoles		μmoles	
K	GDPHexose	0.32*	0.13	0.31	0.18
L	"	0.33	0.14	0.35	0.21
M	"	0.17	0.07	0.18	0.11
N	"	0.19	--	--	--
N_1	"	0.27	0.13	0.32	0.19
SUM	GDPHexose	1.28			
N	UDPHexose	1.84+	0.17	1.87	1.70
N_1	"	0.61	0.02	0.62	0.60
SUM	UDPHexose	2.45			

*Calculated on a molar extinction coefficient for guanosine of 13,700 at pH 7.0.

+Calculated on a molar extinction coefficient for uridine of 10,000 at pH 7.0.

++Analysis by the Park-Johnson method, hydrolysis consisting of 0.1 N HCl for 15 minutes at 100° C.

GMP and GDP.

Figure 6 reveals that GDPM was not eluted off in a discreet area as were other nucleotides. This may be due in part to the overloading of this column which was necessary to obtain sufficient quantities of nucleotides for identification. However, calculated on μmoles per ml eluate basis, GDPM is most concentrated in peak L. It should be mentioned that the preparative chromatograms of these peaks (K through N_1) developed in ethanol-ammonium-acetate, pH 7.5, contained several ultraviolet absorbing bands in addition to GDPM and UDPG. One of these was also ninhydrin positive.

The main components of other peaks eluted from the column as revealed by spectral analysis and chromatography in ethanol-ammonium acetate, pH 7.5, isobutyric acid-ammonium hydroxide-water, pH 4.3, and isobutyric acid-ammonium hydroxide-water, pH 3.7, are given in Fig. 6. Further rigorous characterization of these compounds was not undertaken.

Enzymes Involved in Glucose and Mannose Metabolism

Preparation of Cell Extracts

Hansenula holstii NRRL Y-2448 was grown on a medium consisting of 6.0% glucose, 0.1% corn steep liquor, 0.1% bactotryptone, 0.5% KH_2PO_4, and 0.5% salt solution as

described by Anderson et al. (39), with 200 ml of medium in each Fernbach flask inoculated with 10 ml of a 24 hour culture. The cultures were incubated at 28° C with shaking for various lengths of time, depending on the nature of the experiment for which the yeast was to be used. Cells were harvested in either a refrigerated Lourdes or Servall SS-1 centrifuge at 2° C, washed twice with cold water, and frozen until used.

Various methods of attaining soluble extracts were tried. Usually the cells were acetone dried by adding 20 volumes of acetone at -10° C to the wet cell paste and blending in an Omni-mixer for 2 minutes. After filtration, the powder was reextracted with cold acetone and filtered again. The dry powder was then worked on a filter paper until no acetone vapors were detected. The dried cells were extracted with 10 volumes of buffer (0.1 M Tris-0.01 M $MgCl_2$-0.001 M EDTA-0.001 mercaptoethanol, pH 7.6) at 0° C for 2 hours. Ammonium sulfate was added to the supernatant to 70% of saturation and the precipitate dissolved in buffer.

An alternative procedure consisted of blending the wet cell paste with 4 times the weight of glass beads (Superbrite size No. 110, Minnesota Mining and Manufacturing Company) and 1 volume of buffer at 0° C in the Omni-mixer at 5,000 rpm for one hour. Remaining whole cells and cell debris were removed by centrifugation at

15,000 g for 10 minutes. The supernatant was used directly or fractionated with ammonium sulfate as previously described. In some cases, the extract was used without centrifugation to remove cell debris.

Hexokinase

Spectrophotometric evidence for the presence of hexokinase was obtained by the reduction of TPN in the presence of glucose, ATP, G-6-P dehydrogenase and an extract from acetone dried cells. As seen in Fig. 8, reduction of TPN is dependent on the presence of ATP and glucose. To test the specificity of the kinase, extracts obtained from cells ruptured with glass beads were treated with ammonium sulfate and the precipitated protein assayed by measuring DPNH oxidation in the presence of hexose, ATP, phosphoenolpyruvate, and crystalline lactic dehydrogenase (containing pyruvate kinase). Table VIII shows that mannose is phosphorylated 71% as rapidly as is glucose. However, when both hexoses were present in the incubation mixture, no increase or additive rate was observed above that of glucose alone, indicating that the same enzyme is phosphorylating both hexoses. That mannose will serve as substrate for phosphomannan production as well as glucose will be shown later.

Dehydrogenases

Glucose-6-phosphate may be oxidized to 6-PGA in the presence of TPN and acetone powder extract. Fig. 9

Figure 8. Hexokinase activity.

Complete system in 0.5 ml total volume contained: 1.0 μmole glucose, 1.0 μmole ATP, 0.5 μmole TPN, 0.5 μmole $MgCl_2$, 0.01 ml G-6-P dehydrogenase, 41.0 μmoles glycylglycine, pH 8.0, and enzyme (approximately 0.02 mg protein).

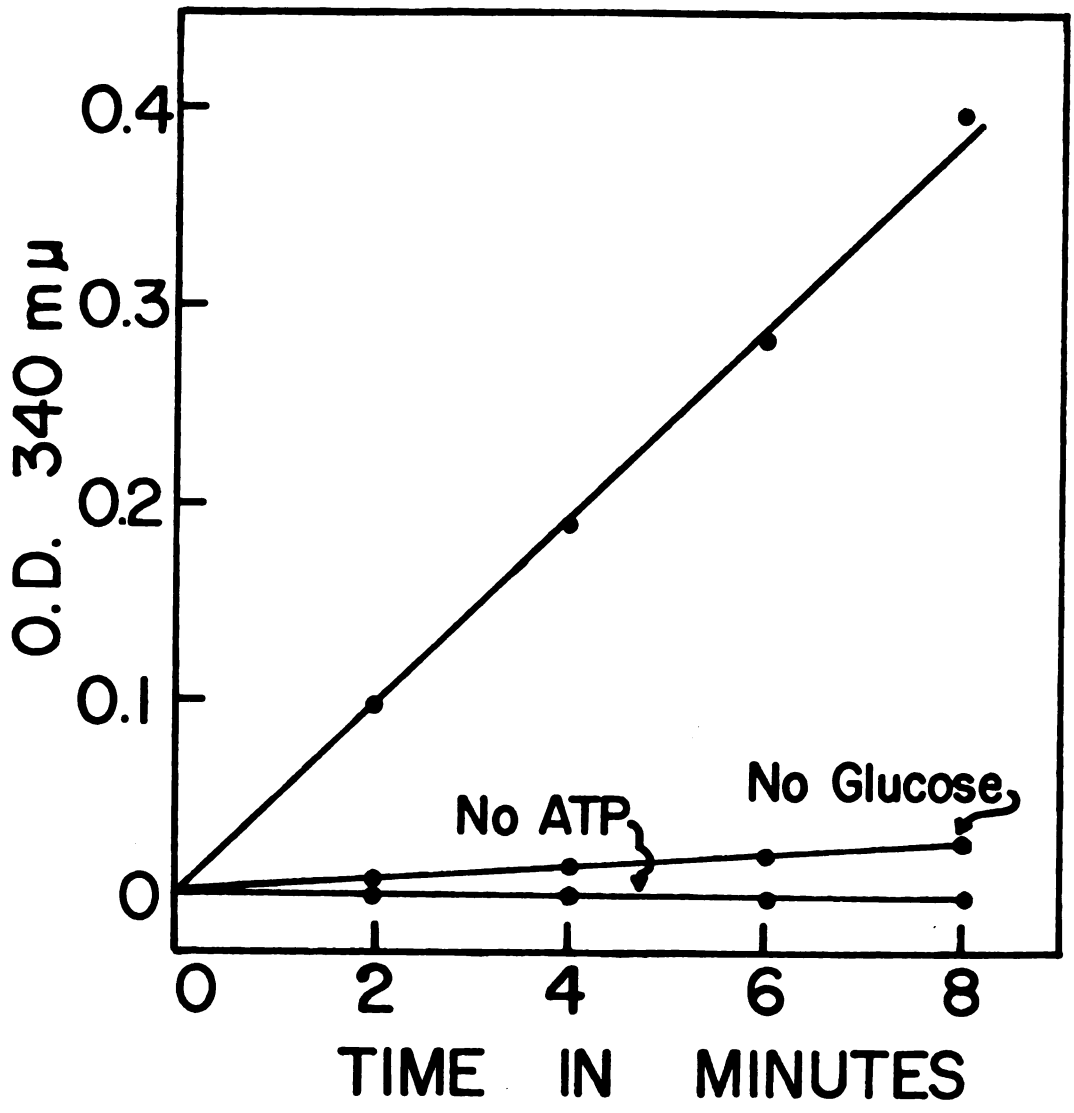

0.4
0.3
0.2
0.1
0
O.D. 340 mμ
No ATP
No Glucose
0
2
4
6
8
TIME IN MINUTES

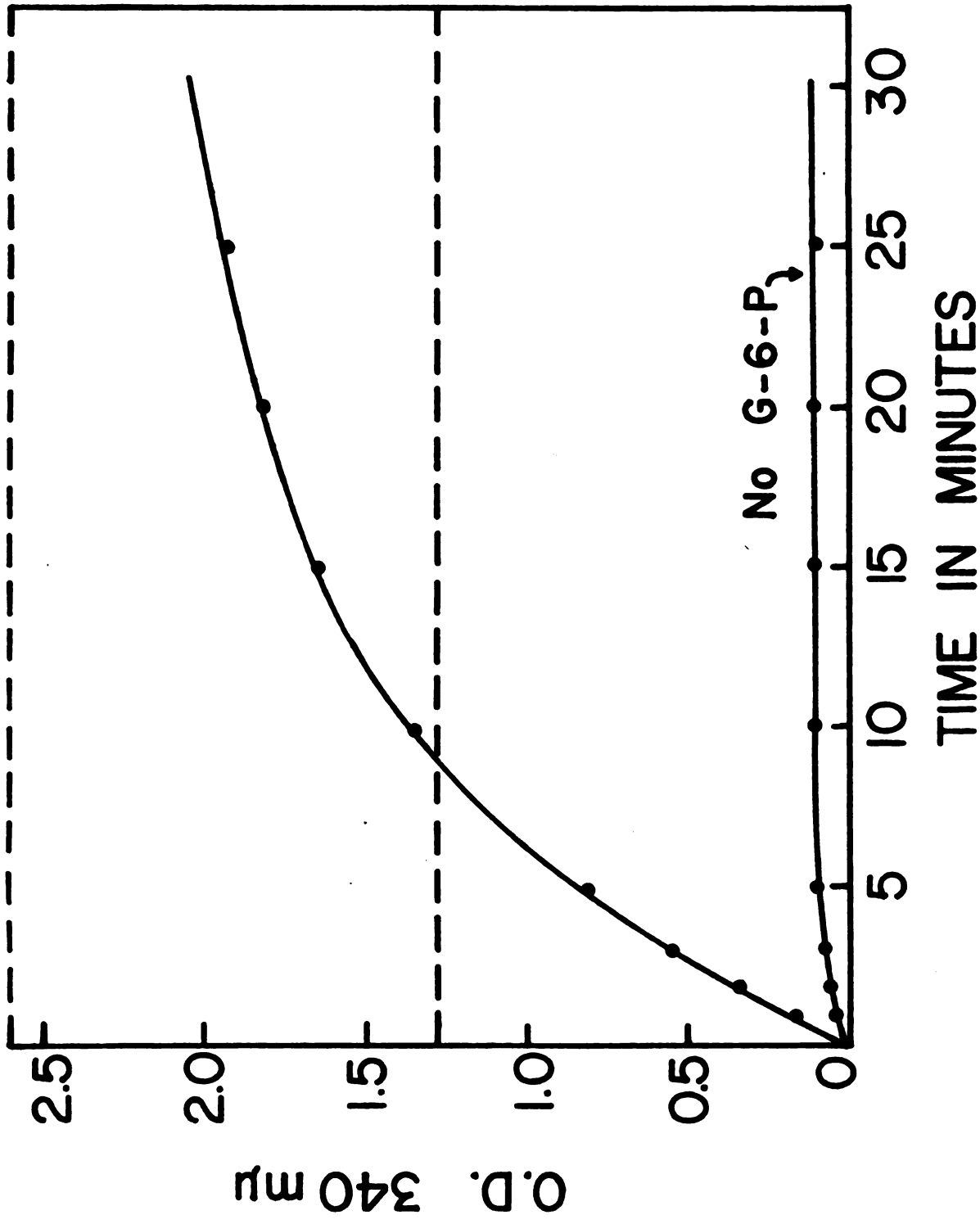
Table VIII. Nonspecific Hexokinase Activity

Hexose	Δ OD_{340}/min
--	0.09
Mannose	0.15
Glucose	0.21
Mannose + Glucose	0.21

The complete system contained in a 0.5 ml total volume: 0.5 μmole each hexose, 0.5 μmole phosphoenolpyruvate, 0.5 μmole DPNH, 0.01 ml crystalline lactic acid dehydrogenase diluted 1:10, 0.5 μmole $MgCl_2$, 2.5 μmoles KF, 0.5 μmole ATP, 42.5 μmoles glycylglycine, pH 8.0, and enzyme (approximately 0.05 mg protein).

Figure 9. Glucose-6-phosphate and 6-phosphogluconic acid dehydrogenase activity.

Complete system in 0.5 ml total volume contained: 0.1 μmole G-6-P, 0.5 μmole TPN, 0.5 μmole $MgCl_2$, 46.0 μmoles Tris, pH 7.6, and enzyme (approximately 0.1 mg protein). The dash lines indicate the theoretical end point for 1.0 mole of TPN reduced (bottom line) and 2.0 moles of TPN reduced (top line) per mole of G-6-P added.


O.D. 340 mμ
2.5
2.0
1.5
1.0
0.5
0
5
10
15
20
25
30
TIME IN MINUTES
No G-6-P

demonstrates that per mole of G-6-P, almost 2 moles of TPN are reduced, indicating that 6-PGA dehydrogenase is also present. The latter enzyme was confirmed by showing that 6-PGA is oxidized by the same extract, producing 1 mole of TPNH per mole of 6-PGA (Fig. 10).

Isomerases

The possibility that G-6-P may be isomerized to F-6-P was tested by measuring ketohexose formation from G-6-P by acetone powder extract (Table IX). Isomerization of the F-6-P formed to M-6-P by phosphomannoisomerase is minimized by running the reaction at pH 9.0 (64). The presence of the latter enzyme was shown by incubating M-6-P with acetone powder extract and measuring ketohexose formation. As expected, more F-6-P is formed at pH 5.5 than at pH 7.6; however, phosphomannoisomerase is present in much lower amounts than phosphoglucoisomerase. Comparable activities of the two isomerases at pH 8.0 may be seen in Fig. 11.

Mutases

Evidence for the ability of M-1-P to be metabolized via M-6-P, F-6-P, G-6-P and 6-PGA by acetone powder extract was obtained (Fig. 12). As the extract had previously been shown to contain phosphomannoisomerase, phosphoglucoisomerase, and G-6-P dehydrogenase, it was not necessary to add further quantities of these enzymes. The initial step in this sequence of reactions (conversion

Figure 10. Six-phosphogluconic acid dehydrogenase activity.

Complete system in 0.5 ml total volume contained: 0.5 μmole TPN, 0.1 μmole 6-PGA, 0.5 μmole $MgCl_2$, 45.0 μmoles Tris, pH 7.6, and enzyme (approximately 0.1 mg protein). The dash line indicates the theoretical end point for 1.0 mole of TPN reduced per mole of 6-PGA oxidized.

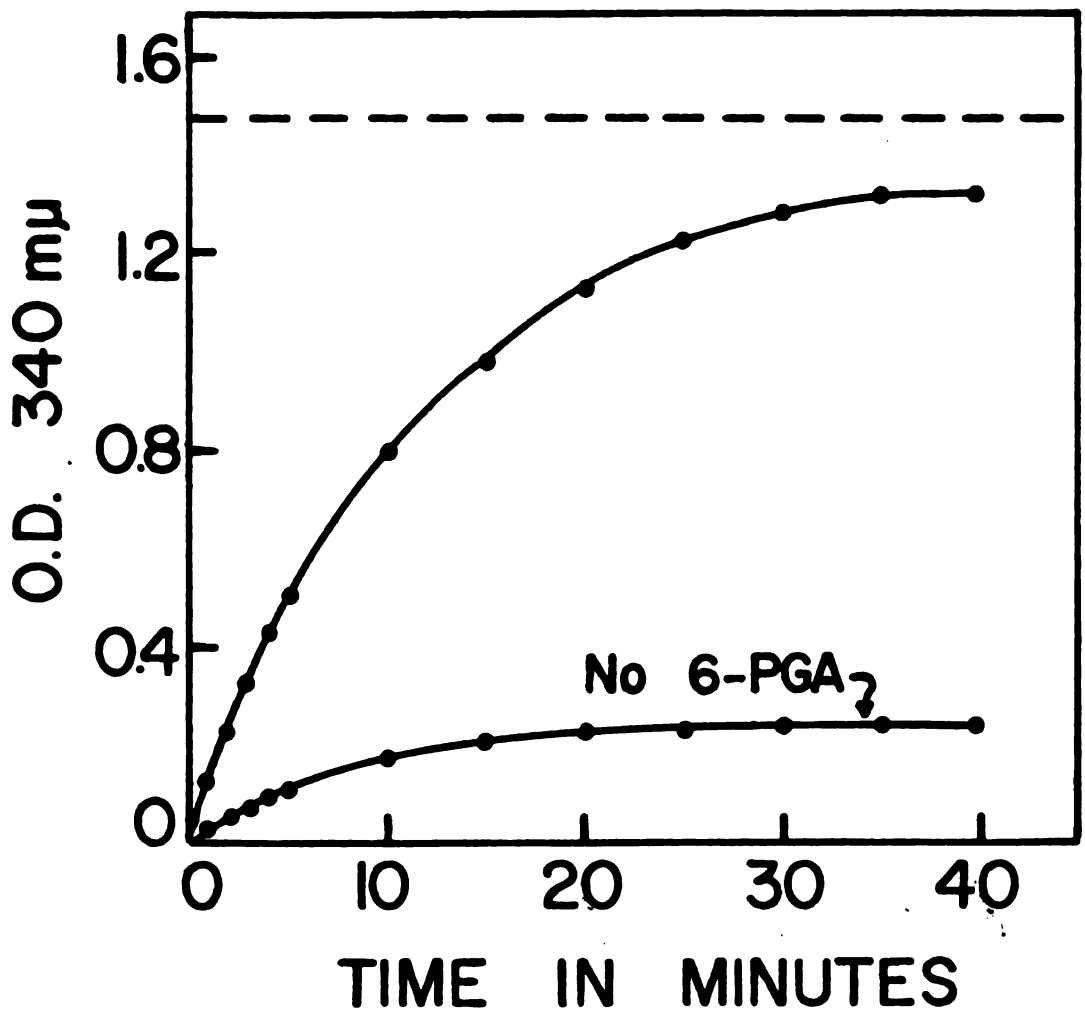

1.6
1.2
0.8
0.4
0
O.D. 340 mμ
No 6-PGA2
0
10
20
30
40
TIME IN MINUTES

Table IX. Ketohexose Formation from Hexose-6-Phosphate

Substrate	Time (min)	F-6-P formed (μmoles) pH 5.5	pH 7.6	pH 9.0
G-6-P	0			0
	10			0.22
	20			0.39
M-6-P	0	0	0	
	30	0.13	0.07	

Separate incubations were run for each time and each pH. The mixture contained in a 0.5 ml total volume: 1.0 μmole hexose-6-phosphate, 21.6 μmoles acetate, pH 5.5, or 36.0 μmoles Tris, pH 7.6 or 44.0 μmoles Tris, pH 9.0, and enzyme (approximately 0.04 mg protein for the G-6-P incubations and 0.2 mg protein for the M-6-P incubations). At the time intervals shown, ketohexose was determined using F-6-P as standard.

Figure 11. Phosphohexoisomerase activity.

Complete system in 0.5 ml total volume contained: 0.5 μmole $MgCl_2$, 0.5 μmole TPN, 43.0 μmoles glycylglycine, pH 8.0, 0.5 μmole F-6-P or 0.5 μmole M-6-P, and enzyme (approximately 0.04 mg protein).

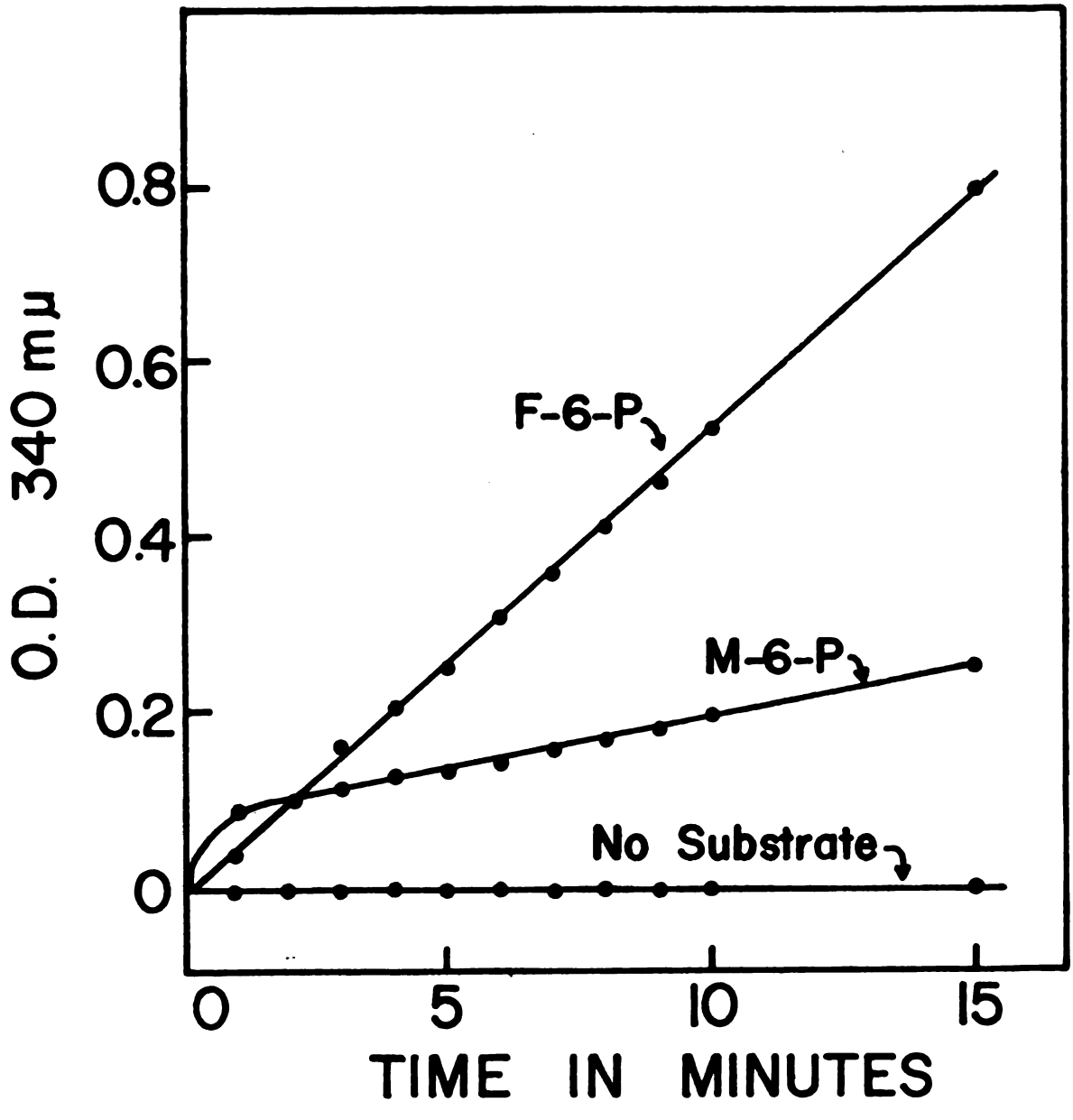
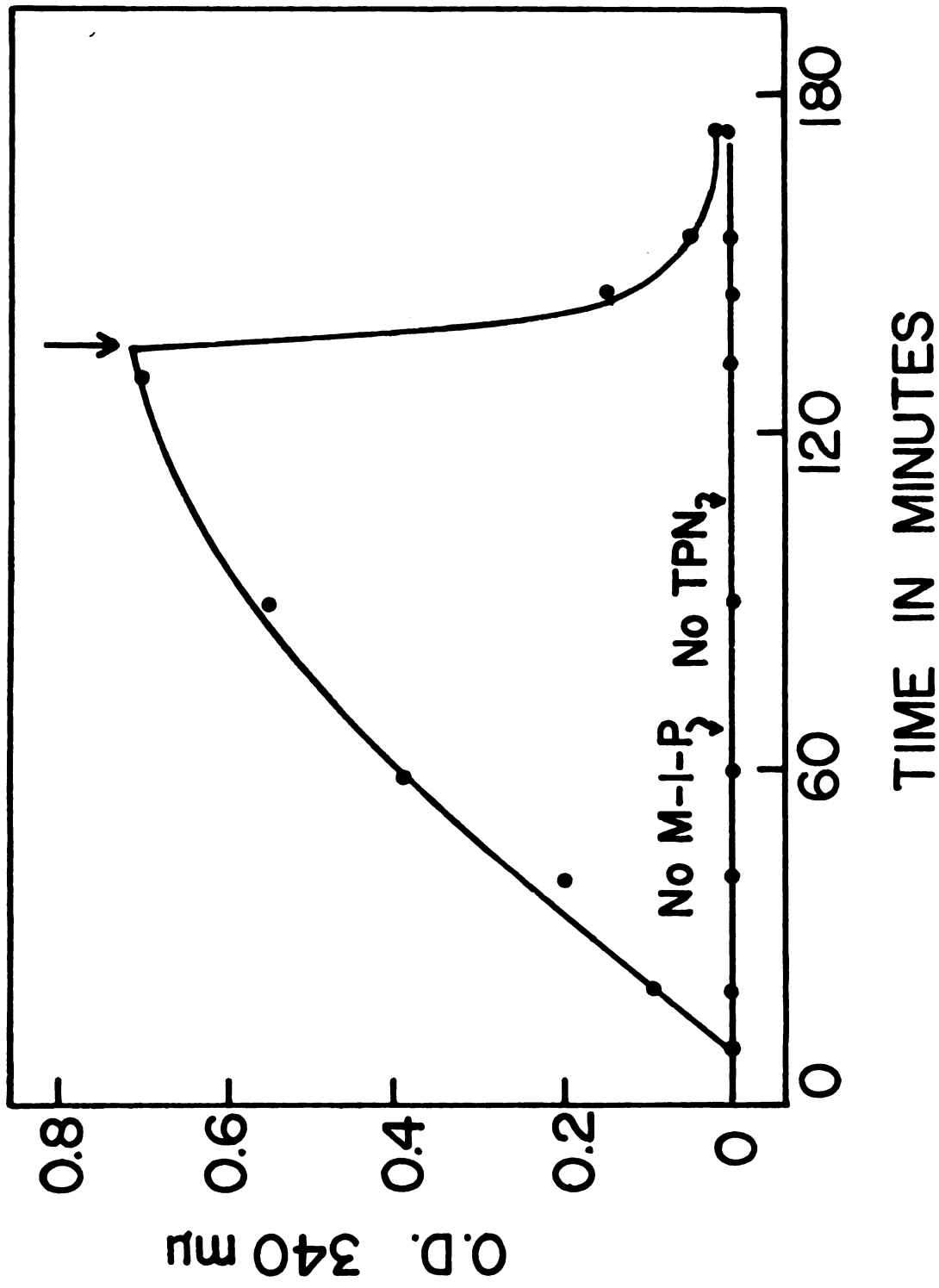

0.8
0.6
0.4
0.2
0
O.D. 340 mμ
F-6-P
M-6-P
No Substrate
0
5
10
15
TIME IN MINUTES

Figure 12. Phosphomannomutase activity.

Complete system in 0.5 ml total volume contained: 1.0 μmole M-1-P, 0.5 μmole TPN, 0.5 μmole $MgCl_2$, 42.0 μmoles Tris, pH 7.6, and enzyme (approximately 0.4 mg protein). Optical density was recorded at various time intervals. At the arrow (135 minutes) 1.0 μmole potassium pyruvate and 0.1 mg crystalline lactic acid dehydrogenase was added to each cuvette.


O.D. 340 mμ
0.8
0.6
0.4
0.2
0
0
60
120
180
TIME IN MINUTES
No M-I-P3
No TPN3

of M-1-P to M-6-P) was shown to be limiting by a greatly increased rate of TPN reduction upon addition of M-6-P. The initial lag period of approximately 10 minutes is probably the time required to build up intermediates to make the chain of enzymic reactions proceed through G-6-P dehydrogenase. That the slow reaction was actually resulting in TPN reduction is substantiated by the rapid oxidation of TPNH upon addition of potassium pyruvate and crystalline lactic dehydrogenase. In one experiment, acid labile phosphorus was determined in the incubation mixture at the end of the spectrophotometric assay. The decrease in the amount of acid labile phosphorus (M-1-P) corresponded very well to the amount of TPNH formed, thus confirming the conversion of M-1-P to acid stable hexose-6-phosphates. No apparent effect of G-1,6-diP on the reaction rate was observed.

A very active phosphoglucomutase was demonstrated in acetone powder extracts by adding G-1-P, TPN, and additional G-6-P dehydrogenase (Fig. 13). A slight stimulation by G-1,6-diP was observed.

Pyrophosphorylases

The phosphoglucomutase reaction provides a source of G-1-P for the synthesis of UDPG. The presence of UDPG pyrophosphorylase was detected by measuring DPN reduction in the presence of UTP, G-1-P, and excess UDPG dehydrogenase. As seen in Fig. 14, this enzyme is very active in acetone powder extracts and was also active in one

Figure 13. Phosphoglucomutase activity.

Complete system in 0.5 ml total volume contained: 0.5 μmole G-1-P, 0.5 μmole $MgCl_2$, 0.01 ml G-6-P dehydrogenase, 1.0 μmole TPN, 5.0 μmoles L-cysteine, pH 7.5, 0.2 mμmole G-1,6-diP, 40.0 μmoles Tris, pH 7.5, and enzyme (approximately 0.02 mg protein).

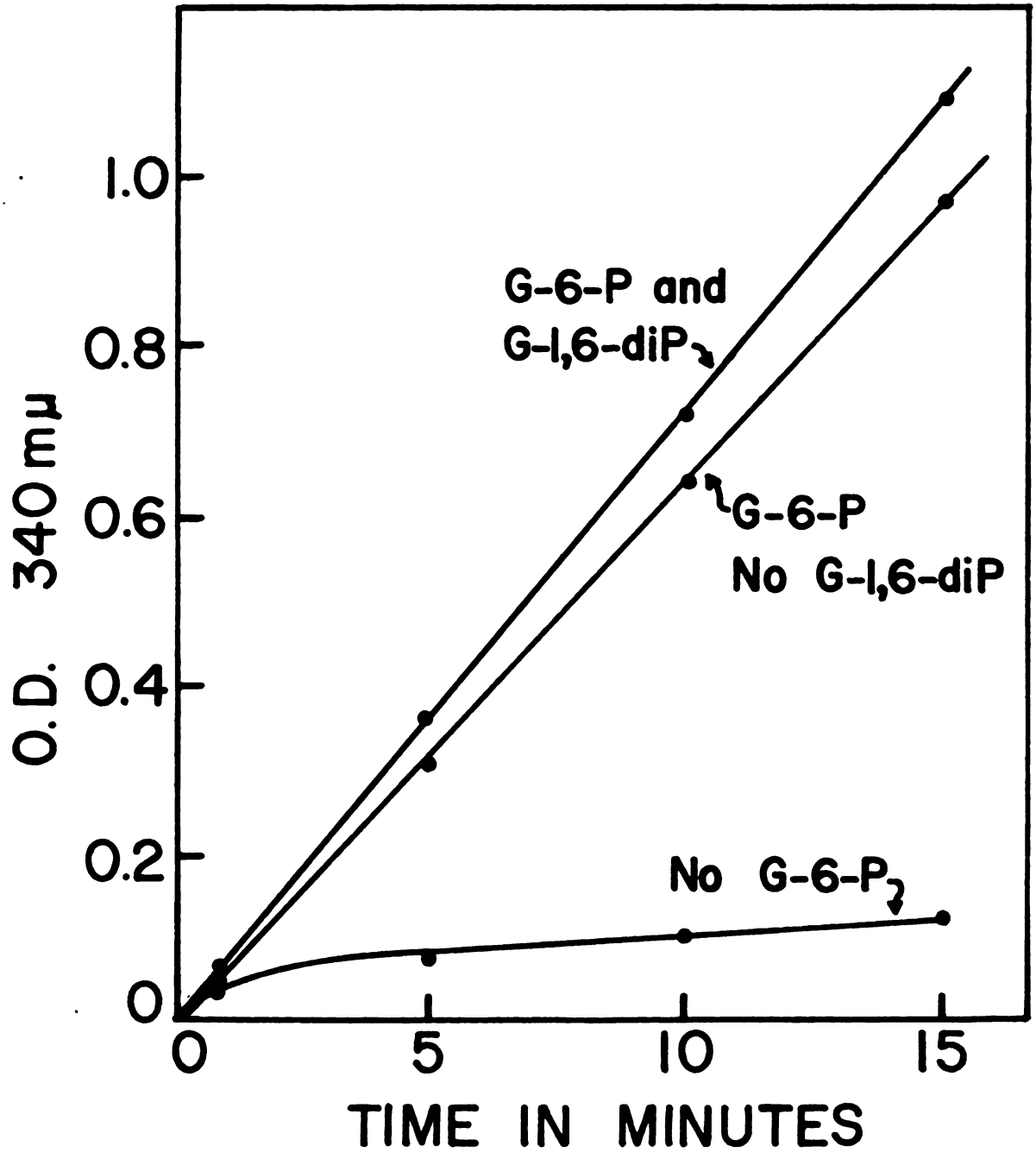
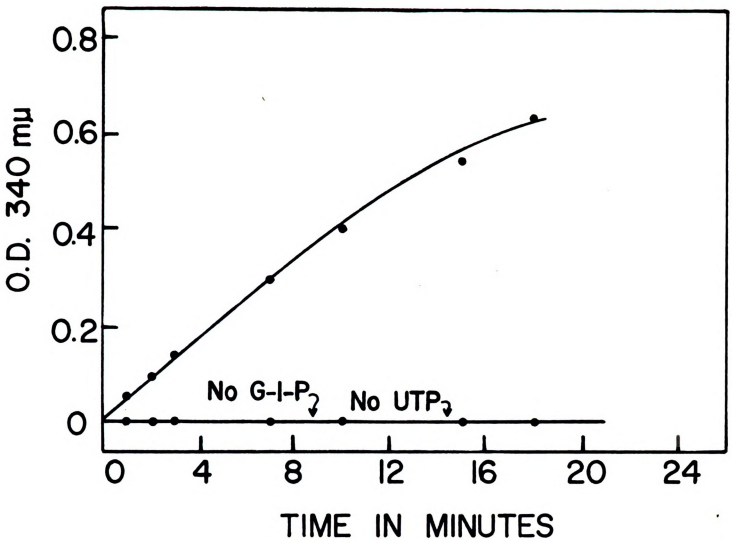

1.0
0.8
0.6
0.4
0.2
0
O.D. 340mμ
G-6-P and
G-1,6-diP
G-6-P
No G-1,6-diP
No G-6-P
0
5
10
15
TIME IN MINUTES

Figure 14. Uridine diphosphate glucose pyrophosphorylase activity.

Complete system in 0.5 ml total volume contained: 0.4 μmole UTP, 1.0 μmole G-1-P, 0.5 μmole $MgCl_2$, 0.25 μmole DPN, 5.0 μmoles NaF, 39.0 μmoles Tris, pH 7.6, and enzyme (approximately 0.2 mg protein).


0.8
0.6
0.4
0.2
0
O.D. 340 mμ
No G-1-P
No UTP
0
4
8
12
16
20
24
TIME IN MINUTES

preparation of air dried cells extracted with 0.1 M Tris buffer, pH 7.6.

Attempts to demonstrate UDPG dehydrogenase spectrophotometrically were consistently negative, both in acetone powder extracts and cells ruptured with glass beads. This is consistent with the absence of UDPGA in the acid soluble nucleotides from this yeast.

The formation of GDPM from GTP and M-1-P by acetone powder extracts was detected chromatographically. After incubation of the extract with GTP and M-1-P, the reaction mixture was heated in a boiling water bath for one minute to denature protein. The supernatant was lyophilized and chromatographed on Whatman No. 1 filter paper in ethanol-ammonium acetate, pH 7.5. For better separation of GDPM and GMP, the GDPM was eluted with water, lyophilized, and rechromatographed in isobutyric acid-ammonium hydroxide-water, pH 4.3. The GDPM was then eluted with water and quantitated by ultraviolet absorption, using a molar extinction coefficient of 13,700 at pH 7.0.

Resolution of the various nucleotides in ethanol-ammonium acetate, pH 7.5, was enhanced by first adsorbing the nucleotides on charcoal after the protein denaturation step, then washing the charcoal with water before eluting the nucleotides with 15% pyridine. Following extraction of the pyridine with chloroform, the aqueous layer was lyophilized and chromatographed. Results of such an experiment

are shown in Fig. 15. No GDPM was detected in the absence of M-1-P.

For further characterization of the product of this enzymic reaction, larger scale incubations consisting of 6.0 μmoles GTP, 12.0 μmoles M-1-P, 60.0 μmoles NaF, 108.0 μmoles Tris, pH 7.6, 10.8 μmoles $MgCl_2$, 1.1 μmole EDTA, 1.1 μmole mercaptoethanol, and 2.4 mg protein in a total volume of 1.8 ml were incubated two hours. The GDPM formed was isolated by chromatography in ethanol-ammonium acetate and isobutyric acid-ammonium hydroxide-water, pH 4.3 as described previously. In this manner, 0.5 μmole of chromatographically pure GDPM was obtained which exhibited a typical guanosine spectrum at pH 1, 7 and 11. Reducing sugar analysis by the Park and Johnson method resulted in a ratio of reducing sugar to base of 0.7:1. An aliquot containing 0.15 μmole was hydrolyzed in 0.1 N HCl for 15 minutes at 100° C and then deionized with Amberlite IR-120 (H) and IR-45 (OH). The sugars obtained were chromatographed in phenol-water and the spots detected with alkaline $AgNO_3$. A heavy mannose spot was observed along with lighter spots of glucose and fructose. Hydrolysis of an equal amount of chemically synthesized GDPM resulted in the detection of only mannose (Fig. 16).

The occurrence of glucose and fructose in hydrolyzates might arise by interconversion of the intact GDPM molecule or alternatively by formation of the respective GDPHexoses

Figure 15. Guanosine diphosphate mannose pyrophosphorylase activity.

Complete system in 0.3 ml total volume contained: 1.0 μmole GTP, 2.0 μmoles M-1-P, 10.0 μmoles NaF, 16.0 μmoles Tris, pH 7.6, 1.6 μmoles $MgCl_2$, 0.16 μmole EDTA, 0.16 μmole mercaptoethanol, and enzyme (approximately 0.6 mg protein). The control lacked M-1-P. Incubation was for 1.5 hours at 25° C. The tubes were then heated in a boiling water bath for one minute, cooled, and centrifuged. The nucleotides were adsorbed onto charcoal, eluted off, and chromatographed in ethanol-ammonium acetate, pH 7.5. Lane 1, control incubation; lane 2, complete incubation; lane 3, standards.

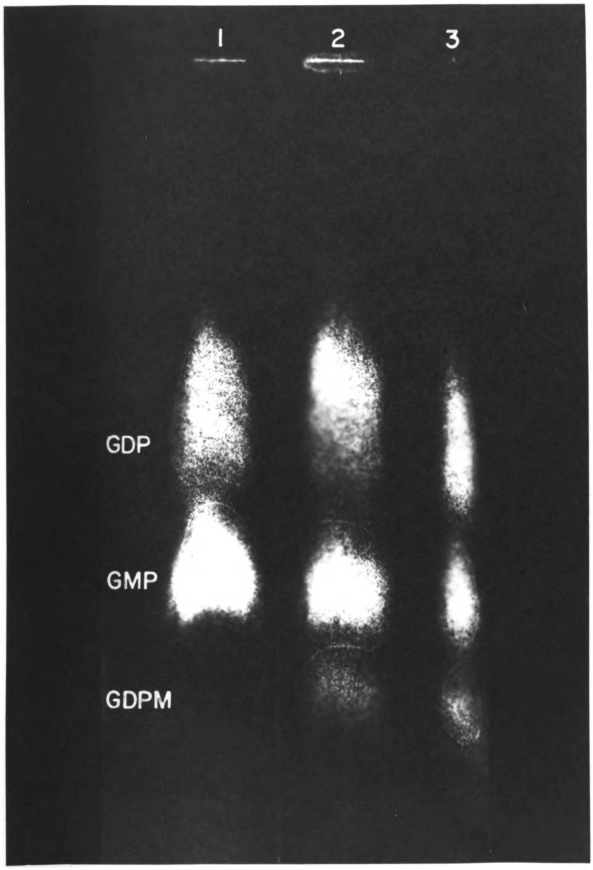
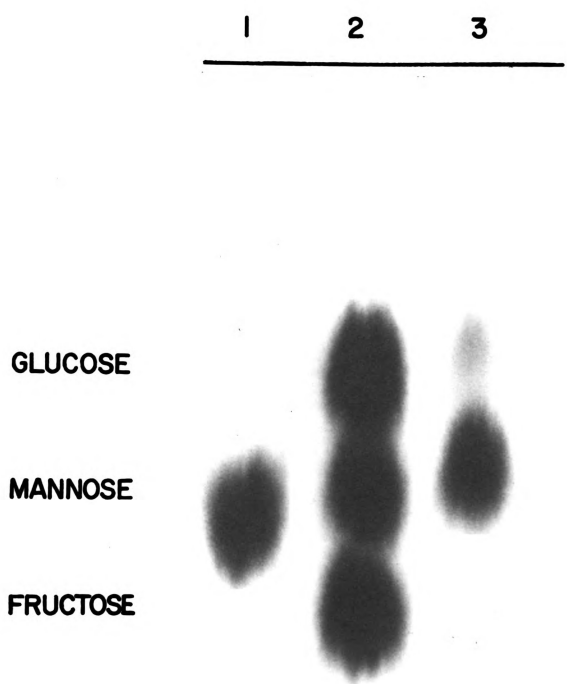

1
2
3
GDP
GMP
GDPM

Figure 16. Sugars from hydrolyzed guanosine diphosphate hexoses.

GDPHexose synthesized enzymically from GTP and M-1-P was hydrolyzed, deionized, and the sugars chromatographed in phenol-water. Chemically synthesized GDPM was treated identically. Lane 1, sugars from chemically synthesized GDPM; lane 2, standards; lane 3, sugars from enzymically synthesized GDPHexose.


1
2
3
GLUCOSE
MANNOSE
FRUCTOSE

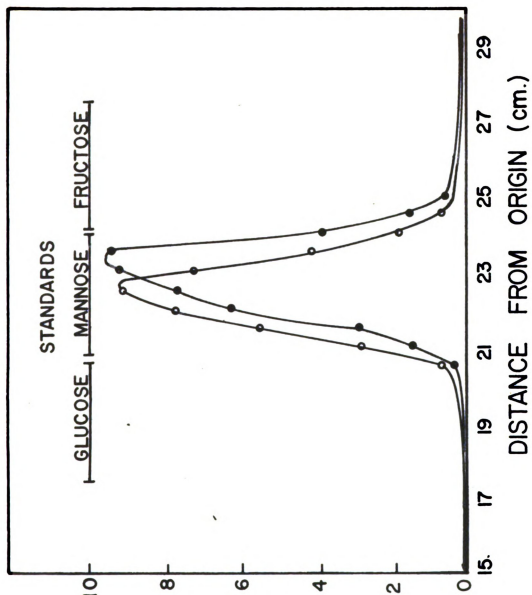
from GTP and hexose-1-phosphate. In an attempt to elucidate the mechanism of formation of these GDPHexoses, an experiment was carried out as follows: 4.0 μmoles C^{14}-GDPM, 10.0 μmoles NaF, 18.0 μmoles Tris, pH 7.6, 1.8 μmoles $MgCl_2$, 0.2 μmole EDTA, 0.2 μmole mercaptoethanol, and 1.1 mg protein in a total volume of 0.33 ml were incubated three and a half hours at room temperature before heating two minutes in a boiling water bath. An identical mixture was heated without incubation. About 2.5 μmoles of GDPHexoses in each case were reisolated by chromatography in the two solvents previously used. An aliquot of each sample was counted in the liquid scintillation spectrophotometer and identical specific activities were observed (51,800 dpm/μmole). Apparently a dilution due to endogenous GDPHexose occurred as the specific activity of the C^{14}-GDPM used initially was 80,000 dpm/μmole. Another aliquot (0.5 μmole) of each sample was hydrolyzed in 0.1 N HCl for 15 minutes at 100° C and deionized. Half of each hydrolyzate was chromatographed in phenol-water and developed with alkaline $AgNO_3$. A heavy mannose spot and faint spots of glucose and fructose were observed for each sample. The remaining half of each hydrolyzate was chromatographed in phenol-water under identical conditions as the preceding run. The vertical areas corresponding to authentic glucose, mannose and fructose were cut into 4.0 mm cross sections and each placed in 10 ml of scintillating fluid. The counting

pattern observed (Fig. 17) shows that only mannose is labeled. It is therefore concluded that the intact GDPM was not being enzymically converted to GDPG or GDPFructose.

To decide if GDPG pyrophosphorylase was present, the following experiments were performed: 4.0 μmoles GTP, 40.0 μmoles NaF, 72.0 μmoles Tris, pH 7.6, 7.2 μmoles $MgCl_2$, 0.7 μmole EDTA, 0.7 μmole mercaptoethanol, and 0.44 mg protein were incubated with 1) 2.0 μmoles C^{14}-M-1-P, 2) 2.0 μmoles C^{14}-G-1-P, and 3) neither hexose-1-phosphate for a control. After three hours, the samples were treated and chromatographed as described for the previous experiment. No ultraviolet absorbing material which corresponded to GDPM was observed in the control sample, but the appropriate areas on the chromatograms were cut out, eluted, and treated the same as for the other two. About 0.17 μmole of GDPHexose from GTP and C^{14}-M-1-P and about 0.06 μmole from GTP and C^{14}-G-1-P were isolated. All three samples were hydrolyzed, deionized and chromatographed in phenol-water. A radioaudiogram revealed only labeled mannose from the hydrolyzed GDPHexose formed from GTP and C^{14}-M-1-P, and only labeled glucose from the hydrolyzed GDPHexose formed from GTP and C^{14}-G-1-P. The C^{14}-glucose area was eluted and rechromatographed in butanol-pyridine-water. Radioactivity was detected with a gas flow counter only in the glucose area and upon development with alkaline $AgNO_3$, a heavy glucose spot was visible. The remainder

Figure 17. Retention of label in mannose in guanosine diphosphate hexoses.

Details of the experiments are described in the text. o—o , GDPMannose, 0 time; ●—● , GDPMannose, 3.5 hours. The difference in peak locations is due to a slight difference in rate of solvent flow.


STANDARDS
GLUCOSE
MANNOSE
FRUCTOSE
c.p.m. x 10⁻²
0
2
4
6
8
10
15
17
19
21
23
25
27
29
DISTANCE FROM ORIGIN (cm.)

of the chromatogram from which the C^{14}-glucose area was eluted was developed with alkaline $AgNO_3$. Spots observed were 1) mannose from the hydrolyzed product of GTP and C^{14}-M-1-P, the spot corresponding exactly to the spot on the radioaudiogram, and 2) glucose from the control sample which lacked hexose-1-phosphate. This latter glucose spot would suggest that GDPG, in an amount too small to detect with the ultraviolet lamp, was synthesized in the control incubation from endogenous G-1-P and added GTP. These experiments demonstrate the formation of GDPM and GDPG from GDPM pyrophosphorylase and GDPG pyrophosphorylase, respectively, and indicate that the two sugar nucleotides are not interconverted.

Experiments Concerned with Precursors of Phosphomannan

Phosphomannan Synthesis by Washed, Resting Cells

A flask containing 200 ml of 6% glucose medium was inoculated with 5 ml of a 24 hour culture. After 68 hours, the cells were harvested by centrifugation and washed four times with 200 ml of cold water. The final wash contained negligible phenol-sulfuric acid reacting material. The cells were resuspended in water to a total volume of 36 ml. To each of seven flasks, 4 ml of this cell suspension were added, together with glucose and phosphate as indicated in Table X. After shaking for 91 hours, the phosphomannan

Table X. Synthesis of Phosphomannan by Washed, Resting Cells

Flask No.	%Glucose	M PO_4, pH 6.0	mg Phosphomannan
1	0	0	9.2
2	2.14	0	49.7
3	2.14	0.007	74.2
4	2.14	0.014	69.6
5	2.14	0.028	93.2
6	2.14	0.043	99.6
7	2.14	0.057	98.6

Flasks contained in a total of 14 ml: 300 mg glucose, phosphate buffer pH 6.0 as indicated, and 4 ml of cell suspension prepared as described in the text. Flasks were shaken 91 hours at 27° C followed by isolation of the extracellular phosphomannan.

was collected by adding 1/100 volume of saturated KCl to the cell-free supernatant and then adding two volumes of 95% ethanol. Following reprecipitation, the polymer was dialyzed 60 hours with distilled water at 3° C. Ten volumes of 95% ethanol and 1/100 volume of saturated KCl were added to precipitate the dialyzed phosphomannan. After washing with absolute ethanol, the samples were dried in vacuo. Table X shows that although some polymer is released from the washed cells suspended in water alone, or in 2.14% glucose, addition of phosphate buffer, pH 6.0, up to 0.043 M greatly stimulates phosphomannan production. Flask 6 represents 29% conversion of glucose and 19% conversion of phosphate to phosphomannan. In other experiments, higher phosphate concentration resulted in inhibition of polymer production. The production of approximately 50 mg of polymer in the absence of phosphate indicates an extremely high endogenous supply of phosphate. However, it is apparent that no other metabolite except glucose and phosphate are necessary for phosphomannan production in resting cells.

Synthesis of Phosphomannan by Whole Cultures

Polysaccharides, such as levan (65), dextran (66), and possibly cellulose (17), are synthesized by extracellular enzymes. An attempt to detect extracellular synthesis of phosphomannan was carried out by adding radioactive substrates to resting cells which were actively producing polymer.

As no accumulation of hexose-phosphate or nucleotides could be detected in the medium by paper chromatography, it was assumed that compounds of this type were impermeable to the cell wall, and any incorporation of label into the polymer would be of an extracellular nature. Cultures were grown for 36 hours on a 1% glucose medium at which time approximately half of the glucose was utilized. Glucose was added to each flask to make the medium again 1% and radioactive glucose, mannose, GDPM, and M-1-P were added as described in Table XI. In addition, radioactive glucose was added to cell-free medium alone. As shown in Table XI, after 24 hours, radioactive phosphomannan is produced in the presence of radioactive glucose and mannose. The amount of phosphomannan increased approximately 50% during the 24 hour incubation period. Assuming a 1% glucose medium at the time of addition of the radioactive substrate and a 50% increase in polymer concentration, the specific activity of the polymer is very close to theoretical. However, no radioactivity from C^{14}-GDPM was incorporated into the polymer above that of the 0 time control. Some incorporation of radioactivity from C^{14}-M-1-P was observed, but based on the 50% increase in polymer concentration, any preferential utilization of this substrate should have resulted in highly labeled polymer. No incorporation of label from C^{14}-glucose incubated with media alone was detected. These experiments imply that there are no extra-

Table XI. Synthesis of Phosphomannan in Whole Cultures

Conditions	Radioactive Substrate	Time	Radioactivity in Phosphomannan
		hrs	cpm/mg
Whole culture	Glucose	0	0
		24	1970
Whole culture	Mannose	0	0
		24	1990
Whole culture	GDPM	0	25
		24	26
Whole culture	M-1-P	0	0
		24	183
Medium	Glucose	24	0

To 20 ml cultures grown 36 hours on 1% glucose were added 100 mg glucose (making cultures again 1% glucose) and radioactive substrates as follows: glucose-1-C^{14}, 10^6 cpm; mannose-1-C^{14}, 1.3×10^6 cpm; GDPM-U.L., 2×10^5 cpm; M-1-P-U.L., 2×10^5 cpm. After shaking for 24 hours at 27° C, phosphomannan was isolated and purified as described in the text for the previous experiment (Table X) and counted.

cellular enzymes capable of producing phosphomannan from the substrates tested.

Studies on Synthesis of Phosphomannan by Cell-Free Extracts

Attempts were made to synthesize phosphomannan by cell-free extracts using various radioactive substrates. Extracts were prepared as previously described by disrupting the cells with glass beads and spinning out most of the remaining whole cells in an International Clinical Centrifuge. These extracts contained all of the enzymes previously described for the conversion of glucose to GDPM. Radioactive substrates used in the experiments were glucose, M-1-P, and GDPM. In addition, many cofactors were added in various combinations such as M-1-P, M-6-P, GTP, ATP, and UTP. Primer phosphomannan and small amounts of the cell-free growth medium were also added to the incubation mixture. As growing or resting cultures have been reported to produce phosphomannan most efficiently at pH 5 (39), both this pH and an alkaline pH (8 to 9) were maintained in various experiments. In no case was label incorporated into polysaccharide which was stable to dialysis.

Extensive dialysis (60 hours) was found to be necessary to remove radioactive GDPM from phosphomannan in 0 time controls. To eliminate the possibility of short chain oligosaccharides being dialyzed out, the dialysis water from an experiment in which C^{14}-GDPM was used was concentrated and chromatographed in butanol-acetic acid-water (4:1:5 v/v).

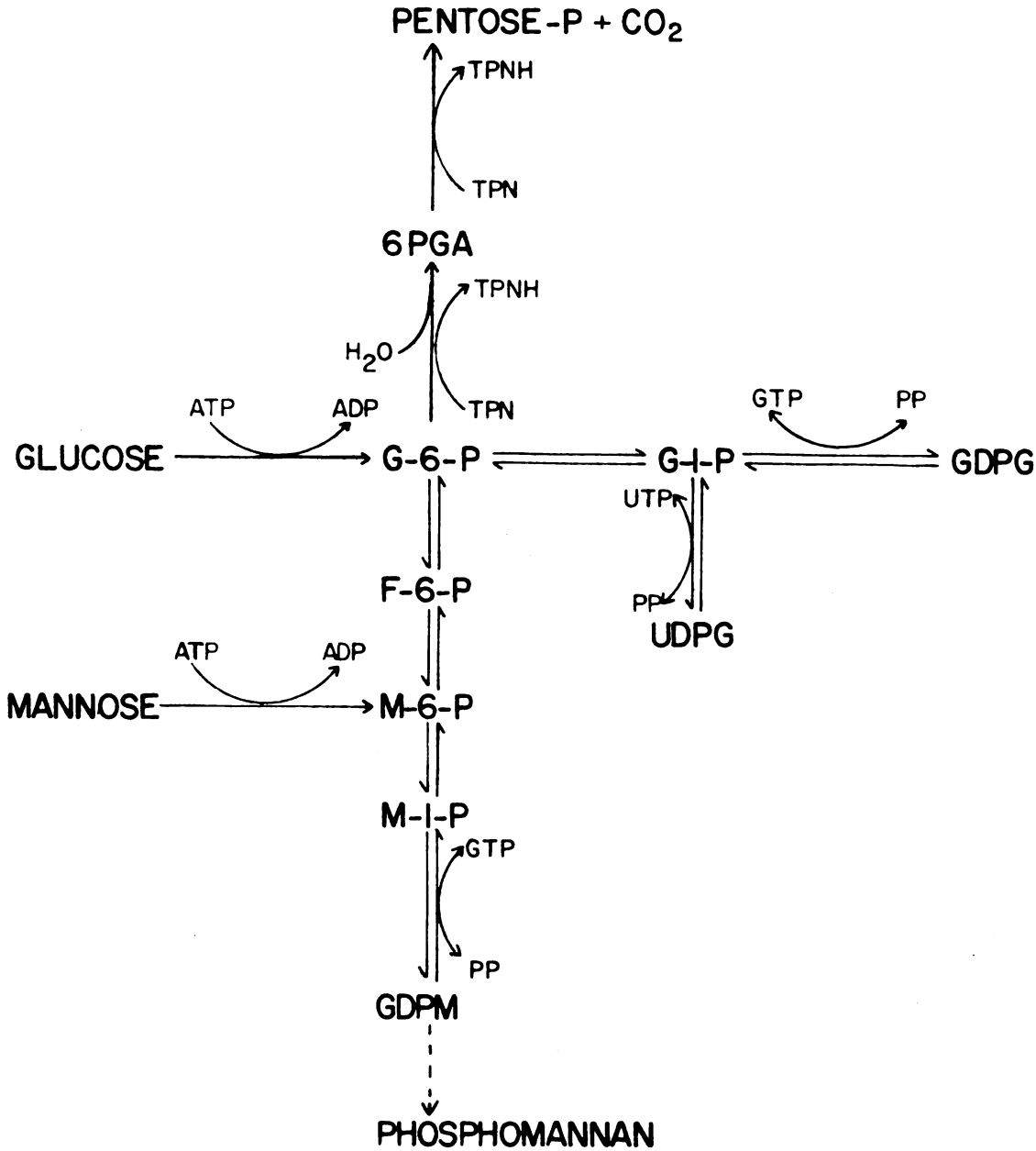
All the counts were detected at the origin coincident with an ultraviolet absorbing spot. Authentic GDPM remained similarly at the origin. Dialysis of phosphomannan synthesized from labeled glucose by whole cultures resulted in removal of only radioactive glucose, no di- or oligosaccharides being detected.

DISCUSSION

Previous studies by D. R. Wilken (unpublished data) have revealed that cultures of Hansenula holstii NRRL Y-2448 incorporate glucose intact into extracellular phosphomannan and it was suggested that GDPHexoses may be involved in this synthesis. The presence of sugar nucleotides in perchloric acid extracts of this yeast has now been adequately established. GDPHexoses and UDPHexoses were found in a ratio of 1:2. GDPM and UDPG were isolated from bakers yeast in a ratio of approximately 1:50 (67,68). The comparatively high proportion of GDPHexoses present in Hansenula may be of significance in relation to the synthesis of mannose-containing polymers. Although the hexose moieties of the GDPHexose fraction consisted of some glucose and fructose, the main component was mannose.

In an attempt to elucidate the steps involved in the biosynthetic route of phosphomannan, the metabolism of glucose and mannose by this organism was investigated. Figure 18 shows the enzymic reactions demonstrated in crude extracts. It has been shown that both mannose and glucose are phosphorylated and are capable of supporting polysaccharide synthesis with equal efficiency. This suggests that the two hexoses proceed through a common pathway leading to phosphomannan. The presence of phosphohexoisomerases

Figure 18. Summary of enzymic reactions demonstrated in Hansenula holstii.


PENTOSE-P + CO_2
TPNH
TPN
6PGA
TPNH
H_2O
TPN
ATP
ADP
GLUCOSE
G-6-P
G-1-P
GTP
PP
GDPG
UTP
PP
UDPG
F-6-P
ATP
ADP
MANNOSE
M-6-P
M-1-P
GTP
PP
GDPM
PHOSPHOMANNAN

results in the interconversion of M-6-P and G-6-P through the intermediate, F-6-P; thus common intermediates are easily visualized. The presence in crude extracts of enzymes capable of converting M-1-P and G-1-P to G-6-P implies the presence of phosphoglucomutase and phosphomannomutase. Whether or not the observed conversion of M-1-P to G-6-P is initiated by a specific phosphomannomutase as was demonstrated in bakers yeast (69) or is due to an unspecific phosphoglucomutase cannot be definitely answered. The rate of formation of G-6-P with M-1-P as substrate is about 1/500 of that with G-1-P as substrate. Pasternak and Rosselet (70) have reported that yeast phosphoglucomutase in the presence of G-1,6-diP acts on M-1-P about 1/7000 the rate as on G-1-P. Comparatively then, the rate of M-1-P mutation observed in these studies is about fourteen times that observed by the aforementioned investigators.

The presence of pyrophosphorylases capable of catalyzing the formation of GDPM, GDPG, and UDPG from the respective nucleoside triphosphates and hexose-1-phosphates was established. Interconversion of GDPM and GDPG did not occur in extracts capable of synthesizing both nucleotides. GDPG pyrophosphorylase has previously been demonstrated only in mammary gland extracts (71); however, the functional significance of this enzyme and GDPG itself remains obscure.

The presence of enzymes capable of catalyzing the

transformation of hexoses to sugar nucleotides, along with the isolation of GDPHexoses, lends some support to the suggested role of GDPM in phosphomannan synthesis. These sugar nucleotides may also function as glycosyl donors in the synthesis of yeast cell wall glucans and mannans.

Attempts were made to synthesize phosphomannan from C^{14}-GDPM or C^{14}-M-1-P, both by whole cultures and extracts. The use of whole cultues is probably limited by permeability of the cell wall to these compounds. No significant incorporation of label was achieved in either case, demonstrating at least that these two compounds are not used extracellularly for synthesis of the polymer. The ability of washed resting cells to produce phosphomannan from glucose alone in amounts close to 50% of that produced with glucose and Pi implies that the cells must contain large amounts of endogenous phosphate compounds which serve as precursors for phosphomannan production.

Assuming that GDPM does participate in the synthesis of this polymer, various mechanisms may be postulated, one being (where M is mannose):

$$\text{GDPM} + \text{M} \longrightarrow \text{M-M} + \text{GDP}$$

$$\text{GDPM} + \text{M-M} \longrightarrow \text{M-P-M-M} + \text{GMP}$$

$$\text{GDPM} + \text{M-P-M-M} \longrightarrow \text{M-M-P-M-M} + \text{GDP}$$

The first reaction, similar to that of sucrose synthesis from UDPG and fructose, results in the synthesis of the

β-1,2-linked disaccharide. The second reaction involves a transfer of the internal cyclic hemiacetal-linked mannose-phosphate residue to carbon 6 of the nonreducing moiety of the disaccharide, resulting in the 1,6-linked phosphodiester trisaccharide. More mannose residues may then be added to the nonreducing end group, followed by addition of another mannose-phosphate residue. Thermodynamically, this mechanism should be favorable. It is possible that other high energy phosphate compounds donate phosphate to the polymer and GDPM serves only to donate the mannose residues.

SUMMARY

The metabolism of mannose by Hansenula holstii NRRL Y-2448 has been investigated in relation to the extracellular phosphorylated mannan produced by this organism. Mannose is phosphorylated and isomerized to F-6-P, as is glucose. The presence of mutases was demonstrated by the conversion of G-1-P and M-1-P to the corresponding hexose-6-phosphates. Oxidation of G-6-P and 6-PGA occurred in the presence of TPN. The presence of pyrophosphorylases capable of catalyzing the formation of GDPM, GDPG, and UDPG from the respective nucleoside triphosphates and hexose-1-phosphates was established. No interconversion of GDPHexoses or oxidation of UDPG was detected.

Acid soluble nucleotides, including GDPHexoses and UDPHexoses, were isolated and identified. The GDPHexoses contained mannose and minor amounts of glucose and fructose.

The synthesis of phosphomannan by washed, resting cells from glucose and Pi and by whole cultures from C^{14}-mannose is described. No incorporation of radioactivity from C^{14}-GDPM or C^{14}-M-1-P into phosphomannan was achieved.

The participation of sugar nucleotides in polysaccharide synthesis is discussed.

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