STUDIES ON METHODS FOR THE ISOLATION AND IDENTIFICATION OF URIDINE DIPHOSPHATE HEXOSES IN LIVER TISSUE OF RATS FED DIETS HIGH IN GALACTOSE

by

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I

INTRODUCTION

A. Historical

Galactose as an etiologic factor in the production of cataracts in rats was first described by Mitchell[1,2] in 1935 and later confirmed by Day[3] and Yudkin[4].

Mitchell[5] described the earliest ophthalmoscopical changes in the lens cortex as occurring in five days in susceptible rats on a 35 per cent galactose diet. The induction time of cataract development was decreased by increasing the galactose content of the diet from 15 to 35 per cent or by decreasing the protein content from 15 to 5 per cent. Altering the type and amount of fat did not change the induction time if the galactose consumed was taken into account.

Craig and Maddock[6] fed twenty-one day old rats a diet containing 70 per cent galactose. Nitrogen balance studies performed during the experiment showed that galactose fed animals excreted two to six times as much urinary nitrogen as control fed animals. The urinary amino acid nitrogen was also increased. Post-mortem examination was done after seventy-five to seventy-eight days. These authors suggested that the observed pathological changes, including growth failure, corneal vascularization, formation of cataracts, hydronephrosis and testicular, prostatic and seminal vesicular atrophy were a result of protein and amino acid deficiency.

When Handler[7] fed 45 gram rats of the Vanderbilt strain, diets containing more than 60 per cent lactose or 40 per cent galactose he found that they lived only three to seventeen days. In moribund rats the blood galactose was found to be as high as 600 mg per cent and blood glucose as low as 40 mg per cent while liver glycogen was virtually exhausted. He concluded that galactose interferes with normal carbohydrate (glucose or glycogen) metabolism.

Dam[8] fed young chicks and weanling rats a diet containing 54.6 per cent galactose. The chicks developed a quivering syndrome leading to convulsions and death. Chicks sacrificed before death exhibited high blood galactose, normal blood glucose and very low liver glycogen. The rats sacrificed on the thirty-fourth day of feeding exhibited cataracts, high blood galactose, normal blood glucose and liver glycogen.

The discrepancy between the data of Dam and of Handler is difficult to reconcile. However, there must have been some inadequacy in Handler's diet or increase susceptibility of his strain of rats, for other investigators[3,6] have fed higher galactose diets without death of their animals.

Bellows and Chinn[9] and Buschke[10] suggest that osmotic disturbances play an important role in the development of galactose cataracts. Kirby, Estey and Wiener[11] found that galactose was toxic to tissue cultures of lens epithelium at a much lower level than was either glucose or fructose

Kosterlitz[12] in 1937 found that galactose phosphate accumulates in the livers of rabbits fed large amounts of galactose. He later

proved this ester to be galactose-1-phosphate and that it was also present in livers of rats assimilating galactose[13].

Schwartz et al[14] using red blood cells from normal and galactosemic humans studied the effect of galactose in vivo and in vitro. They found that respiration of normal red cells with galactose as the substrate was only 5-6 per cent of the respiration when glucose was used. They also found that galactosemic red cells, in contrast to normal red cells, did not respire with galactose as the substrate. These authors also demonstrated that galactosemic erythrocytes accumulated large amounts of galactose-1-phosphate, both in vivo and in vitro, when exposed to galactose; that normal red cells accumulated small amounts of galactose-1-phosphate, in vitro, on high concentrations of galactose; and that after exposure to galactose the O_2 uptake of galactosemic erythrocytes was reduced, while that of normal cells was enhanced. Shortly after finding the accumulation of galactose-1-phosphate in galactosemic red cells they demonstrated the presence of galactose-1-phosphate in cataractous lenses of galactose fed rats.

B. Galactose-glucose Interconversion

The reversible transformation of galactose into glucose in biological systems is important both for utilization of galactose as a source of energy and for synthesis of complex galactose containing compounds. The first step in metabolism of galactose in yeast and animal tissues is the phosphorylation of galactose in the one position to form alpha-galactose-1-phosphate (Gal-1-P) [13,15,16]. The transformation of the galactose-1-phosphate to glucose-1-phosphate (G-1-P) then takes place according to the following equations, in

which UDPG and UDPGal refer to uridine diphosphoglucose and uridine diphosphogalactose:

(1) Gal-1-P + UDPG $\leftrightharpoons$ UDPGal + G-1-P

(2) UDPGal $\leftrightharpoons$ UDPG

Caputto and coworkers[17] in 1950 isolated a uridine nucleotide from yeast and identified it as UDPG. The structure of UDPG is shown herewith.

Reaction 1 was first demonstrated to be an independent reaction by Kalckar[18]. The enzyme catalyzing this reaction was named Gal-1-P uridyl transferase and has been found in yeast, red blood cells[19] and mammalian liver[20,20]. It has not been found in mammary gland, brain or muscle[20].

```
        ┌──────── O ─────┐
        |                |
        |   OH     OH    |     H         OH        OH
        |   |      |     |     |         |         |
        HC - C  -  C  -  C  -  C - O  -  P - O  -  P = O
        |   |      |     |     |         ||        |
        |   H      H     H     H         O         O
        |                                           \
        N  - CH                               H - C ────┐
        |    ||                                   |     |
  O  =  C    CH                               H - C - OH|
        |    |                                    |     |
        HN - C = O                           HO - C - H O
                                                  |     |
                                              H - C - OH|
                                                  |     |
                                              H - C ────┘
                                                  |
                                             H2 - C - OH
```

Structure of uridine diphosphate glucose

Kalckar and coworkers have demonstrated a defect in the enzyme Gal-1-P uridyl transferase in liver and red blood cells of humans with congenital galactosemia[19,21,22,23].

The inversion of configuration at carbon-4 of the UDP bound hexoses occurs in reaction 2. Leloir[24] was the first to investigate this reaction. Using a dialyzed extract of galactose adapted yeast and substrate amounts of UDPG he found that at equilibrium 75 per cent of the hexose moiety was glucose and 25 per cent was galactose. The enzyme catalysing this reaction was called galactowaldenase. Hansen and Craine[25] estimated about 21 to 27 per cent galactose ester and 73 to 79 per cent glucose ester at equilibrium in Lactobacillus bulgaricus.

The inversion of configuration at carbon-4 of the UDP bound hexose has subsequently been shown not to be a Walden type inversion and is most consistant with an oxidation-reduction reaction[26,27,28,29]. Hence, Kalckar and Maxwell[29] called the enzyme UDPGal-4-epimerase. Besides its presence in galactose adapted yeast and L. bulgaricus, the enzyme has been found in calf liver and brain, in rat liver, brain and mammary gland[20], and in human erythrocytes[22]. The enzyme has been purified 200 fold from calf liver acetone powder. It is diphosphopyridine nucleotide (DPN) dependent.

The main pathway of UDPG synthesis in mammalian tissue probably proceeds via the following mechanism[23,30], in which UTP and PP refer to uridine triphosphate and pyrophosphate:

(3) G-1-P + UTP $\rightleftharpoons$ UDPG + PP

The enzyme catalyzing this reaction is called UDPG pyrophosphorylase. UTP is formed from uridine diphosphate (UDP) by transphosphorylation, using ATP as the phosphoryl donor[31]. The pyrophosphorylase has been found in liver[20,34], red blood cells[19,22,32], mammary

gland[20], brain[20], and muscle[20], as well as in microorganisms and plants. It is an important link in the formation of glucuronides. It also links glucose and glycogen metabolism with beta-galactosides and galactolipids.

Isselbacher[33] recently demonstrated the presence of an enzyme in rat, pigeon and human liver, catalyzing the following reaction:

(4) Gal-1-P + UTP ⇌ UDPGal + PP

In conformity with previous nomenclature, this enzyme was named UDPGal pyrophosphorylase. The activity of this enzyme is only about one sixth that of Gal-1-P uridyl transferase.

He also measured the activity of uridyl transferase and pyrophosphorylase enzymes in rat liver at different ages. The results appear in Table 1.

TABLE I

Activity of uridyl transferase and pyrophosphorylase enzymes in rat liver with respect to age of animals. Results are averages of three sets of experiments and are expressed as millimicromoles of reactants converted per milligram of liver protein per 20 min.*

	P-Gal Transferase	UDPG Pyrophosphorylase	UDPGal Pyrophosphorylase
Fetal (18th day of gestation)	6.8	340	0.9
Neonatal (1 day)	7.7	348	1.6
Adult (60 Days)	39.4	98	6.7

*From Isselbacher[33].

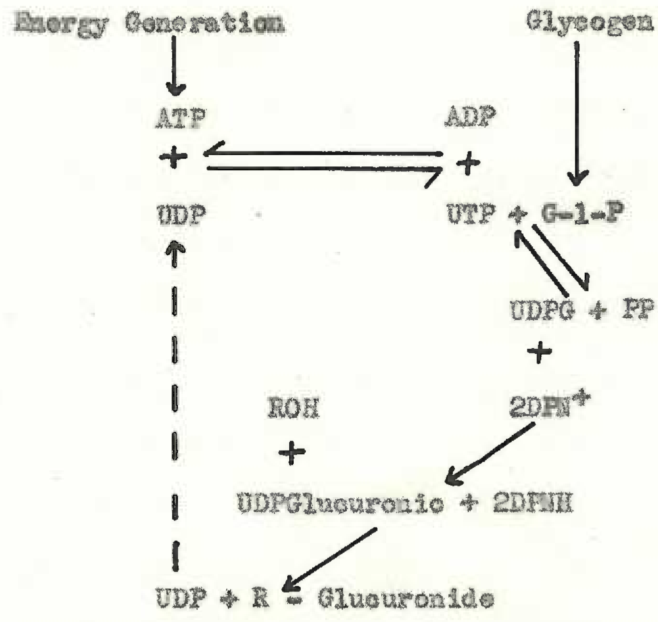
C. Glucuronide Synthesis

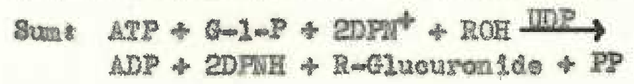
The isolation of uridine diphosphoglucuronic acid (UDPGA) from liver[35,36] raised the problem of biosynthesis. Unlike UDPG, UDPGA would not undergo pyrophosphorolysis. Strominger and coworkers[37] found that particle free supernatant fluid from liver of several animals oxidized UDPG to UDPGA in the presence of DPN^+. The reaction is catalyzed by a two step dehydrogenation of UDPG at carbon-6 of glucose. The enzyme has been purified 200-400 times from calf liver acetone powder[39].

(5) $UDPG + 2DPN^+ \longrightarrow UDPGA + 2DPNH + 2H^+$

The inability to demonstrate a free aldehyde intermediate may indicate that a single enzyme is responsible for both oxidative steps.

With the demonstration of UDPGA and glucuronide synthesis in liver a lucrative field of research was opened[35,39]. That UDPGA was actually the donor of glucuronic acid in the synthesis of glucuronides of o-aminophenol and menthol in liver suspensions was shown by Storey and Dutton[40]. It has been demonstrated that the enzyme systems catalyzing the coupling of glucuronic acid of UDPGA to an acceptor resides in the microsomal fraction of liver homogenates, while the enzyme catalyzing the oxidation of UDPG resides in the supernatant[37,38,41]. So far the known acceptors of glucuronic acid from UDPGA are phenols and some related structures[36,37,41,42], carboxylic acids[43,44,45,46,47] and amines[48,49,50]. Among these acceptors are such compounds as thyroxine, corticosteroids and other steroids and bilirubin. Kalckar and Maxwell[23] summarized the overall reactions in glucuronide synthesis as illustrated in Figure 1.


Energy Generation
Glycogen
ATP
ADP
+
+
UDP
UTP + G-1-P
UDPG + PP
+
ROH
2DPN+
+
UDPGlucuronic + 2DPNH
UDP + R - Glucuronide


Sum: ATP + G-1-P + 2DPN+ + ROH UDP
ADP + 2DPNH + R-Glucuronide + PP

It has been demonstrated that bilirubin excreted in bile or urine is a bilirubin glucuronide[44,51]. The glucuronide-forming enzyme system in fetal and new born guinea pig liver, however, is unable to form the bilirubin glucuronide. There is a similar lack of activity in the human fetus and new born[52].

Congenital non-hemolytic, non-obstructive jaundice occurs in a mutant strain of Wistar rats, in Gunn's strain of jaundiced rats and in humans. In this syndrome there is a defect in the glucuronide-forming enzyme system of liver[53,54] that synthesizes bilirubin-glucuronide. There is also a deficiency in transferase activity in liver microsomes of patients with constitutional hepatic dysfunction[55,56].

The "glucuronyl transferase enzyme system" has been demonstrated in guinea pig, rabbit, mouse and rat liver in decreasing amounts. It is also present in sheep, pigeon and frog liver homogenates[57].

Grodsky and Carbone[58] demonstrated activity in homogenates of rat kidney and brain, as well as liver. Kidney activity was slightly more than one third that of liver, while brain was only about one tenth as active as liver.

D. UDPG and UDPGal as Glycosyl Donors

Lactose Synthesis: Gander, Petersen and Boyer[59,60] have shown that cow's udder contains an enzyme system which catalyzes the in vitro synthesis of lactose-1-phosphate according to reaction 6.

(6) UDPGal + G-1-P ⟶ lactose-1-P + UDP

They have named the enzyme galactosyl transferase.

Schambye[62a] and coworkers determined the C^{14} distribution in glucose and galactose moieties of lactose after intravenous injection of acetate-1-C^{14} into cows. The glucose and galactose contained nearly

equal activity. Wood and coworkers[62b] perfused isolated cow's udder with acetate-1-C^{14} and found 16 to 47 times more activity in the galactose than in the glucose of lactose. Wood and coworkers[61] injected acetate-1-C^{14} into the arterial supply of the left half of a cow's udder. They then measured the activity of the glucose and galactose moieties of lactose from the right and left sides. The galactose in the injected side contained 90 per cent of the C^{14} activity of lactose. The C^{14} activity of the glucose and galactose from the non-injected side was equal. Wood and coworkers concluded that free glucose was the galactose acceptor. This would not be compatible with the enzyme system of Gander et al.

Free fructose as a glucose acceptor from UDPG has been demonstrated in the formation of sucrose in plants[63,64]. The same plants can also form sucrose phosphate from glucose and fructose-6-phosphate. The enzymic formation of sucrose and sucrose phosphate is believed to be catalyzed by two different enzyme systems. This type of mechanism may account for the discrepancy in the in vivo and in vitro work in lactose synthesis.

Glycogen Synthesis: Leloir and Cardini[66] found that equal amounts of UDP and glycogen were formed when UDPG, a small amount of glycogen and an enzyme from the soluble fraction of liver were incubated together. An increase in glycogen could be detected only when the liver preparations were free from amylase. Several mono, di and oligosaccharides were found to be inactive in this system. These authors concluded that UDPG acts directly as a glucose donor to glycogen and that the reaction is similar to polysaccharide

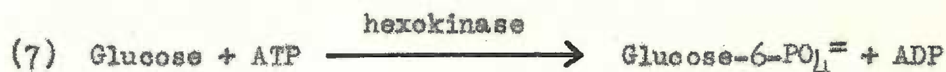
formation from glucose-1-phosphate with animal phosphorylase, a reaction which also requires a primer of high molecular weight.

Galactolipid Synthesis: Burton and coworkers(67) found that both D-glucose-1-C^{14} and D-galactose-1-C^{14} were readily incorporated into the neutral galactolipid fraction of rat brain tissue in vivo. They found that the in vitro incorporation of free hexose into the neutral galactolipid was dependent upon the presence of ATP. However, both 1-C^{14}-D-galactose-1-phosphate and uridine diphospho-D-galactose-1-C^{14} could be incorporated into the galactolipid fraction in the absence of ATP.

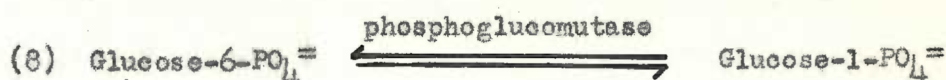
They proposed the following scheme for the incorporation of glucose and galactose into galactolipids of brain. This scheme is based on their observations and the known enzyme activity previously demonstrated in brain tissue.

I. The Incorporation of Glucose:*

A. Glucose is activated by ATP in presence of hexokinase and phosphoglucomutose (equations 7 and 8)

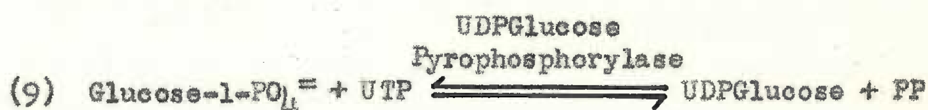


$$(7)\quad \text{Glucose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{Glucose-6-}PO_4^{=} + \text{ADP}$$

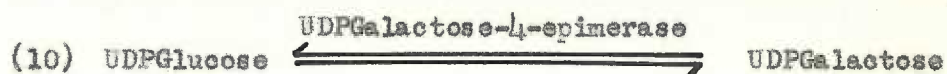


$$(8)\quad \text{Glucose-6-}PO_4^{=} \underset{}{\overset{\text{phosphoglucomutase}}{\rightleftharpoons}} \text{Glucose-1-}PO_4^{=}$$

B. The glucose-1-phosphate is incorporated into uridylic nucleotide and converted to UDPGalactose (equations 9 and 10)



$$(9)\quad \text{Glucose-1-}PO_4^{=} + \text{UTP} \overset{\text{UDPGlucose Pyrophosphorylase}}{\rightleftharpoons} \text{UDPGlucose} + \text{PP}$$



$$(10)\quad \text{UDPGlucose} \overset{\text{UDPGalactose-4-epimerase}}{\rightleftharpoons} \text{UDPGalactose}$$

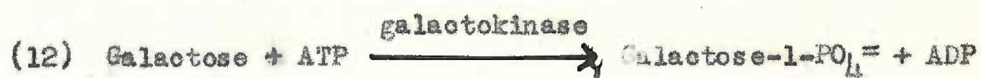
*Modified from Burton et al(67)

C. The UDPGalactose may donate the galactose moiety to a lipid acceptor to form the neutral galactolipid (equation 11)

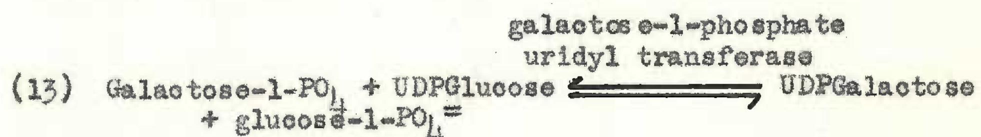
(11) UDPGalactose + lipid acceptor $\longrightarrow$ galactolipid + UDP

II. The Incorporation of Galactose

A. Galactose is activated by ATP and incorporated into the uridylic nucleotide (equations 12 and 13)



(12) Galactose + ATP $\xrightarrow{\text{galactokinase}}$ Galactose-1-$PO_4^{=}$ + ADP



(13) Galactose-1-$PO_4^{=}$ + UDPGlucose $\xrightleftharpoons{\text{galactose-1-phosphate uridyl transferase}}$ UDPGalactose + glucose-1-$PO_4^{=}$

B. The UDPGalactose formed can then donate the galactose moiety to form the galactolipid (equation 11)

II

PROBLEM AND APPROACH

The problem was two fold. (1) Development of a method for the isolation of UDPhexose from rat liver that would give quantitative results and yet allow for the determination of multiple samples without being excessively time consuming. (2) Determination of the amount of UDPhexose in livers of rats fed high galactose diets and the amount of UDP bound hexose represented by glucose and galactose.

The problem was approached as follows:

(1) Extraction of rat liver with perchloric acid.

(2) Since uridine nucleotides are somewhat unique in that they do not contain a free amino group in the pyrimidine base, their ionization characteristics differ from the nucleotides that do possess a free amino group. The nucleosides and nucleotides that possess a free amino group become cationic at a low pH range while uridine does not. Cation exchange resin treatment of the liver extracts at a low pH was therefore used in retaining many of the bases, nucleosides and nucleotides, as well as amino acids and other compounds that are cationic at the pH range used.

(3) Since norite is relatively specific in adsorbing nucleotides it was used in the adsorption of uridine nucleotides. The nucleotides can then be removed by washing the norite with ethanol containing small concentrations of ammonia.

(4) Utilization of paper chromatography allowed for the separation of the nucleotides remaining after adsorption and elution from norite. By chromatographing known uridine nucleotides along with the unknown, UDPhexose could be located and eluted from the chromatograms.

(5) By the use of ultra violet absorption spectra the UDPhexoses could further be identified and quantitated.

(6) The hexoses of UDPhexoses were hydrolyzed and the resulting UDP precipitated with barium hydroxide and zinc sulfate leaving the hexose in solution.

(7) Paper chromatography of the hexoses hydrolyzed from UDPhexose provided a means of identifying the UDP bound hexoses. These hexoses (glucose and galactose) could then be eluted from the chromatograms and determined quantitatively.

Rats were fed high galactose diets and their livers processed as above to determine UDPhexose concentration and amounts of glucose and galactose bound to UDP.

III

MATERIALS AND DEVELOPMENT OF METHODS

A. Animals and Rations

All animals used were rats of the Sprague-Dawley strain obtained from Northwest Rodent Company, Pullman, Washington.

Rats weighing 250 to 400 grams used for the UDPG recovery experiments (vide infra) were fed on a stock ration. Rats weighing 85 to 110 grams used in the galactose feeding experiments were fed the following synthetic diets:

	Control Diet (Per Cent)	Galactose Diet (Per Cent)
Casein	18	18
Salt Mixture (68)	4	4
Cod Liver Oil	2	2
Wesson Oil	6	6
Brewers Yeast	10	10
Dextrin	60	30
Galactose		30

The only difference in the above rations is that some of the experimental animals received 30 per cent of their diet as galactose in place of dextrin.

All animals were allowed food and water ad libitum.

The animals fed the experimental diets were divided into two groups. Groups of animals were fed either the control or the galactose diets for five or ten days.

B. Excision and Extraction of Liver

The animals were anesthetized with nembutal given intraperitoneally (3-5 mg per 100 g body wt.). The livers were rapidly excised, blotted between paper towels to remove excess blood and immediately frozen in dry ice. About 5 g samples were quickly weighed in the frozen state and replaced in dry ice.

All of the following procedures were carried out in a cold room at 6°C. unless stated otherwise.

Liver samples were homogenized in two volumes of cold 0.6 N perchloric acid (PCA) in a Potter Elvehjem type homogenizer. The homogenate was centrifuged and the residue reextracted twice with two volumes of cold 0.2 N PCA. Potter and coworkers[69] found that the nucleotides of liver were about 90 per cent extracted with two volumes of 0.6 N PCA and two volumes of 0.2 N PCA. After each extraction the supernatant was neutralized to pH 6-7 with concentrated KOH, using phenol red as an indicator. While adding KOH the supernatant was cooled nearly to freezing in an alcohol-dry ice bath. The extracts were combined and brought to a pH of about 1.2 with concentrated H_2SO_4 using Tropelin OO as an indicator. Extracts were kept cool with an alcohol-dry ice bath as before. Final adjustment to pH 1.2 was made with a Beckman pH meter at 6°C.

In the UDPG recovery experiments (stock ration) the extracts from about 20 gms of liver were combined, the pH adjusted as

indicated above, and made to a volume of 200 ml. with H_2SO_4 at pH 1.2. In the experiments with galactose and control diets, the extracts from the liver of each animal were combined, treated as above and brought to a volume of 50 ml. with H_2SO_4, pH 1.2. Prior to exchange chromatography the precipitated $KClO_4$ was removed by centrifugation.

C. Construction of Resin Columns

Dowex 50 (100-200 mesh 8^x) was made into a slurry and poured into large glass columns fitted with a glass wool plug to retain the resin. Two normal H_2SO_4 was allowed to flow through the column until the optical density of the effluent at 260 millimicra became constant. The H_2SO_4 was followed by distilled water until the effluent approached pH 5. The resin was then dried in room air.

The individual columns used in ion exchange of liver extracts were prepared from glass tubing of 11 mm inside diameter. One end of a piece of glass tubing 15 to 18 inches long was pulled to a fine tip. The columns were prepared for use by inserting a glass wool plug to hold the resin, followed by the addition of 4 g of the washed Dowex 50 made into a slurry with H_2SO_4 at pH 1.2. The resin was allowed to settle by gravitation and was covered with several centimeters of H_2SO_4, pH 1.2 until immediately prior to adding liver extracts, at which time the fluid level was allowed to reach the top level of the resin column. The columns would not run dry even if free flow was allowed. Flow rate of the columns was about 1 ml. per minute at 6°C.

D. Ion Exchange of Liver Extract

Two types of experiments were carried out, 1) recovery of UDPG and 2) isolation of UDPG from livers of experimentally fed animals.

In UDPG recovery experiments a known amount of rechromatographed authentic UDPG* was added to a 20 ml. aliquot of one-half of the liver extracts previously made to a volume of 200 ml. These were thoroughly mixed and quantitatively transferred to the Dowex columns. In the feeding experiments 20 ml. aliquots of the liver extracts were transferred to the Dowex columns. Four liver samples in duplicate were run at a time.

The sides of the columns were washed several times with H_2SO_4, pH 1.2 after the fluid level of the extracts had reached the top of the resin column. They were then filled with 15 to 20 ml. of H_2SO_4, pH 1.2. Tropelin 00 and phenol red were retained tightly by the columns. Effluent was collected in 100 ml. graduate cylinders from the time of adding the extracts until 50 ml. had been collected. A control column was run at the same time, using only H_2SO_4, pH 1.2. A 1:30 dilution of the effluent from this column was used as a blank in making optical density readings. The effluents were thoroughly mixed. One tenth ml. of each sample was diluted to 3 ml. with distilled water (1:30 dilution). The optical densities of these solutions were read at 260 millimicra in a Model DU Beckman Spectrophotometer. A millimolar absorption coefficient of 10 was used to calculate micromoles of nucleotide in each 50 ml. of effluent as follows:

$$\frac{\text{O.D. at } 260 \times 30 \times 50}{10} = \text{total micromoles}$$

The samples were quantitatively transferred to 100 ml. beakers and neutral red added. The solutions were then titrated to a yellow

*Sigma Chemical Company, St. Louis, Missouri or from Pabst Laboratories, Division of Pabst Brewing Co., Milwaukee, Wisconsin

color with concentrated KOH and immediately back titrated to a pH just below 7, at which acidity norite adsorbs UDPG optimally.

E. Norite Preparation and Recovery of UDPG from Water Solutions

Norite A was prepared for use in adsorption of nucleotides by washing with 50 per cent ethanol containing 0.1 per cent ammonia. Ethanol-ammonia treatment was followed by washing with distilled water.

In preliminary experiments it was found that 10 mg of norite, treated as above, would completely adsorb 0.1 micromole of authentic UDPG from distilled water. About 77 to 80 per cent could be eluted from the norite with two ten ml. portions of 50 per cent ethanol containing 0.1 per cent ammonia.

One hundred mg of prepared norite was added to 10 ml. of distilled water containing 1.18 micromoles of UDPG. The solution was stirred intermittantly for 30 minutes, centrifuged in a Servall centrifuge for 15-20 minutes at 12000 rpm and the supernatant decanted. The norite was stirred with 10 ml. of distilled water for 5 minutes, centrifuged and the supernatant decanted. UDPG was eluted from the norite by stirring with 50 per cent ethanol containing 0.1 per cent ammonia for 30 minutes. The norite was centrifuged and the supernatant decanted. The elution was repeated with 10 ml. of ethanol-ammonia. The supernatants from the elution were combined and made to 25 ml. Table 2 demonstrates recovery:

F. Norite Adsorption of Nucleotides from Liver Extracts

These experiments were carried out on extracts as treated in D above. In trial runs 5 mg of norite per 0.1 micromole of nucleotides

TABLE II

Recovery of authentic UDPG (1.18) micromoles adsorbed onto norite and eluted with ethanol-ammonia.

	Micromoles UDPG Calculated from O.D. at 262 millimicra*		
	Sample Number		
	1	2	3
Solution after absorption	.01	.008	.008
Wash water	.000	.000	.000
Ethanol-ammonia elution	.913	.925	.935
Per cent recovery	77.5	78.5	79.5

*Calculation of micromoles of UDPG

$$\frac{\text{O.D. at 262} \times \text{Vol. of Soln.}}{\text{10 (millimolar absorption coefficient)}} = \text{micromoles UDPG}$$

were used to adsorb materials giving absorption at 260 millimicra. By following the O.D. of the effluent from the Dowex columns before and after norite adsorption the completeness of adsorption could be determined. It was found that it was necessary to remove glycogen before reading optical density. All materials absorbing at 260 millimicra were adsorbed by 5 mg of norite per 0.1 micromole. Therefore 5 mg instead of 10 mg (as used with the aqueous UDPG solutions) of norite were used in the experiments to be reported.

To the effluent from the Dowex columns (treated as described in D) was added 5 mg of norite per 0.1 micromole, calculated as UDPG as in D above. These solutions were stirred intermittantly for 30 to

40 minutes. They were then quantitatively transferred to polyethylene centrifuge tubes and spun in a Servall centrifuge for 20 minutes at about 12000 rpm. The supernatant was discarded. The norite was then washed with 10 ml. of distilled water for 5 minutes, centrifuged as before and the wash water discarded. Washing was repeated twice more with 10 ml. of distilled water to complete the removal of impurities. In following the optical density of the wash water in several experiments, it was found that absorption at 260 millimicra fell to zero with the second or third 10 ml. wash and that subsequent ethanol elution contained no salt interfering with chromatography. This washing procedure did not remove UDPG.

G. Elution of UDPG and Other Nucleotides from Norite

Ten ml. of 50 per cent ethanol containing 0.1 per cent ammonia were added to each tube. Each sample was stirred intermittantly for 30 to 40 minutes. The tubes were centrifuged as before for 20 minutes at 12000 rpm and the supernatant was decanted into Dowex 50 columns. The Dowex 50 columns were prepared as described in C except that 1.0 g of Dowex, instead of 4 g was used and the Dowex was in a distilled water, instead of an H_2SO_4 slurry. The water level was allowed to reach the top of the resin column before pouring in the supernatant. The effluent was collected in 50 ml. round bottom flasks containing one drop of neutral red indicator. The norite was treated three more times with 10 ml. portions of ethanol-ammonia and each eluate was decanted into the Dowex columns. After the eluate had passed through the columns the sides were washed twice with ethanol-ammonia. The columns were then blown dry with air under pressure.

The effluent was collected in 50 ml. flasks and titrated with 50 per cent ethanol containing 1.0 per cent ammonia to a pH close to 7. The ethanol-ammonia eluate from the norite was treated with Dowex $50H^+$ to lower the pH.* By back titrating to pH 7 with ethanol-ammonia there was little increase in the salt concentration which might interfere with subsequent paper chromatography. The ethanol-ammonia solutions were evaporated to dryness at 40°C. Evaporation was facilitated by blowing clean dry room air over the surface of the solutions.

H. Paper Chromatography for Separation of Nucleotides

The residue was brought into solution with 0.3 or 0.4 ml. of distilled water. One tenth ml. of each sample was spotted on Whatman No. 1 filter paper. Authentic UDPG and UMP were spotted at either

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*Cardini and Leloir[70] reported the formation of UMP and cyclic 1:2-monophosphoric ester of glucose upon mild alkaline treatment of UDPG. To determine whether the conditions employed in the present procedure would decompose UDPG, the following experiment was carried out: 1:18 micromoles of UDPG were added to each of two flasks containing 40 ml. of 50 per cent ethanol-0.1 per cent ammonia at 6°C. The solutions remained at 6°C for 12 hours. At this time they were treated with Dowex 50 and back titrated to about pH 7 as described in G above. They were then evaporated to dryness and the residue dissolved in 0.2 ml. of distilled water. One tenth ml. of each was spotted on Whatman No. 1 filter paper, along with authentic rechromatographed UMP and UDPG and chromatographed as described in H. Descending chromatography was carried out on one sample for 48 hours and until the solvent front had traveled about 50 cm. on the other. The chromatograms were removed, air dried and ultra-violet adsorbing substances located by the method of Markham and Smith[71], using a mineralight lamp. The chromatograms were then sprayed with the phosphate spray of Hanes and Isherwood[72]. The UV absorbing substances and the blue spots that appeared after spraying were identical. The only spots visualized in the sample lanes were those corresponding to an R_f of authentic UDPG. There were no spots migrating at the same R_f as authentic UMP or migrating with an R_f greater than UDPG. If UMP and the cyclic 1:2 phosphate ester were formed with the above treatment there should have been a spot migrating with an R_f of UMP and a spot migrating with an R_f greater than UDPG. It was concluded that there was no degradation of UDPG with the procedure used.

edge of the same paper. The sample spots were kept to about a 1 cm. diameter by using multiple applications and by blowing a stream of cool air across the paper.

The end of the paper opposite the spots was serrated to insure even flow of solvent from the end. The paper strips were placed in troughs suspended near the top of a glass tank. A piece of filter paper lined the tanks and dipped into solvent of the same composition as that used for development of the chromatograms. The starting line was near the trough. The tank was sealed and the inside atmosphere allowed to equilibrate with the paper for 12 to 18 hours. At the end of this period the solvent for development was transferred to the troughs through a hole in the top of the tank. The hole remained closed except for solvent transfer. The ethanol-ammonium acetate solvent pH 7.5 of Paladini and Leloir(70) was used. The chromatograms were developed for 48-56 hours at room temperature. At the end of development the paper strips were removed and dried at room temperature.

I. Identification and Quantitation of UDPhexose

Nucleotides were located on the paper strips with an UV mineralight lamp. Those spots which migrated at the same R_f as authentic UDPG were cut from the chromatograms along with a control strip of the same size. These paper strips were placed in test tubes with 4 ml. of 0.1 N H_2SO_4. The tubes were agitated intermittantly for 60-90 minutes. The paper strips were then removed and the H_2SO_4 solution transferred to 15 ml. centrifuge tubes which were spun for 5 to 10 minutes at 2000-2500 rpm. Centrifuging completely sedimented

paper fibers which would subsequently interfere with spectrophotometric readings. These solutions were read against a blank at 250, 260, 262 and 280 millimicra in the Spectrophotometer. The spectra of the eluted spots and authentic UDPG were identical (Table 3)

TABLE III

Illustration of the spectra of a sample spot and authentic rechromatographed UDPG.

Wave Length in Millimicra	Optical Density		Ratio of the Optical Densities at 280:260 Millimicra*	
	Sample	Known UDPG	Sample	Known UDPG
220	.034	.000	.37	.37
225	.015	.000		
230	.042	.000		
235	.105	.050		
240	.193	.115		
245	.295	.192		
250	.400	.266		
255	.495	.332		
260	.547	.369		
262	.554	.373		
265	.550	.365		
270	.462	.315		
275	.340	.235		
280	.201	.138		
285	.082	.054		
290	.020	.012		
295	.004	.000		
300	.002	.000		

*The ratio of the optical densities at 250:260 millimicra and 280:260 millimicra is a good indication of the purity of uridine and its nucleotides in relation to other UV absorbing substances such as other nucleosides and nucleotides.

Three ml. aliquots of the material eluted from the chromatograms were transferred to 15 ml. centrifuge tubes. The tubes were stoppered with rubber stoppers penetrated by a piece of capillary

tubing and placed in a boiling water bath for 5 minutes. They were then cooled and the stoppers removed. Barium hydroxide had been prepared, one ml. of which exactly neutralized 3 ml. of H_2SO_4 used in the above hydrolysis. One and two tenths ml. of this barium hydroxide and 0.2 ml. of 5 per cent zinc sulfate were added to each 3 ml. of H_2SO_4 solution. The solutions were thoroughly mixed between additions of barium hydroxide and zinc sulfate and again after addition of zinc sulfate. The precipitate was removed by centrifugation at 2000 to 2500 rpm for 15 minutes. The supernatant contained the hexoses hydrolyzed from the UDP hexose by heating in the boiling water bath and the precipitate contained the UDP.* The final pH was 6.9. Three ml. of each sample were evaporated to dryness at 60°C in a manner similar to that described in G above. The residue was dissolved in 0.15 ml. of distilled water. The tubes were centrifuged for 5 minutes at 2000 to 2500 rpm to facilitate transfer to filter paper for chromatography.

J. Paper Chromatography for Separation of Hexoses

Whatman No. 1 filter paper was used throughout. The ends of each strip were placed between two glass rods held tightly together by rubber bands. These strips were then suspended from a glass rack within a large zinc coated waste can. About ten paper strips could be hung in this apparatus at a time, allowing several cm. of space

*It was found that hydrolysis and barium hydroxide-zinc sulfate treatment (as described) of 0.5 micromole of authentic UDPG removed all of the nucleotide. Since less than 0.5 micromole of UDPG (calculated from absorption at 262 millimicrons) was encountered (in I above) in experiments to be reported, this procedure was considered satisfactory in precipitating nucleotides.

between each strip. The waste can was then placed in a sink and a large hose run from the hot water faucet to the bottom of the tank. The hot water tap was fully opened allowing water at 60-70° to flow from the bottom of the waste can out over the top. The papers were washed in this manner for eight hours. They were then removed, dried at room temperature, and used in subsequent chromatography. Only after this procedure were the reducing substances in the paper decreased sufficiently to allow for elution and determination of the microgram quantities of hexoses encountered in experiments to be reported.

One tenth ml. of each sample was transferred to Whatman No. 1 filter paper washed as above. The paper was ruled and serrated and applications of samples made as described in H. Glucose and galactose were spotted at either edge as a guide to the location of the sample hexoses. A glass tank was lined with filter paper that dipped into solvent of the same composition as that used for chromatographic development. The paper strips were placed in troughs suspended near the top of the tank. The starting line was on the descending limb of the paper several cm. from the trough. The tank was sealed and the paper strips allowed to equilibrate with the atmosphere inside the tank for 12-24 hours. The developing solvent was then added to the troughs through a small hole in the tank top. The chromatograms were developed for 36 hours by the descending method using the solvent of Jermyn and Isherwood(73). The solvent was ethyl acetate-pyridine-water in the ratio 25:1:35. At the end of 36 hours the chromatograms were removed and dried at room temperature in a hood. Guide

strips containing the known glucose and galactose were cut from the chromatograms. These were then sprayed with 0.3 per cent p-aminohippuric acid in 95 per cent ethanol[72] (PAH-ethanol) and placed in an oven at 140°C for 8 minutes. Glucose and galactose appear as yellow orange spots in UV light or orange in visible light if hexose concentration is great enough. In preliminary experiments the entire chromatogram was sprayed with PAH-ethanol. In the sample lanes two spots appeared when viewed in UV light. One was light and corresponded to the R_f of known galactose and the other darker spot corresponded to the R_f of known glucose.

The spots were circled and the centers marked. The guide strips were then placed along side the chromatograms and a line drawn across them between the centers of the hexose spots on the guide strips. It was found that 20 to 40 g of glucose or galactose produced spots by the above method of less than 16 square cm. and that the two chromatographed in the same lane were completely separated by 1 to 2 cm. Sixteen square cm. areas were therefore cut from the chromatograms corresponding to known glucose and galactose. Control squares were also cut. The squares were cut into 1 cm. strips, placed in test tubes containing water and the hexoses eluted for 60 to 75 minutes with frequent vigorous agitation. The eluate was transferred to 15 cm. glass centrifuge tubes which were spun at 2000-2500 rpm for 5 to 10 minutes. Centrifugation was used to sediment the paper fibers since they interferred with the sugar determinations.

Duplicate sugar determinations were done on each sample by the method of Park and Johnson[75]. Known concentrations of glucose and

galactose were chromatographed with each set of experimental samples. These were eluted and the sugars determined as above. The known glucose and galactose data were used to construct a curve for calculating the hexose content of the unknown samples.

IV

RESULTS AND DISCUSSION

A. Some Critical Steps in the Isolation of UDPhexose

During the development of the method used for the isolation of UDPhexose it became apparent that several of the steps were critical.

It was important to cool the perchloric acid (PCA) extracts while adding concentrated KOH during pH adjustment to 6 to 7. If the extracts were not cooled sufficient heat was generated to hydrolyze the hexose from UDPhexose.

Adjusting the pH of the liver extracts to 1.2 prior to ion exchange with Dowex 50 is important. Since most purines, pyrimidines, nucleosides and nucleotides, with the exception of uridine nucleotides, exist as cations at pH 1.2 they will be retained by the Dowex columns, while uridine nucleotides will pass through the columns. Cohn[76] in 1950 calculated the net charge per molecule of some ribonucleotides as a function of pH. He found that adenylic, cytidilic and guanylic acids were cationic below pH 1.5.

During paper chromatography of nucleotides, it was found that the R_f values of UMP and UDPhexose were similar enough that complete separation occurred only after UDPhexose had migrated 35 to 40 cm. Since the absorption spectra in UV light and the micromolar absorption coefficients are the same for UMP and UDPhexose, contamination

of UDPhexose with UMP will give falsely high values for UDPhexose when calculated from absorption at 262 millimicra.

B. Characterization of UDPhexose from Liver Extracts

In the procedure described under materials and development of methods the UDPhexose isolated from each liver sample was characterized during the process of determining the amount of UDPhexose per liver sample and the amount of glucose and galactose bound to the UDP.

(1) Paper chromatography of the norite eluant revealed several UV absorbing spots, one of which migrated at the same R_f as authentic UDPG.

(2) Elution of this spot from the chromatogram and determination of its UV absorption spectrum revealed a spectrum identical with authentic UDPG. (Table 3)

(3) Paper chromatography of the hydrolysis products from this spot after treatment with barium hydroxide and zinc sulfate revealed two spots with the same R_f values as known glucose and galactose. Elution and quantitative determination of total hexose showed a 1 to 1 ratio of hexose to uridylic acid calculated from UV absorption at 262 millimicra.

Since the only uridine nucleotide containing glucose and galactose so far isolated from rat liver is the uridine diphosphate, it is presumed that the above characterization identifies the compounds isolated as uridine diphosphate glucose and uridine diphosphate galactose.

C. Calculations

Explanations of some of the calculations used in arriving at the

data to follow are given below:

(1) Total micromoles of UDPhexose in a liver sample

$$\text{Total micromoles} = \frac{OD \times 4 \times 3}{10}$$

a) OD is the optical density at 262 millimicra which is the wavelength of maximum absorption of uridine.

b) 4 is the number of ml. of 0.1 N H_2SO_4 used to elute the UDPhexose from the chromatogram.

c) 3 is another dilution factor. Of the 0.3 ml. used to dissolve the residue after evaporation of the ethanol-ammonia eluate only 0.1 ml. was chromatographed.

d) 10 is the millimolar absorption coefficient of uridylic acid.

(2) Per cent recovery of UDPhexose in the recovery experiments was calculated by substracting the average micromoles of UDPhexose per liver sample, to which no UDPG had been added, from the total micromoles of UDPhexose recovered from the chromatograms of those samples to which UDPG had been added. This value was divided by the micromoles of UDPG that had been added to the extracts prior to treatment with Dowex 50.

$$\text{Per Cent Recovery} = \frac{\underset{\text{(no UDPG added)}}{\text{Total micromoles UDPhexose}} - \underset{\text{(UDPG added)}}{\text{Total micromoles UDPhexose}}}{\text{Micromoles UDPG added}}$$

(3) In calculating the micromoles of hexose bound to the UDP a number of dilution factors were involved. Of the 4 ml. of H_2SO_4 used to elute the UDPhexose from the chromatograms, 3 ml. were taken for hydrolysis and barium hydroxide-zinc sulfate treatment, which made

a volume of 4.4 ml. Three ml. of this was taken for evaporation and two thirds of the residue was chromatographed. So the theoretical amount of hexose actually chromatographed was known on the basis of the quantity of UDPhexose represented in the aliquot employed.

$$\text{Predicted micromoles of hexose on UDP} = \frac{\text{OD at 262 millimicra}}{10} \times 3 \times \frac{3}{4.4} \times \frac{2}{3}$$

$$\frac{\text{OD at 262 millimicra}}{10} = \text{micromoles of UDPhexose per ml. of } H_2SO_4 \text{ eluate.}$$

(4) In calculating micromoles of hexose recovered, the micrograms of glucose and galactose recovered from the final chromatography were converted to micromoles by dividing by the micromolecular weight of these hexoses (180). The micromoles of glucose and galactose recovered were then added to give the micromoles of hexose recovered.

(5) The per cent of the UDP bound hexose represented by glucose and galactose was calculated by dividing the amount of each recovered by the sum of the two.

D. UDPG Recovery Experiments

In these experiments authentic UDPG was added to one-half of the aliquots from a pooled PCA extract of rat liver. The extracts were carried through the procedure outlined in Section III. The results of these experiments appear in Tables 4, 5 and 6.

The per cent recovery of UDPhexose from rat liver extracts ranged from 70 to 82 per cent as calculated from UV absorption at 262 millimicra. About 77 to 80 per cent of authentic UDPG was recovered from norite after adsorption from distilled water. See Section III, E. The recovery of UDPG from rat liver corresponds well to

TABLE IV

UDPG Recovery (Experiment No. 1) From Livers of Rats Fed a Stock Ration

	Each Sample is the Extract from 2.25 g of Liver					
	No UDPG Added to Samples				UDPG Added to Samples 0.836 micromoles to 5 0.683 micromoles to 6	
	1	2	3	4	5	6
*Ratio of OD at 280:260 millimicra of eluted spot having a peak at 262 millimicra and migrating at same R_f as authentic UDPG	0.37	0.37	0.38	0.37	0.37	0.37
Total micromoles of UDPhexose per liver sample extracted	0.915	0.950	0.960	1.00	1.63	1.45
Average micromoles of UDPhexose Sample 1-4		0.958				
Per cent recovery of UDPhexose calculated from absorption at 262 millimicra					78.0	72.2
Predicted micromoles of hexose on UDP calculated from absorption at 262 millimicra	0.127	0.132	0.133	0.139	0.270	0.201
Micromoles hexose recovered	0.128	0.119	0.142	0.161	0.231	0.204
Per cent of total hexose recovered as a) glucose	72	75	72	72	72	79
b) galactose	28	25	28	28	28	21

*Refer to foot note at bottom of Tabâe III

TABLE V

UDPG Recovery (Experiment No. 2) From Livers of Rats Fed a Stock Ration

	Each Sample is the Extract From 2.16 g of Liver							
	No UDPG Added to Samples				0.448 Micromoles of UDPG Added to Samples			
	1	2	3	4	5	6	7	8
Ratio of OD at 280:260 millimicra of eluted spot having a peak at 262 millimicra and migrating at same R_f as authentic UDPG	0.37	0.37	0.37	0.37	0.37	0.40	0.36	0.41
Total micromoles of UDPhexose per liver sample extracted	0.745	0.733	0.738	0.710	1.045	1.093	1.075	1.100
Average micromoles of UDPhexose, samples 1-4		0.732						
Per cent recovery of UDPhexose calculated from absorption at 262 millimicra					70.0	80.5	76.7	82.0
Predicted micromoles of hexose on UDP calculated from absorption at 262 millimicra	0.084	0.083	0.084	0.081	0.119	0.124	0.122	0.125
Micromoles of hexose recovered	0.082	0.075	0.072	0.069	0.132	0.136	0.139	0.115
Per cent of total hexose recovered as: a) glucose	88	91	78	82	75	76	74	76
b) galactose	12	9	22	18	25	24	26	24

TABLE VI

UDPG Recovery (Experiment No. 3) From Livers of Rats Fed a Stock Ration

	Each Sample is the Extract from 2.12 g of Liver							
	No UDPG Added to Samples				1.2 Micromoles of UDPG Added to Samples			
	1	2	3	4	5	6	7	8
Ratio of OD at 280:260 millimicra of eluted spot having a peak at 262 millimicra and migrating at same R_f as authentic UDPG	0.38	0.38	0.38	0.39	0.38	0.38	0.38	0.38
Total micromoles of UDPhexose per liver sample extracted	0.835	0.859	0.805	0.835	1.730	1.690	1.70	1.68
Average micromoles of UDPhexose, samples 1-4	0.833							
Per cent recovery of UDPhexose calculated from absorption at 262 millimicra					74.7	71.4	72.0	70.5
Predicted micromoles of hexose on UDP calculated from absorption at 262 millimicra	0.114	0.117	0.110	0.114	0.235	0.230	0.232	0.229
Micromoles of hexose recovered	0.106	0.114	0.103	0.110	0.213	0.206	0.218	0.220
Per cent of total hexose recovered as: a) glucose	81	78	79	77	73	74	75	72
b) galactose	19	22	21	23	27	26	25	28

UDPG recovery from water using norite adsorption and elution. It would appear that in the recovery of UDPhexose from liver extracts, the factor limiting quantitative recovery was the adsorption and elution from norite.

The reported ratio of hexose to UDP in UDPhexose is 1:1[17]. The micromoles of hexose recovered ranged around the theoretical ratio of 1:1.

Except for samples 1 and 2 in Table 5 the UDP bound hexose consists of about 18 to 28 per cent galactose and 72 to 82 per cent glucose. These results are similar to those found by Leloir[24] and by Hansen and Craine[25] using in vitro systems from yeast and bacteria. The per cent of glucose and galactose in the UDPhexose recovered from samples to which UDPG had been added was nearly the same as from samples to which no UDPG was added. The authentic UDPG added in the recovery experiments evidently contained a mixture of UDPG and UDPGal in about the same ratio as UDPhexoses of rat liver. If the authentic UDPG had actually contained only glucose as the hexose, then in those samples to which UDPG was added there would have been a greater per cent of the hexose recovered as glucose and less as galactose.

E. UDPhexose in Livers of Rats Fed Control and Galactose Diets

Data for rats fed control and galactose diets appear in Tables 7 and 8.

There was little difference in the concentration of UDPhexose in the livers of rats fed control and galactose diets for 5 days. The micromoles of hexose recovered from the UDPhexose were consistently lower than the predicted micromoles of bound hexose. The most

TABLE VII

UDPhexose in Livers of Rats Fed Control or Galactose Diets for 5 Days

	Control Fed Rats				Galactose Fed Rats			
	1	2	3	4	1	2	3	4
Wt. of liver extracted in grams	2.35	2.10	2.05	1.90	1.75	2.19	2.02	1.92
Ratio of OD at 280:260 millimicra of eluted spot having a peak at 262 millimicra and migrating at same R_f as authentic UDPG	0.38 0.38	0.37 0.37	0.37 0.36	0.36 0.37	0.38 0.37	0.38 0.38	0.37 0.38	0.38 0.37
Total micromoles of UDPhexose per liver sample extracted	0.940 0.956	0.687 0.708	0.768 0.775	0.840 0.756	0.530 0.558	1.04 1.11	0.907 0.890	0.897 0.890
Micromoles of UDPhexose per g of liver	0.400 0.407	0.328 0.337	0.375 0.378	0.440 0.398	0.303 0.318	0.475 0.507	0.449 0.440	0.472 0.462
Predicted micromoles of hexose on UDP calculated from absorption at 262 millimicra	0.106 0.108	0.078 0.080	0.087 0.088	0.095 0.086	0.060 0.062	0.117 0.125	0.130 0.110	0.120 0.110
Micromoles of hexose recovered	0.088 0.084	0.063 0.076	0.083 0.079	0.086 0.071	0.056 0.056	0.103 0.107	0.090 0.095	0.094 0.089
Per cent of hexose recovered Ratio glucose;galactose	77-23 74-26	80-20 78-22	76-24 76-24	76-24 89-11	68-32 72-28	57-43 55-45	77-23 80-20	59-41 62-38

TABLE VIII

UDPhexose in Livers of Rats Fed Control or Galactose Diets for 10 Days

	Control Fed Rats			Galactose Fed Rats		
	1	2	3	1	2	3
Wt. of liver extracted in grams	2.00	2.07	2.05	2.07	2.03	2.17
Ratio of OD at 280:260 millimicra of eluted spots having a peak at 262 millimicra and migrating at same R_f as authentic UDPG	0.37 0.36	0.36 0.36	0.37 0.36	0.37 0.37	0.36 0.36	0.37 0.38
Total micromoles of UDPhexose per liver sample extracted	0.572 0.552	0.384 0.389	0.600 0.615	0.840 0.790	0.713 0.682	0.885 0.925
Micromoles of UDPhexose per g of liver	0.286 0.277	0.186 0.189	0.293 0.300	0.403 0.382	0.351 0.336	0.408 0.426
Predicted micromoles of hexose in UDP calculated from absorption at 262 millimicra	0.049 0.047	0.033 0.033	0.051 0.052	0.071 0.067	0.061 0.058	0.076 0.079
Micromoles of hexose recovered	0.046 0.048	0.030 0.031	0.048 0.053	0.063 0.059	0.055 0.058	0.070 0.078
Per cent of hexose recovered Ratio glucose:galactose	71-29 70-30	72-28 70-30	76-24 70-30	33-67 32-68	39-61 36-64	40-60 41-59

likely explanation is that the UDPhexose eluted from the chromatograms contained a small amount of UMP. If UMP were present in the UDPhexose there would be no difference spectrophotometrically from pure UDP hexose, but it would be evident in the isolation procedure, since there is no hexose in UMP. Only two of the four galactose fed animals exhibited an increase in the per cent of the hexose represented by galactose. In these two animals galactose represented about 40 per cent of the UDP bound hexose compared to a maximum 26 per cent in the control fed rats.

The UDPhexose concentrations of livers in rats fed control and galactose diets for 10 days showed a greater range than the rats fed for 5 days. The value for UDPhexose concentration in control rat No. 2 was low. Neither finding can be explained at this time. The quantities of hexose recovered were closer to the theoretical in the 10 day experiments than in the 5 day experiments. One significant finding is the partial reversal of the quantities of glucose and galactose bound to UDP in the rats fed galactose for 10 days. The highest per cent galactose in the control fed animals was 30 while the lowest in the galactose fed animals was 59. This indication of reversal of the UDPGlucose-UDPGalactose ratio in two of four animals fed galactose for 5 days is confirmed by the results of the 10 day feeding.

F. Galactose Toxicity in Rats

Hansen et al[77] fed newly hatched chicks a diet containing 15 per cent galactose. They found that the UDPhexose content of liver from these chicks had doubled in 6 to 10 days in comparison to

control fed chicks and that galactose made up a greater portion of the hexose. It was inferred that the normal 75:25 UDPG-UDPGal equilibrium established by UDPGal-4-epimerase (galactowaldenase) does not occur in chicks fed galactose.

The liver concentration of UDP hexose in rats fed galactose (discussed in E above) did not change as it did in the chicks of Hansen et al. Although the UDPG:UDPGal ratio changed in only 2 of 4 experiments in rats fed galactose 5 days, the shift in the ratio is quite evident in the animals fed ten days. This partial reversal of the UDPG:UDPGal ratio in rats fed a galactose diet for 10 days supports the findings of Hansen et al in chicks.

The role of galactose in galactose toxicity in rats was partially discussed in the introduction. The accumulation of Gal-1-P in rat liver[13] and lens[14], the toxicity of galactose to lens epithelium in tissue culture[11] and the inhibition of phosphoglucomutase and glucose-6-phosphate phosphatase[23] enzymes by Gal-1-P all point to Gal-1-P as the toxic agent in galactose toxicity. However, there are other facets to galactose toxicity which merit discussion.

The only known pathways for the entrance of Gal-1-P into the metabolic scheme lie in the enzymatic reactions 1 and 4 illustrated in the introduction. In both of these reactions Gal-1-P is conjugated to form UDPGal which may then be transformed to UDPG (through the 4-epimerase enzyme). The main pathway of UDPG synthesis in mammalian tissue is through the reaction[25,30] G-1-P + UTP $\rightleftharpoons$ UDPG + PP. One of the functions of UDPG is in glucuronide synthesis after its

oxidation to UDPGA. Since many metabolic products are detoxified through glucuronide formation a decrease in the availability of UDPG might well allow for accumulation of toxic products. If the ratio of UDPG to UDPGal is upset, as indicated above, then the availability of UDPG in glucuronide synthesis may well be decreased. This appears very likely in that UDPG is utilized in the Gal-1-P uridyl transferase reaction for the formation of UDPGal and that this enzyme activity is some six times greater (Table 1) than that of the enzyme involved in the formation of UDPGal through the UDPGal pyrophosphorylase reaction which requires no UDPG.

Another factor concerns the activity of the UDPGal-4-epimerase which functions in the UDPGal to UDPG conversion. Evidently the activity of this enzyme is not sufficient to maintain the normal UDPG-UDPGal ratio in rats fed galactose.

V

SUMMARY

Two types of experiments have been carried out.

The first constitutes a method for the isolation and recovery of added UDPhexose from rat liver. The method involved, (1) liver extraction with perchloric acid, (2) treatment of the extract with Dowex 50 H^+ at a pH of 1.2 (3) adsorption and elution of nucleotides from norite, (4) paper chromatography for separation of nucleotides and identification of UDPhexoses, and (5) paper chromatography of the hydrolyzed UDPhexoses for the quantitative determination of liberated hexoses (glucose and galactose). The factor preventing quantitative recovery of added UDPG appears to be incomplete elution of UDPhexoses from norite.

In the second set of experiments UDPhexose was determined in livers of rats fed high galactose or control diets. It was found that the normal UDPG:UDPGal ratio was altered in livers of rats fed high galactose diets.

This altered ratio is discussed in relation to galactose toxicity in rats.

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