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NUCLEOTIDE INHIBITION OF MAMMALIAN LIVER GALACTOSE-1-PHOSPHATE URIDYLYLTRANSFERASE

STANTON SEGAL AND SHIRLEY ROGERS

Division of Biochemical Development and Molecular Diseases, Children's Hospital of Philadelphia and Department of Pediatrics, Medical School of the University of Pennsylvania, Philadelphia, Pa 19146 (U.S.A.)

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SUMMARY

Mammalian liver hexose-1-phosphate uridylyltransferase (UDP-glucose α-D-galactose-1-phosphate uridylyltransferase, EC 2.7.7.12) has been shown to be inhibited by various nucleotides. The nature of the inhibition has been examined with regard to the structural requirements of the inhibitory compounds and the possible regulatory function of intracellular nucleotides on enzyme activity. The most potent inhibitors were UTP and UDP which produced significant inhibition of the enzyme at concentrations normally found in liver tissue. UTP, UDP and UMP were linear competitive inhibitors of UDP-glucose with K_i of 0.13, 0.35, and 2.3 mM, respectively, and uncompetitive inhibitors of galactose 1-phosphate. UDP-glucuronic acid and UDP-mannose caused similar inhibition.

The uridine moiety appeared necessary for competition with substrate UDP-glucose since substitution of cytidine, adenine or guanosine in the nucleotide as in CDP-glucose, ADP-glucose or GDP-glucose produced uncompetitive inhibition of UDP-glucose. Nucleotides containing glucose were competitive inhibitors of galactose 1-phosphate while those containing other sugars or without a sugar moiety caused uncompetitive inhibition of the sugar phosphate site.

INTRODUCTION

Although many recent studies have centered about alternate pathways of D-galactose metabolism in mammalian tissue[1,2], our knowledge of the conventional pathway involving uridine nucleotide sugars[3,4] whereby most galactose is metabolized under normal circumstances is far from complete. The importance for understanding the regulation and control of the nucleotide sugar pathway stems not only from the fact that D-galactose is a major nutrient in most mammals but also from the existence of the devastating syndrome of galactose toxicity in the human inherited disorder, congenital galactosemia, where there is a deficiency of the enzyme, galactose-1-phosphate uridylyltransferase (UDP-glucose α-D-galactose-1-phosphate uridyltransferase, EC 2.7.7.12)[5]. This enzyme catalyzes the reversible reaction

α-D-galactose-1-*P* + UDP-glucose ⇌ UDP-galactose + α-D-glucose-1-*P*

Since the liver is the principle organ involved in galactose metabolism, we have been concerned with the developmental, regulatory and kinetic characteristics of the enzyme in rat and human liver[6,7]

Our previous studies sought to determine nucleotide sugar substrate specificity and showed that only UDP-glucose and UDP-galactose fulfilled this role, there being no reactivity of ADP-glucose, CDP-glucose, TDP-glucose, GDP-glucose, UDP-xylose, UDP-mannose or GDP-mannose with galactose 1-phosphate The work described here focuses on the inhibitory effects of nucleotide compounds on galactose-1-phosphate uridylyltransferase activity This was done from two points of view, first, to determine the structural requirements of inhibitory compounds in relation to the substrates, and secondly, to assess the possible role of normally occurring intracellular nucleotides in regulation of enzyme activity Our findings indicate that uridine nucleotides are potent inhibitors of the reaction at physiological concentrations, suggesting the enzyme may be tightly regulated by the metabolic state of cellular nucleotides

METHODS AND MATERIALS

The enzymatic procedure employed which has been described in detail by BERTOLI AND SEGAL[6] involves the determination of radioactive UDP-galactose formed by interaction of [^{14}C]galactose 1-phosphate with UDP-glucose The incubation mixture contained 0 25 mM UDP-glucose, 0 35 mM galactose 1-phosphate (0 0056 μC), 100 mM glycine buffer pH 8 2 and 10 mM mercaptoethanol in a total volume of 200 μl The reaction was started by adding enzyme, incubated at 37° for 5 min with liver supernatants and 2 min with purified enzyme, and stopped by boiling for 90 sec 20 μl was spotted on 21 cm DEAE-cellulose ion exchange paper strips for chromatography with 0 5 M LiCl by the descending method The product radioactivity was determined by counting the area of the paper containing the [^{14}C]UDP-galactose with a liquid scintillation spectrometer using diluted Liquifluor as the scintillator The amount of product formed could be calculated using the specific activity of radioactive substrate Mercaptoethanol replaced dithiothreitol used previously as a sulfhydryl reagent

Several different enzyme preparations were utilized (1) A 30 000 × g supernatant with activity of 10 nmoles UDP-galactose formed per min per mg protein prepared after homogenization of livers of 35–45-day-old Sprague–Dawley rats at a 1 20 dilution in 20 mM KCl as described previously[6] BERTOLI AND SEGAL[6] using this preparation demonstrated the stoichiometric conversion of [^{14}C]galactose 1-phosphate to [^{14}C]UDP-galactose Such supernatants under the assay conditions described above have no UDP-glucose or UDP-galactose pyrophosphorylase and no UDP-galactose-4-epimerase activity Since glucose 1-phosphate is a known transferase inhibitor[6] incubation mixtures were tested for glucose 1-phosphate after the addition of the various glucose containing nucleotide inhibitors None was found as indicated by the inability to detect glucose oxidase after treatment with alkaline phosphatase In addition, after incubation of inhibitory amounts of [^{14}C]ADP-glucose for 5 min with this preparation, no labeled compound other than ADP-glucose was found on chromatography of the mixture (2) A 100-fold partially purified rat liver enzyme, 1150 nmoles UDP-galactose formed per min per mg protein, prepared by minor modifications of

the method of MAYES AND HANSON[8] The steps involved precipitation of a 20 mM KCl homogenate with $(NH_4)_2SO_4$ at 50% saturation, dialysis against distilled water, adsorption and elution from calcium phosphate gel, chromatography of a gel eluate on DEAE-cellulose using batches of 20 and 80 mM phosphate buffer The enzyme at this stage was diluted again, placed on DEAE-cellulose column, and eluted with 80 mM KCl This enzyme was very unstable and deteriorated at $-45°$ within two days (3) A 50-fold purified preparation made according to MAYES AND HANSON[8] from human liver obtained at autopsy, 327 nmoles UDP-galactose formed per min per mg protein[7] (4)A partially purified calf liver enzyme purchased from Boehringer, Mannheim This preparation had about 70% of transferase activity per mg protein as our 100-fold purified rat liver but the extent of purification could not be ascertained from the supplier The method of LOWRY *et al* [9] was used for determination of protein

All assays were determined using linear initial rates of reaction and protein concentrations within the range of proportionality of the reaction Data from the inhibition studies were plotted according to the LINEWEAVER–BURK[10], HOFSTEE[11], CLELAND[12], and DIXON[13] methods The type of inhibition was discerned from these plots according to the criteria of WEBB[14] Apparent K_i values were calculated from the abscissa intercepts of the LINEWEAVER–BURK plot and CLELAND replot (slope or intercept *vs* 1) Where the inhibition was competitive the intersection of lines in the DIXON plot was employed Values obtained were comparable

Various nucleotides were purchased from Calbiochem, Los Angeles, Calif, or Sigma, St Louis, Mo Solutions of nucleotides were adjusted to pH 9 2 before addition to incubation mixtures [^{14}C]Galactose 1-phosphate (specific activity 32 mC/mmole was obtained from International Chemical and Nuclear Corp , City of Industry, Calif Chromatography on DEAE-cellulose anion exchange paper (Whatman DE81) revealed less than 2% neutral sugar impurity

RESULTS

Nucleotide inhibition of galactose-1-phosphate uridylyltransferase

The inhibition of transferase activity of rat liver homogenate by a large group of nucleotides is shown in Table I At concentrations of 5 mM, uridine and adenine nucleotides, and diverse sugar nucleotides containing both pyrimidine and purine bases and a variety of sugars are potent inhibitors The inhibition appears to be dependent on inhibitor concentration in those cases tested The most potent inhibitors appear to be UTP and UDP with inhibition seemingly related to the number of phosphate groups in the molecule This relationship is also observed with adenine nucleotides UDP-glucuronic acid which is of physiological importance also shows significant inhibition at lower concentrations Transferase activity in the supernatant liver preparation was not inhibited by 5 mM of inorganic phosphate or comparable amounts of Na^+, K^+ or Cl^-

The inhibition observed by these compounds in the 30 000 $\times$ *g* supernatant fraction of liver homogenates was seen to be of the same extent in partially purified enzyme prepared from rat, calf and human liver (Table II) The percent inhibition seen at the 5 mM inhibitor concentration in Table II for 100-fold purified rat liver enzyme is essentially the same for eight of the inhibitors tested as shown in Table I for the liver supernatant prepararion The only exception is the effect of ATP and ADP The

TABLE I

GALACTOSE-1-PHOSPHATE URIDYLYLTRANSFERASE INHIBITION BY NUCLEOTIDES

Incubation conditions described under METHODS employing 20 μl (60 μg protein) of a 30 000 $\times$ g supernatant fraction of rat liver homogenates Values are averages of at least three determinations Assessment of inhibitory activity of ATP and ADP was done both with and without equal molar amounts of Mg^{2+} without any difference in results

Inhibitor	*% Inhibition*			
	5 mM	*1 mM*	*0 5 mM*	*0 25 mM*
UTP	99	99	56	47
UDP	65	64	46	30
UMP	48	9	3	
ATP	36		20	
ADP	32		18	
AMP	0		0	
UDP-glucuronic acid	80	46	33	14
UDP-mannose	54			
UDP-xylose	79			
TDP-glucose	90	30	18	4
CDP-glucose	75	33	16	
ADP-glucose	74	45	30	18
GDP-mannose	50		28	
GDP-glucose	64			
Uracil	12			
Uridine	32			
Adenine	16			
Adenosine	17			
Glucuronic acid	5			

TABLE II

NUCLEOTIDE INHIBITION OF PARTIALLY PURIFIED PREPARATIONS OF LIVER GALACTOSE-1-PHOSPHATE URIDYLYLTRANSFERASE

Enzyme preparations and incubation conditions are described in METHODS 10 μg of protein was used and incubation time was 2 min Values are average of three determinations Assessment of inhibitory activity of ATP and ADP was done both with and without equal molar amounts of Mg^{2+} without any difference in results

Inhibitor (5 mM)	*% Inhibition*		
	Enzyme preparation		
	Rat liver 100-fold purified	*Calf liver Boehringer*	*Human liver 50-fold purified*
UTP	90		
UDP	65		
ATP	0	−9	
ADP	0	−4	
TDP-glucose	87	91	77
CDP-glucose	72	71	77
GDP-mannose	62	15	40
UDP-xylose	75	79	77
ADP-glucose	74		
UDP-glucuronic acid	80		

inhibition observed with these compounds with the cruder enzyme preparation was not seen with the partially purified preparations This was not the case with the adenine sugar nucleotide, ADP-glucose, however Over a broad ADP-glucose concentration range from 0 25 to 5 mM the percent inhibition of purified and unpurified supernatant enzymes was the same The purified rat liver enzyme was more sensitive to inorganic phosphate than the homogenate supernatant fraction, 5 mM causing a 20% inhibition of activity of the former but no inhibition of the latter

Nature of the inhibition

The type of inhibition was studied by analysis of initial velocity changes caused by several inhibitors at varying substrate concentrations with the 30 000 × *g* liver supernatant preparation At least three inhibitor concentrations were used The fixed substrate concentrations were 0 25 mM for UDP-glucose and 0 35 mM for galactose 1-phosphate The apparent K_m for these substrates in this preparation is 0 16 mM for UDP-glucose and 0 14 mM for galactose 1-phosphate[6] Higher substrate levels in relation to the apparent K_m cannot be employed because of substrate inhibition

TABLE III

TYPE OF INHIBITION AND K_i VALUES OF VARIOUS NUCLEOTIDES WITH GALACTOSE-1-PHOSPHATE URIDYLYLTRANSFERASE

All inhibitors were studied in at least three concentrations 20 µl of a 30 000 *g* supernatant of rat liver homogenate prepared as described under METHODS was used When UDP-glucose was varied, the galactose 1-phosphate level was 0 35 mM UDP-glucose was kept at 0 25 mM when galactose-1-phosphate was varied

Inhibitor	*Type of inhibition and K_i (mM) (variable substrate)*			
	UDP-Glucose		*Galactose 1-phosphate*	
UMP	Competitive	2 3	Uncompetitive	6 9
UDP	Competitive	0 35	Uncompetitive	0 45
UTP	Competitive	0 13	Uncompetitive	0 08
UDP-glucuronic acid	Competitive	0 40	Uncompetitive	0 09
UDP-mannose	Competitive	1 6	Uncompetitive	5 7
TDP-glucose	Noncompetitive	1 0	Competitive	0 28
CDP-glucose	Uncompetitive	0 88	Competitive	0 75
ADP-glucose	Uncompetitive	0 70	Competitive	0 67
GDP-mannose	Uncompetitive	5 3	Uncompetitive	3 6

The results of these are shown in Table III which also indicates the apparent K_i value for each inhibitor The K_i value for the inhibitor was acceptable only when there was agreement of the result of the determination of K_i from the LINEWEAVER–BURK, DIXON and CLELAND plots of the data The uridine nucleotides UMP, UDP and UTP were linear competitive inhibitors when UDP-glucose was the variable substrate and uncompetitive inhibitors of galactose 1-phosphate A typical experiment is shown in Fig 1 where UTP is the inhibitor and the data plotted according to LINEWEAVER–BURK[10]

The uridine containing sugar nucleotides, UDP-mannose and UDP-glucuronic acid exhibit the same types of inhibition as the nucleotides being competitive with UDP-glucose and uncompetitive with galactose 1-phosphate Fig 2 shows the

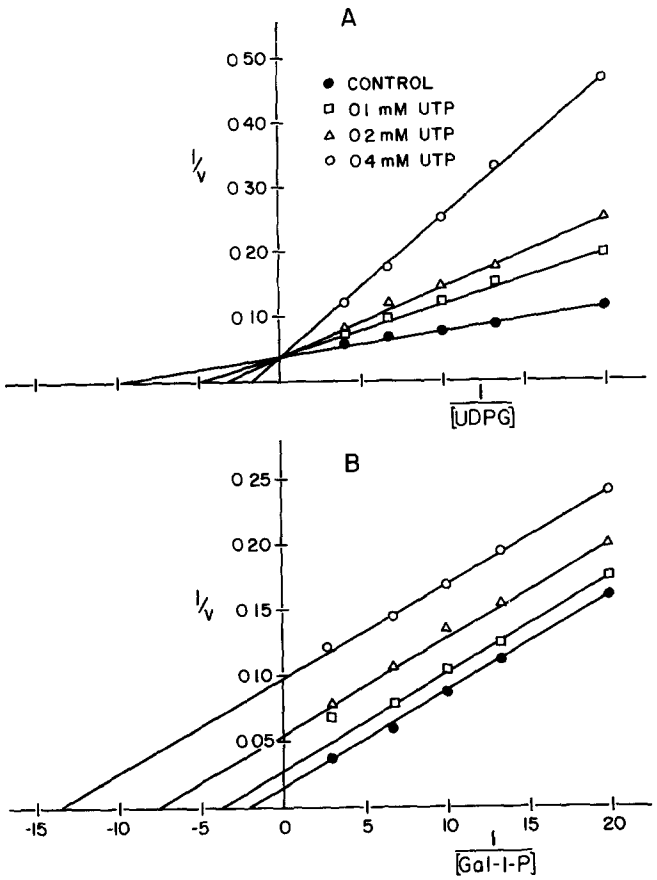

Fig 1 Effects of UTP on galactose-1-phosphate uridylyltransferase velocities A With respect to UDP-glucose (UDPG) when galactose 1-phosphate was 0 35 mM B With respect to galactose-1-phosphate (Gal-1-*P*) when UDP-glucose was 0 25 mM UTP concentration is denoted by the same symbols in both graphs Initial velocities (v) are nmoles of UDP-galactose formed per min per mg soluble protein Substrate concentrations are mM 20 μl of the 30 000 g liver supernatant fraction was incubated for 5 min under experimental conditions described in METHODS

velocity data with UDP-glucuronic acid as inhibitor Substitution of cytosine or a purine base for uracil in the sugar nucleotide as in CDP-glucose, ADP-glucose or GDP-mannose changes the inhibition with UDP-glucose as substrate to the linear uncompetitive type This is shown in Fig 3 where ADP-glucose is the inhibitor TDP-glucose was the only sugar nucleotide tested which showed linear non-competitive inhibition of UDP-glucose

The configuration of the sugar moiety appears to determine the type of inhibition when galactose 1-phosphate is the variable substrate In those instances where glucose is the sugar component of the nucleotide the compound is a linear competitive inhibitor of galactose 1-phosphate (Fig 3) When both the uracil structure is altered and the sugar configuration is not that of glucose such as GDP-mannose, the inhibition becomes uncompetitive for both substrates Current experiments in progress to

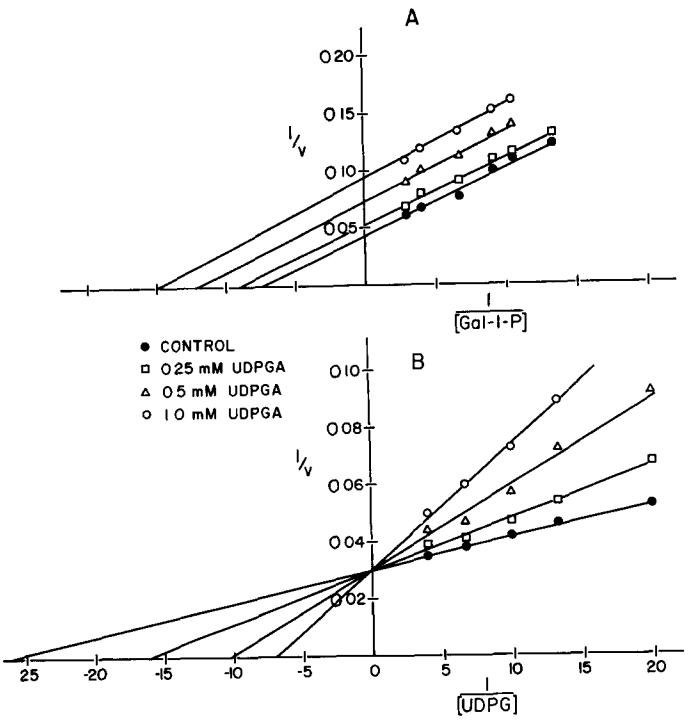

Fig 2 Effects of 0 25, 0 5 and 1 0 mM UDP-glururonic acid (UDPGA) on enzyme activity. A Activity with respect to galactose 1-phosphate (Gal-1-*P*) at 0 25 mM UDP-glucose B Activity with respect to UDP-glucose (UDPG) at 0 35 mM galactose 1-phosphate Inhibitor concentration is denoted by the same symbols in both graphs Substrate concentrations are mM and initial velocity (v) is nmoles of UDP-galactose formed per min per mg soluble protein 20 μl of the 30 000 $\times$ g liver supernatant fraction was incubated 5 min under conditions described in METHODS

determine the nature of previously reported substrate inhibition of the enzyme[6] indicate that UDP-glucose is a competitive inhibitor of galactose 1-phosphate

The ATP and ADP inhibition mechanism was studied but not as extensively as the other nucleotides At 5 mM concentration these adenine nucleotides were competitive inhibitors of galactose 1-phosphate and non-competitive inhibitors of UDP-glucose The K_i for the ATP inhibition of galactose 1-phosphate was relatively high at 8 mM

DISCUSSION

The present observations furnish some insight regarding the stereospecificity of the enzyme and the structural requirements of nucleotides for interference with enzyme activity Of the various nucleotides and sugar nucleotides examined, only those with a pyrimidine base with carbonyl function at positions 2 and 4 as in uracil are competitive inhibitors of UDP-glucose The potency of the competitive inhibition of UDP-glucose also depends on the phosphorylation of the ribose moiety of uridine since uracil and uridine have only little inhibitory capacity even at high concentrations, while UMP, UDP and UTP have a progressive increase in apparent affinity for

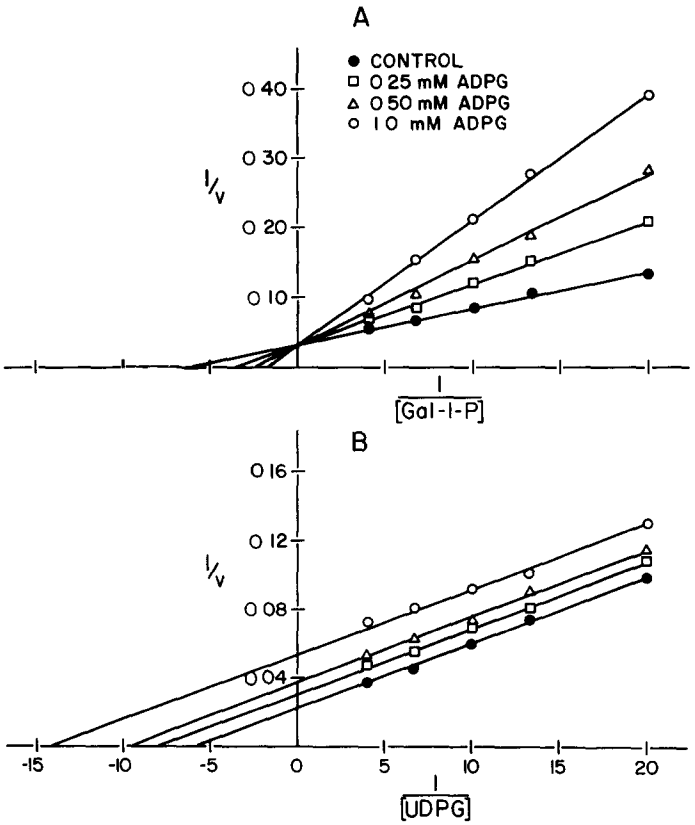

Fig 3 Effects of ADP-glucose (ADPG) on galactose-1-phosphate uridylyltransferase activity A With respect to galactose 1-phosphate (Gal-1-*P*) at 0 25 mM UDP-glucose B With respect to UDP-glucose (UDPG) at 0 35 mM galactose 1-phosphate Inhibitor concentration is denoted by the same symbols in both graphs Initial velocity (v) is nmoles UDP-galactose formed per min per mg soluble protein The concentrations of substrates are mM 20 μl of the 30 000 $\times$ g liver supernatant fraction was incubated 5 min under conditions described in METHODS

the enzyme Substitution of sugars such as mannose for the terminal phosphate of UTP to give UDP-mannose appears to increase the K_i of the inhibitor compound The fact that UDP-mannose or UDP-xylose cannot act as a substrate for the enzyme[6] must indicate that the configuration of the hydroxyl at C-2 of the hexose carbon chain, as well as the 6-carbon chain length, are critical structural requirements of the sugar nucleotide to participate in the transferase reaction An alteration of the pyrimidine moiety as in CDP-glucose or substitution of a purine such as in GDP-mannose changes the inhibition of UDP-glucose from a competitive to an uncompetitive type where though the apparent affinity for UDP-glucose is greater, the velocity of the reaction is slower

Glucose 1-phosphate has been reported to be a potent inhibitor of galactose-1-phosphate uridylyltransferase[6] It is perhaps not surprising then that nucleotide sugars with a glycosyl unit such as ADP-glucose, TDP-glucose and UDP-glucose are competitive inhibitors of galactose 1-phosphate Substitution of mannose for glucose

changes the inhibition of galactose 1-phosphate to the uncompetitive form It should be noted in Table III that competitive inhibition of one substrate is generally associated with uncompetitive inhibition of the other substrate in the reaction

The nature of the inhibition by ATP of the homogenate enzyme preparation does not fit the patterns described above with ATP being a competitive inhibitor of galactose 1-phosphate The reason for the disappearance of the ATP effect when the enzyme is purified is unexplained and remains to be determined

The assignment of physiological significance to nucleotide inhibition of transferase is open to speculation The assessment of a physiologic role can only be tentative, especially in view of possible compartmentation of substrates within cells The amount of inhibition would be dependent on the normal cellular concentration of substrates and inhibitors as well as the K_i of the inhibitor Hepatic cellular levels of compounds such as UDP-mannose, TDP-glucose and ADP-glucose are probably so low that it is unlikely that they exert any control on the reaction This is probably not the case with the uridine nucleotides, UMP, UDP, UTP as well as UDP-glucuronic acid

KEPPLER *et al* [15–18] have accurately determined the rat liver cell concentration of various uridine nucleotides as well as galactose 1-phosphate They found normal UDP-glucose levels of 0 32 μmoles/g wet wt , UTP + UDP of 0 34 μmole/g wet wt , and UMP of about 0 04 μmole/g wet wt Loading an animal with galactose[18] produces galactose 1-phosphate levels of 0 18 μmole/g wet wt These UDP-glucose and galactose 1-phosphate levels are comparable to those used in our standard assay for the inhibitions shown in Table I Under these conditions a concentration of UTP and UDP of 0 34 μmole/g or about 0 43 μmole/ml cell water (assuming 80% of wet weight is water) would give an enzyme inhibition of about 50% The K_i for UTP (Table III) is about 0 10 mM, well below the normal intracellular level One of the interesting findings of KEPPLER *et al* [18] is that galactose loading causes liver UTP + UDP to decrease to about 0 1 μmole/ml This suggests that the inhibition of transferase is diminished as the need to metabolize galactose increases and may be a significant regulatory mechanism Liver cell levels of UDP-glucuronic acid of about 0 3 mM (ref 16) are within the inhibitory range of the compound and below the observed K_i Also, ATP levels in liver have been reported to be about 3 mM (refs 18,19) which is also in the inhibitory range of ATP shown in Table I

Galactose-1-phosphate uridylyltransferase has been reported to show both substrate and product inhibition which has possible regulatory features[6] From the data presented here it appears likely that this transferase activity may also be regulated by cellular nucleotides and be dependent on multiple factors of cell metabolism Our present findings underscore the need for examination of galactose metabolism in liver under physiological circumstances where cellular nucleotide levels may be altered

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