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SOME CHARACTERISTICS AND DEVELOPMENTAL ASPECTS OF RAT URIDINE DIPHOSPHOGALACTOSE 4-EPIMERASE

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SUMMARY

UDP-Gal 4-epimerase (EC 5.1.3.2) activity in developing rat liver, gut, kidney and brain was studied. The assay involved the thin-layer chromatographic separation of sugar nucleotides on polyethyleneimine-impregnated cellulose. UDP-[^{14}C]Gal, the substrate, was converted to UDP-[^{14}C]Glc by the epimerase reaction. Since both the reactant and the product have the same R_F value, the UDP-Glc is converted by UDP-Glc dehydrogenase to UDP-GlcUA which is easily distinguished from the UDP-Gal on these plates.

Differential centrifugation localized epimerase activity from newborn and adult rat liver in the soluble fraction. Newborn liver had greater specific activity than any other tissue examined, while adult gut had the highest activity of any adult tissue. The activity of the newborn liver declined at Day 2, remaining at the same level until Day 16 when a precipitous decline to adult levels of activity by Day 20 occurred. Brain and kidney had lower specific activity than found in liver. No differences in activity of liver enzyme were noted between male and female newborn or adults.

The newborn enzyme had a v_{max} five times greater than the adult, but both enzyme preparations had the same (a) stability characteristics, (b) K_m values for UDP-Gal (0.153 mM) and for UDP-Glc (0.5 mM), (c) pH optimum (8.3–8.65) and (d) requirement for exogenous NAD^+. Further, both were inhibited by NADH, *p*-chloromercuribenzoate, UDP-Man, UMP and TDP-Glc, and both exhibited product inhibition with UDP-Glc.

INTRODUCTION

Previous communications from this laboratory have been concerned with the development and kinetic characteristics of rat liver galactokinase and galactose-1-phosphate uridyltransferase, as they relate to the metabolism of galactose in the developing animal[1,2]. UDP-Gal 4-epimerase, which catalyzes the conversion of UDP-

Abbreviation: PCMB, *p*-chloromercuribenzoate.

Gal to UDP-Glc, is the terminal reaction in the conventional sugar nucleotide pathway which enables galactose to enter glucose metabolic pathways. Although some aspects of this reaction have been studied in mammals[3,4] and microorganisms[5-7], developmental variations have not been examined. The present experiments, employing a new highly sensitive method for the measurement of the epimerase enzyme, continue our investigation into the development of the galactose-metabolizing capabilities of the rat by evaluation of the variation of the kinetics and age-dependent changes in epimerase activity in rat tissues.

EXPERIMENTAL PROCEDURES

Materials

AMP, ADP, CDP-Glc, NAD^+, GDP-Glc, GDP-Man, TDP-Glc, UMP, UDP-Gal, UDP-Glc, UDP-GlcUA, UDP-Man, UDP-xylose and UDP-Glc dehydrogenase (Type III) were products of Sigma or Calbiochem. UDP-[^{14}C]Gal, uniformly labeled, specific activity 34 mC/mmole and 276 mC/mmole, were products of International Chemical and Nuclear Corp. and New England Nuclear Corp., respectively. UDP-[^{14}C]Glc, uniformly labeled, specific activity 216 mC/mmole, was the product of New England Nuclear Corp., and UDP-[^{14}C]GlcUA, uniformly labeled, specific activity 125 mC/mmole, was the product of International Chemical and Nuclear Corp. [^{14}C]Gal-1-*P*, uniformly labeled, specific activity 22 mC/mmole, and [^{14}C galactose, 32 mC/mmole, were products of International Chemical and Nuclear Corp. Liquiflor was from New England Nuclear Corp. Thin-layer cellulose plates were obtained from Eastman–Kodak Co. Polyethyleneimine was obtained from Chemirad Corp.

Animals

All animals employed were Sprague–Dawley rats and were obtained from either Charles River Breeding Farms or Huntingdon Farms. They were between 35 and 45 days old, unless otherwise indicated. Male animals were employed except where noted. These animals as well as those over 20 days (weanlings) were fed a Purina rat chow diet and water *ad libitum* until killed. Gestational age of pregnant animals was estimated from dates of mating.

Enzyme preparations

The animals were sacrificed by decapitation and exsanguination. In adults, liver was obtained from the right lobe, while in neonates several lobes were employed. Adult gut specimens were obtained 3–4 cm distal to the ligament of Treitz, while in neonates gut specimens were taken distal to the pylorus. Brain tissue was obtained from the frontal cortex. Entire neonatal kidneys were employed, but cortices only were employed with adult animals.

Approx. 200–500 mg of tissue were homogenized with ground glass grinders on ice with 4 vol. of 0.1 M KCl. Adult homogenates were then diluted 1:6 with quartz-distilled water. Neonatal liver homogenates were diluted 1:8 with quartz-distilled water, but other neonatal tissues were diluted 1:6. The tissue preparations were then centrifuged at 30 000 $\times$ *g* for 30 min at 4°. The approximate protein concentrations of 20 μl of the clear supernatants were 60–65 μg for adult liver; 40–45 μg for neonatal

liver; 35 μg for gut; 45 μg for kidney and 33 μg for brain. Proteins were determined by the method of LOWRY *et al.*[8].

Differential centrifugation studies at 30 000 and 100 000 $\times$ *g* were performed with newborn and adult preparations. Assays of activity conducted with the crude preparation and supernatants from the centrifuged fractions confirmed the presence of the enzyme activity in the soluble fraction in both the adult and newborn tissues. Crude uncentrifuged preparations possessed almost no activity in the routine assay procedure, probably reflecting the presence of a potent NADase which inactivates the NAD^+ employed in the assay[9].

Assay procedure

The epimerase assay is based on an anion-exchange, thin-layer chromatographic separation of sugar nucleotides on polyethyleneimine-impregnated cellulose, as described by RANDERATH AND RANDERATH[10]. We employed commercially prepared 20 cm $\times$ 20 cm thin-layer cellulose plates obtained from Eastman–Kodak Co. They were impregnated with 5% polyethyleneimine by ascending flow, allowed to dry and subsequently developed by ascending flow with quartz-distilled water to remove impurities. After drying the plates were wrapped in aluminum foil and stored at -10 to $-20°$. The assay procedure involves the formation of UDP-[^{14}C]Glc during the incubation of the enzyme with UDP-[^{14}C]Gal. Since both of these nucleotide sugars possess the same R_F value (0.34) with the 0.2 M LiCl solvent system employed, it is necessary to convert the UDP-[^{14}C]Glc to UDP-[^{14}C]GlcUA (R_F 0.04) by means of the enzyme UDP-Glc dehydrogenase, thus effecting a separation of the initial reactant from the product.

The routine incubation mixture contained 20 nmoles UDP-[^{14}C]Gal (0.036 μC), 0.2 μmole NAD^+, 40 μl 1 M glycine buffer (pH 9.1), and 20 μl of the enzyme preparation brought to a final volume of 0.2 ml with quartz-distilled water and to a final pH of 8.65. Initiation of the reaction was accomplished by addition of the enzyme preparation, incubation was carried out at 37°, and termination of the reaction was brought about by placing the tube in boiling water for 60 sec. After the tube cooled to room temperature, 0.2 μmole of NAD^+ was added as coenzyme for the UDP-Glc dehydrogenase reaction. This reaction was initiated with the addition of 150 units of UDP-Glc dehydrogenase and was carried out at room temperature for 15 min. At that time a 20-μl aliquot (about 8000 counts/min) was streaked at the origin of the polyethyleneimine-impregnated thin-layer cellulose plates which were scored in the following manner: the origin was denoted lightly with pencil 2.5 cm above the bottom of the sheet. This line was then marked off in 2-cm sections, and the plate was then scored perpendicularly to the origin line with a sharp edge, thereby dividing each one of the 2-cm sections by a clear furrow. After the spots had dried, the cellulose plate was placed in the chromatographic tank containing 0.2 M LiCl at a height of 0.7 to 1.0 cm. Alignment of the plates should be in the same direction as impregnation with polyethyleneimine and washing with water in order not to interfere with the discreteness of the separation of the nucleotides.

The chromatogram was removed from the tank when the solvent front had reached the top of the sheet but was not allowed to over-run. The plate was then dried in a stream of cool air with a hair dryer and was scored with a pencil into four sections of 5, 2, 6 and 3.5 cm each, from the origin. These sections were then counted

separately in a liquid scintillation spectrometer at a 50% efficiency, utilizing a liquifluor–toluene system at a dilution of 1:20.

The UDP-GlcUA in the first section (commencing at the origin) equalled the decrease in the UDP-Gal that migrated to the third section. Demonstration of 97% conversion of authentic UDP-Glc to UDP-GlcUA by UDP-Glc dehydrogenase confirms that the epimerase activity is represented by the indicator enzyme assay. Thus Section 1 was taken as product, and the percentage of counts in Section 1 of the total counts in Sections 1 and 3 was used to determine the nmoles UDP-Glc formed per μg per min. A product correction was applied from the analysis of the complete system which was boiled immediately after the addition of the enzyme.

In reactions employing UDP-Glc as the substrate, the UDP-glucuronic acid found in the first section of the chromatogram represented the unreacted portion and the UDP-Gal was again found in Section 3. In K_m studies where more than 20 μmoles of UDP-Glc was employed, it was necessary to utilize an aliquot of the incubation mixture for the UDP-Glc dehydrogenase reaction, since complete conversion of the relatively larger amounts of UDP-Glc was not otherwise accomplished under the normal assay conditions.

Product identification

This division of the plate was arrived at after fluorescent identification confirmed the presence of authentic UDP-GlcUA in the first section and of UDP-Glc and UDP-Gal in the third. Similarly, scintillation counting of 1-cm sections of the channels on the thin layer substantiated the findings of the fluorescent identification with ultraviolet light, although the labeled material occupied a somewhat larger area than the circumscribed fluorescence region. Accordingly, plates were cut in consonance with the radioactive distribution pattern on the polyethyleneimine cellulose plates. The other two sections (2 and 4) were found to contain a consistent amount of radioactivity regardless of the counts found in Sections 1 and 3.

Purity specifications furnished by the companies supplying the UDP-[^{14}C]Gal indicated 1–2% contamination with [^{14}C]Gal-1-*P* and [^{14}C]galactose. To confirm this, chromatography with ^{14}C-labeled Gal-1-*P* and galactose was performed and demonstrated migration of the former to the second section and the latter to the fourth section. Of greater moment as a source of contamination of the initial material was a variable amount of UDP-[^{14}C]Glc found in the UDP-[^{14}C]Gal. This amount constituted the blank since it was constant either with a zero time control or with a boiled enzyme control. In most lots of UDP-[^{14}C]Gal received, this contamination amounted to approx. 1–1.5% of the total radioactivity and was therefore of no concern. However, in a few instances, the degree of contamination with UDP-Glc was considerably greater, and accordingly that material was not employed.

Boiling did not alter the position of the nucleotides or interfere with their separation. Further, the NAD^+ used in the assay did not cause smearing on the plates. Initial studies were undertaken with Tris buffer in the incubation mixture, but smearing of the labeled material on the chromatagram was observed and hence the use of Tris was discontinued.

Assays of epimerase activity performed by both the radioactive method and the spectrophotometric method[11] gave identical values. However, the sensitivity with

the radioactive assay appears to be greater since one is able to measure levels of activity not readily discernible with the spectrophotometric method.

Enzyme stability

The frozen enzyme preparation is stable for more than one month when maintained at $-45°$. The adult and neonatal enzyme had similar stability characteristics, both possessing marked sensitivity to heat inactivation at 50° for as little as 30 sec and both demonstrating a marked protective influence of the substrate and lesser protective effect of their obligatory cofactor, NAD^+.

Inital velocity

Initial velocity of the adult enzyme (60 μg of liver protein) showed a first-order course for 10 min and the newborn (40–45 μg) for about 8 min. 6 min was therefore chosen as the time period for routine incubations in order to ensure proportionality of conversion against time incubated. Enzyme specific activity is expressed as nmoles product formed per min per μg of soluble protein.

Linearity is maintained by the adult up to 100 μg of liver protein, and by the newborn up to 80 μg. The routine assays were performed with 20 μl of the supernatant preparation of adult and newborn liver which represented 60 and 40 μg of protein, respectively.

RESULTS

pH optimum

Optimal activity was exhibited in the range of pH 8.3–8.65 for both the newborn and the adult. The UDP-Glc dehydrogenase has a pH optimum of 8.7, thus obviating the necessity for an additional quantity of buffer. All pH values were determined with a Corning pH meter.

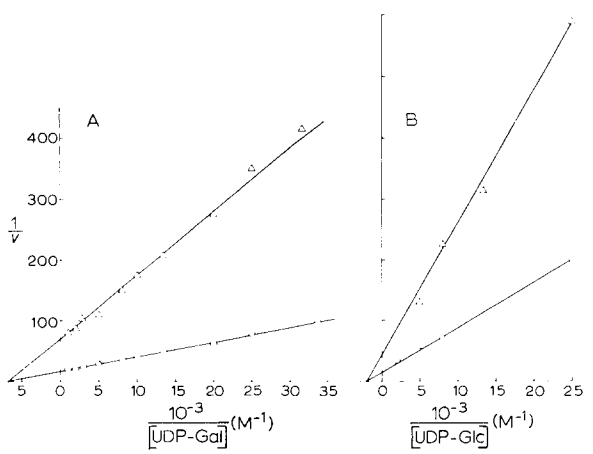

Fig. 1.A. Initial velocity pattern with UDP-Gal as the varied substrate. Initial velocities are expressed as nmoles of UDP-Glc formed per min per μg of newborn (○—○) and adult (△—△) rat liver protein. B. Initial velocity pattern with UDP-Glc as the varied substrate. Initial velocities are expressed as nmoles of UDP-Gal formed per min per μg of newborn (○—○) and adult (△—△) rat liver protein.

Substrate concentration

Fig. 1A shows the Lineweaver–Burk plot of the effect of UDP-Gal concentration on the reaction kinetics of both the adult and newborn enzyme. The K_m for both the adult enzyme and the newborn is 0.153 mM. The newborn enzyme is approx. 5 times more active than the adult, their respective v_{max} values being 0.078 nmole/μg per min and 0.015 nmole/μg per min.

In the reverse direction (Fig. 1B), employing UDP-Glc as the variable substrate, the K_m values for the newborn and adult preparations were both 0.5 mM, and the v_{max} values were 0.072 nmole/μg per min for the newborn and 0.025 nmole/μg per min for the adult.

Nucleotide inhibition

With UDP-Gal as substrate, competitive inhibition was observed with the product, UDP-Glc, as depicted in Fig. 2. AMP, ADP, ATP, CDP-Glc, GDP-Glc and GDP-Man at 1 mM concentration did not exert any inhibitory effect on either the newborn or adult enzyme extracts. Galactose had no inhibitory effect, while UMP at 0.5 mM demonstrated 75% inhibition with the adult and 60% inhibition with the newborn. The latter extent of inhibition was observed when UMP and galactose were added together. UDP-Man at 0.15 mM concentration exhibited 50% inhibition of both the adult and newborn preparations. TDP-Glc at 1 mM concentration exhibited 50% inhibition of the adult and 32% of the newborn. UDP-xylose effect on epimerase

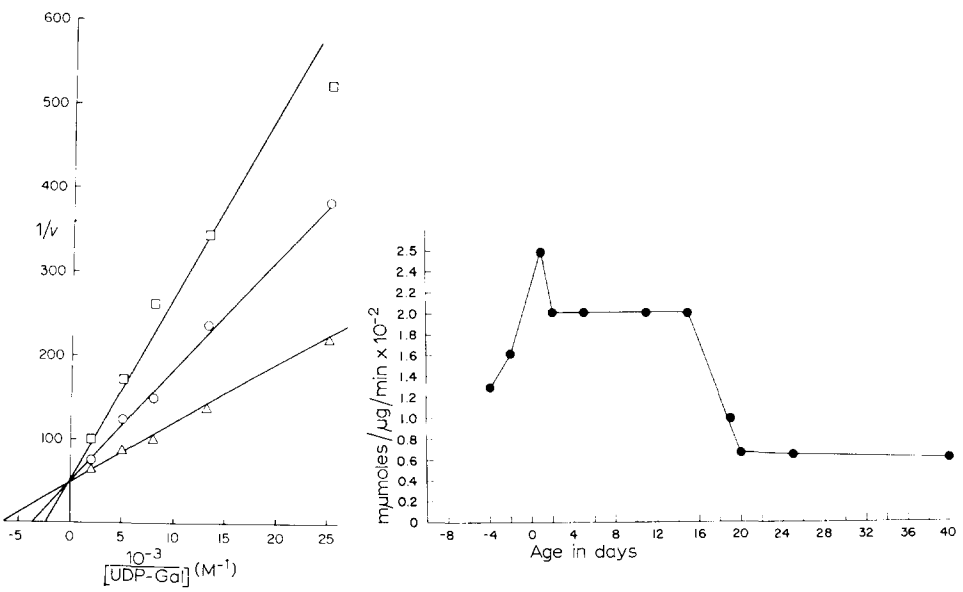

Fig. 2. Reciprocal plots with UDP-Gal as the varied substrate and UDP-Glc as inhibitor. UDP-Glc concentrations: △ △, none present; ○—○, 0.1 mM; □—□, 0.2 mM. Initial velocities are expressed as nmoles UDP-Glc formed per min per μg of adult rat liver protein.

Fig. 3. Developmental changes in UDP-Gal 4-epimerase activity in rat liver. Each point represents an average of 3–5 determinations, except the 1-day-old and the adult rats which are each the average of 10 determinations. Experimental conditions are described in the text. UDP-Glc formation is the ordinate.

could not be assayed with this system since it inhibits completely the UDP-glucose-dehydrogenase-catalyzed reaction[12]. No inhibitory effect on the indicator reaction was exhibited by UMP or UDP-Man.

Effect of inhibitors

Assays performed without added NAD^+ (1 mM) revealed a complete lack of activity in both the adult and newborn preparations. Moreover, studies employing 0.15 mM NADH caused 95% inhibition of normal activity. *p*-Chloromercuribenzoate (PCMB) at 20 μM concentration exerted 50% inhibitory influence on both preparations, but this effect was overcome by cysteine. No such reversal of PCMB inhibition was demonstrable with dithiothreitol. The protective effect on the enzyme of cysteine suggests that sulfhydryl groups are essential for activity, and indeed PCMB causes a loss of catalytic properties when half of the sulfhydryl groups are blocked[13].

TABLE I

UDP-Gal 4-EPIMERASE ACTIVITY IN VARIOUS RAT TISSUES

Except as indicated for liver, all adult and newborn tissues were from male animals. The conditions described for the routine assay were employed for these studies. The values presented are average specific activity ± S.E.

Tissue	*Number of animals*	*Specific activity*
Adult		
Liver		
Male	9	0.0063 ± 0.0002
Female	7	0.0060 ± 0.0001
Gut	5	0.022 ± 0.0015
Kidney	6	0.0018 ± 0.0001
Brain	4	0.0033 ± 0.0002
Newborn		
Liver		
Male	11	0.026 ± 0.0009
Female	7	0.026 ± 0.0009
Gut	4	0.00065 ± 0.00005
Kidney	5	0.0036 ± 0.0005
Brain	4	0.0032 ± 0.0005

Enzyme-activity development in rat tissues

Table I records levels of epimerase activity found in various tissues and Fig. 3 depicts the epimerase activity in developing rat liver. Newborn liver possessed greater activity than fetal or adult liver. The liver from the 1-day-old rats had a peak activity which decreased to a plateau at Day 2, remaining at that level until Day 16 when there was a sharp, rapid decline to the adult levels of activity by Day 20. The newborn gut had negligible activity, while the adult gut activity was quite high; indeed it was the highest of any adult tissue examined. Lower activity was found in kidney and brain preparations from both newborn and adult. Significant differences were not found between male and female liver extracts.

Mixtures of adult enzyme with fetal and with newborn were used to ascertain whether an inhibitor might account for the decreased activity seen with adult prepa-

rations. The data obtained gave results anticipated from the average of the individual specific activities of these preparations, thus making it improbable that inhibitors accounted for the differences in enzyme activity at these different stages of development.

DISCUSSION

The data presented indicate that the newborn liver possesses significantly greater UDP-Gal 4-epimerase activity than the adult organ. Thus this study underscores the greater galactose-metabolizing capabilities of neonatal animals, as previously demonstrated in the studies on galactokinase and transferase reported from this laboratory. The time course of achievement of maximal epimerase activity in the livers differs somewhat from both the galactokinase and the transferase. In the case of the galactokinase, increase in activity commences 4 days pre-partum and continues to rise, reaching its apogee at 5 days (ref. 1). Again in the case of the transferase, increasing activity is seen prior to birth, with a maximum achieved at Day 10 post partum[2]. It is therefore noteworthy that the epimerase activity rises post partum, maintaining a maximum at Day 1, dipping somewhat at Day 2, and thereafter continuing on a plateau until adult levels are achieved by a sharp decline in activity between Days 16 and 20.

Since both the neonatal and adult liver epimerase had the same K_m values in both directions and the same stability and inhibitor characteristics, it is highly probable that the enzyme from both sources is the same. The greater v_{max} value of the newborn may be the result of increased amount of enzyme protein, but such a suggestion must await enzyme purification for confirmation.

Studies with NADH revealed an inverse correlation between activity and levels of NADH in the system. Moreover, both the newborn and adult enzyme evinced significant dependence on NAD^+ for their action. The crude preparation employed contains a NADase and consequently an excess of NAD^+ was employed to ensure persistence of the cofactor. Assays performed employing lower levels of NAD^+ showed decreased activity. In addition, studies involving an initial pre-incubation period with NAD^+ demonstrated a decrease in activity with increasing pre-incubation time, suggesting destruction of the cofactor. Taken together with other published reports of NAD^+ dependence[3,14], these observations strongly support a compulsory role for NAD^+. ROBINSON, KALCKAR AND TROEDSSON[4] and KALCKAR AND ROBINSON[15] have presented data which suggest that glycolysis very likely exerts an important controlling effect on epimerase activity through the production of NADH.

The lack of inhibitory action by CDP-Glc, GDP-Man, ADP-Glc and GDP-Glc, non-pyrimidine nucleotides, in conjunction with the pronounced inhibitory effect of UDP-Man, UMP and TDP-Glc, accentuates the special role of uridine and like nucleotides as a substrate for the epimerase. Such a role has been clarified by recent studies by BODOWSKY *et al.*[16] who employed eight synthetic UDP-Glc analogues possessing modifications in the uracil nucleus and demonstrated the need for an acylamido-group for maximal reactivity. They proposed a secondary structure involving several hexose–uracil hydrogen bonds, upon which they consider specificity to reside. SALO *et al.*[17] have demonstrated a strict specificity of yeast epimerase for the galactose moiety. However, studies with calf epimerase[16] and the present studies

with rat epimerase suggest that sugars other than glucose and galactose possess sufficient specificity to act in an inhibitory manner when combined with the proper nucleotide.

The inhibition of epimerase by UDP-Glc when UDP-Gal is the substrate suggests that when galactose is being metabolized and UDP-Gal formed *in vivo*, there may be regulation of epimerization by UDP-Glc. Intracellular concentrations of UDP-Glc in liver are normally much higher than those by which epimerase activity was inhibited in these studies[18].

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