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DEVELOPMENTAL ALTERATIONS IN HEPATIC UDP-GLUCURONOSYLTRANSFERASE

A COMPARISON OF THE KINETIC PROPERTIES OF ENZYMES FROM ADULT SHEEP AND FETAL LAMBS

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Abstract—The kinetic properties of hepatic microsomal UDP-glucuronosyltransferase were studied in sheep in the perinatal period, using acetaminophen as the aglycone. Kinetic analyses indicated that activity at V_{max} was significantly less in fetal microsomes (113, 135 or 141 days) as compared with the adult sheep. However, these differences between fetal and adult animals were not due simply to smaller amounts of UDP-glucuronosyltransferases catalyzing conjugation of acetaminophen in fetuses versus adults. Thus, the kinetic properties of UDP-glucuronosyltransferase(s) were different in fetus and adult. The "fetal" versus "adult" enzyme had a higher affinity for UDP-glucuronic acid, but a poorer affinity for acetaminophen. Furthermore, enzyme in fetal liver (113 days of gestation) was activated about 30% by the allosteric effector UDP-*N*-acetylglucosamine, whereas enzyme in adult liver was activated by 500%. These differences between fetal and adult enzymes diminished just prior to parturition (141-day fetus). Enzyme in microsomes from the 141-day fetus responded to UDP-*N*-acetylglucosamine-like enzyme in adult microsomes and had affinities for substrates that were similar to "adult" enzymes. These data indicate that maturation of the system that glucuronidates acetaminophen is a complex process. It may involve the expression in fetuses of a type of UDP-glucuronosyltransferase that is different from that expressed in the adult. An alternative but not mutually exclusive possibility is that maturation of the glucuronidation system involves modification of enzyme function by alteration of the phospholipids in the immediate environment of UDP-glucuronosyltransferase within the microsomal membrane.

Glucuronidation is one of the most common and important drug detoxification pathways in mammals [1, 2]. The fetus and neonate, however, appear to have limited capacities for glucuronidating endogenous and exogenous compounds [3, 4], which could be important in determining the toxicities of xenobiotics administered to the fetus and newborn [5]. Perinatal development of the ability to glucuronidate different compounds, therefore, has been studied extensively using hepatic microsomal preparations from fetal and neonatal rodents [4, 6]. There appear to be two distinct patterns of development of UDP-glucuronosyltransferase activities in rat [7, 8]. Changes in rates of glucuronidation of different aglycones, as a function of gestational age, fall into two categories: the so-called late-fetal group of activities and the neonatal group. Rates of glucuronidation of compounds in the late fetal group, as for example 2-aminophenol, reach values just before birth that are greater than corresponding activities in adults. The glucuronidation activity of this group returns to adult levels by a few days after birth. In contrast, compounds in the neonatal group, as for example bilirubin, have negligible rates of glucuronidation (10% of adult levels or less) before birth, and the rate then begins to increase slowly after birth.

An implicit assumption in the studies of perinatal development of UDP-glucuronosyltransferase (EC 2.4.1.17) function is that the observed activities at different gestational ages are measurements of the amounts of enzymes present. Unfortunately, measurements of the activities of hepatic microsomal UDP-glucuronosyltransferase during the perinatal period have been limited to assays at arbitrary concentrations of substrates. Since it has been shown that there are qualitative as well as quantitative changes in the functions of UDP-glucuronosyltransferases during development [9], it is important to characterize developmental changes by measuring the kinetic constants of UDP-glucuronosyltransferases at different stages of fetal development or after birth. Despite numerous investigations of the perinatal development of hepatic microsomal UDP-glucuronosyltransferase activities, the kinetic properties of these enzymes have not been characterized in fetal or neonatal animals. This has been done in the present study, using acetaminophen as the aglycone and hepatic microsomes from adult sheep or fetal lambs as the sources of UDP-glucuronosyltransferase. Acetaminophen was chosen as the aglycone because it is a therapeutic agent that is administered to pregnant women, and in some instances to newborns. The data show that there are complex changes during fetal development in the

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kinetic properties of UDP-glucuronosyltransferase(s) catalyzing the glucuronidation of this substrate and that these changes cannot be explained simply on the basis of variable amounts of enzyme as a function of gestational age.

METHODS

Materials. UDP-glucuronic acid (ammonium salt), acetaminophen, Trizma hydrochloride (pH 7.8), UDP-*N*-acetylglucosamine (sodium salt) and sucrose were purchased from the Sigma Chemical Co. (St. Louis, MO). Authentic acetaminophen glucuronide was supplied by the McNeill Consumer Products Co. (Fort Washington, PA). 3-Acetamidophenol was obtained from the Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were reagent grade except acetonitrile, which was high performance liquid chromatography (HPLC) grade.

Preparation of hepatic microsomes. The procedure applied here was similar to that described previously [10]. Fresh livers were obtained from time-mated adult sheep of mixed breeds and their fetal lambs during the third trimester at 113, 135 and 141 days of gestation. Term in this species is 145 ± 5 days. Portions of the adult liver (80 g) and the whole fetal liver (weighing 80–105 g) were homogenized, respectively, with 3 vol. of a medium of 0.25 M sucrose and 0.25 M sucrose containing 5 mM EDTA. The homogenate was centrifuged at 10,000 *g* for 20 min. The supernatant fraction then was centrifuged at 100,000 *g* for 60 min. The microsomal pellet was resuspended in 0.25 M sucrose and centrifuged again at 100,000 *g* for 60 min. The final microsomal pellet was suspended and homogenized in 20 ml of 0.25 M sucrose. All of the above processes were carried out at 0–4°. A portion of the freshly made microsomal preparation was used immediately to determine its stability at 0–4° on ice by measuring the activities of UDP-glucuronosyltransferase (see below). The remaining microsomal preparation was stored at −80°. The stability of the microsomes under such storage conditions was determined at different periods of time (see below). Microsomal protein was determined by the method of Bradford [11] using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA).

Stability of microsomal preparation. The stabilities of both adult and fetal microsomal preparations were examined after storage at 0–4° (on ice) for 0, 1, 3 and 6 hr after they were prepared freshly. The frozen microsomes from adult and fetal sheep were also examined for their stabilities after storage at −80° for 1, 3, 5 and 7 days and at 2, 3 and 4 weeks up to 3 months. Stability was determined by measuring initial rates of transferase activities in the microsomes at 2 mM UDP-glucuronic acid and 0.25, 0.5, 1.25 and 2.5 mM acetaminophen respectively (see below for details). When the freshly prepared microsomes and the frozen microsomes were compared, the activities of UDP-glucuronosyltransferase in both the adult and fetal microsomal preparations were the same. Nor did the activities change when the fresh microsomes (adult and fetal) were kept on ice for up to 6 hr or when the microsomes were frozen at −80° for periods as long as 3 months.

Determination of UDP-glucuronosyltransferase activity. UDP-glucuronosyltransferase activity with acetaminophen as the aglycone was measured by modifying the method described by Zakim and Vessey [10]. The incubation mixture contained the microsomal fraction (0.5 to 1 mg protein), 50 mM Tris/HCl buffer (pH 7.8), 1 mM $MgCl_2$, various concentrations of UDP-glucuronic acid and acetaminophen as indicated in the figures. Water was added to bring the final volume to 0.5 ml. The mixture was warmed to 37° for 2 min, and the reaction was started by adding UDP-glucuronic acid. Aliquots of 100 μl were taken from the assay mixture at 0, 3, 6 and 10 min. Protein was precipitated with 100 μl of 6% perchloric acid containing 0.1 mM 3-acetamidophenol as the internal standard for HPLC analysis (see below). After centrifugation, the supernatant fraction was neutralized with 30 μl of 2 M K_2HPO_4 and injected onto a reversed-phase column (Altex C-18, 5 μm, 4.6 × 250 mm). The mobile phase was 7% acetonitrile (v/v) in 50 mM phosphate buffer (pH 2.4). The flow rate was 1.4 ml/min. The column effluent was monitored by a u.v. detector set at 254 nm. The retention times of acetaminophen glucuronide, acetaminophen and internal standard were 4.5, 8.7 and 15.2 min respectively. Activities of UDP-glucuronosyltransferase are expressed as micrograms of acetaminophen glucuronide formed per minute per milligram of microsomal protein.

Kinetic analysis and kinetic constants. Kinetic analyses of UDP-glucuronosyltransferase in both the adult and fetal liver microsomes were carried out by determining initial rates of activity as a function of UDP-glucuronic acid concentrations (6, 7.5, 10, 14 and 15 mM) at five different fixed acetaminophen concentrations (0.25, 0.33, 0.5, 0.75 and 1.25 mM). Kinetic constants (V_{max}, K_{UDPGA}, K'_{UDPGA}, K_A, K'_A) for UDP-glucuronosyltransferase were determined for an enzyme with a rapid-equilibrium random-order mechanism [10, 12, 13]. Linear least-squares regression analysis was used to calculate the slopes and intercepts of both the primary and secondary double-reciprocal plots. The notations for the kinetic constants are as described previously [10, 12]. The methods for obtaining these parameters are reported in detail in references [10, 12].

RESULTS

Comparison of activities at V_{max} for adult and fetal sheep. Figure 1 shows the double-reciprocal plots of initial rates of UDP-glucuronosyltransferase activity as a function of acetaminophen concentrations (0.05 to 2.5 mM) with adult or fetal microsomes (135 days) as the source of enzyme. Both double-reciprocal plots were linear over a 50-fold range of acetaminophen concentrations. Double-reciprocal plots of initial rate of UDP-glucuronosyltransferase activity as a function of UDP-glucuronic acid concentration, at a fixed concentration of acetaminophen, were not linear but bent concave downward at UDP-glucuronic acid concentrations greater than 6 mM (Fig. 2). This nonlinearity of double-reciprocal plots, showing a non-Michaelis–Menten type of kinetics, is observed in other animal species such as rat, guinea pig, and beef [10, 14, 15]. To determine

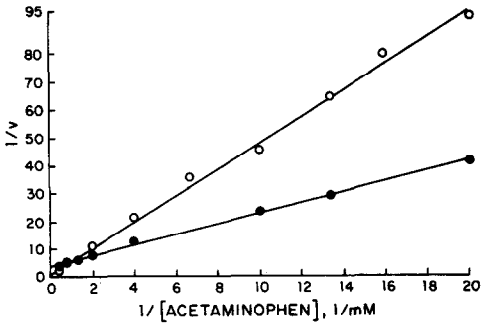

Fig. 1. Linear double-reciprocal plots of initial rates of UDP-glucuronosyltransferase activity as a function of acetaminophen concentration for hepatic microsomes from adult and fetal sheep. Initial rates of activity were determined with 2 mM UDP-glucuronic acid, 1 mM $MgCl_2$, 0.05 to 2.5 mM acetaminophen and 50 mM Tris/HCl, pH 7.8, at 37° for both the adult (●) and fetal (○) microsomes (135 days). Activities are expressed as μg of acetaminophen glucuronide formed per min per mg of protein.

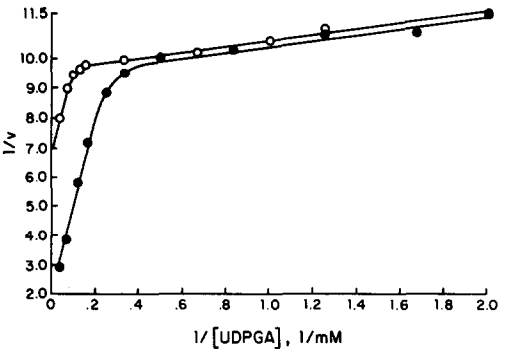

Fig. 2. Nonlinear double-reciprocal plots of initial rates of UDP-glucuronosyltransferase activity as a function of the concentration of UDP-glucuronic acid for hepatic microsomes from adult and fetal sheep. Initial rates of activity were determined at 0.25 and 1.25 mM acetaminophen, respectively, for the adult (●) and fetal (○) microsomes (135 days). Activities are expressed as μg of acetaminophen glucuronide formed per min per mg of protein.

activity at V_{max}, assays were carried out at concentrations of UDP-glucuronic acid above 6 mM. A typical set of double-reciprocal plots for activity as a function of the concentration of UDP-glucuronic acid are shown in Fig. 3A at several different, fixed concentrations of acetaminophen. The slopes and 1/v intercepts of these plots were plotted further as a function of reciprocal concentration of acetaminophen to yield the secondary plots shown in Fig. 3B.

Listed in Table 1 are the kinetic constants for UDP-glucuronosyltransferase in liver microsomes from four adult sheep and three fetal lambs at gestational ages of 113, 135 or 141 days. The values of K_{UDPGA} and $K_{aglycone}$ are for binding of substrates to free enzyme. The K' terms are for binding of substrate to enzyme saturated with the other substrate. It is obvious from Table 1 that the activity at V_{max} of hepatic UDP-glucuronosyltransferase did not change significantly among the three fetuses, whose gestational ages ranged over a 30-day period. Thus, the adult sheep had a much larger capacity for glucuronidation of acetaminophen versus the fetal lambs at any age of gestation. The enzyme in fetal lamb as compared with adult sheep had a higher affinity for UDP-glucuronic acid but a poorer affinity for acetaminophen. These results indicate that the different capabilities of adult and fetal animals for glucuronidation of acetaminophen are not due simply to different amounts of UDP-glucuronosyltransferase(s). This conclusion is emphasized by time-dependent changes in K terms as a function of gestational age, enzyme in fetus becoming more like adult enzyme in the absence of changes in activity at V_{max}. One final point of interest in Table 1 is that the difference between capabilities for glucuronidation by fetal and adult animals will depend on conditions used for assaying activity. Thus, determination of activity at V_{max}, by bisubstrate kinetic analysis, is the only accurate method for describing the difference in catalytic capacity between adult and fetal animals.

Comparison of allosteric activation of UDP-glucuronosyltransferase by UDP-N-acetylglucosamine in adult and fetal sheep. UDP-*N*-acetylglucosamine is

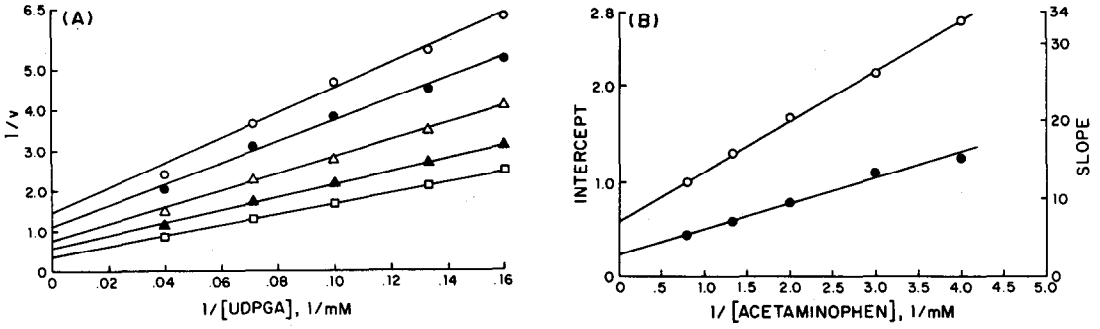

Fig. 3. Bisubstrate enzyme kinetic analyses of hepatic microsomal UDP-glucuronosyltransferase. (A) Primary plot: double-reciprocal plots of initial rates of transferase activity as a function of the concentration of UDP-glucuronic acid at 0.25 (○), 0.33 (●), 0.5 (△), 0.75 (▲) and 1.25 (□) mM acetaminophen. Activities are expressed as μg of acetaminophen glucuronide formed/min/mg protein. (B) Secondary plot: plots of 1/v intercept (●) and slopes (○) obtained from the primary plot as a function of reciprocal acetaminophen concentration. The units of intercept are 1/μg/min/mg protein and the units of slope are mM/μg/min/mg protein.

Table 1. Kinetic constants for UDP-glucuronosyltransferase in hepatic microsomes from adult sheep and fetal lambs at different gestational ages

	Gestational age (days)	V_{max} (nmoles/min/mg)	K_{UDPGA} (mM)	K'_{UDPGA} (mM)	K_A (mM)	K'_A (mM)
Adult		10.74 ± 1.07	21.52 ± 2.87	24.08 ± 3.77	1.03 ± 0.38	1.14 ± 0.40
Fetus	141	1.81	10.70	15.55	1.17	2.49
Fetus	135	1.92	6.63	3.61	4.13	2.25
Fetus	113	2.07	3.47	2.05	7.89	4.67

Kinetic constants were determined by bisubstrate enzyme kinetic analyses as described in Methods and the legend of Fig. 3. V_{max} is expressed as nmoles of acetaminophen glucuronide formed/min/mg protein. Values for the adult sheep are mean ± S.D. obtained from four adult liver microsomes. Term in this species is 145 ± 5 days.

an allosteric effector of UDP-glucuronosyltransferase [15, 16]. Prior work with rat liver microsomes has shown, however, that enzyme in fetal and neonatal rats has only limited sensitivity to activation by UDP-*N*-acetylglucosamine [9]. The data in Fig. 4A show that this nucleotide sugar activated enzyme in liver microsomes from adult sheep and that the extent of activation depended on the concentration of UDP-glucuronic acid, i.e. activation was greatest at the lowest concentrations of UDP-glucuronic acid. By contrast, UDP-*N*-acetylglucosamine was a much less effective activator of fetal enzyme at 113 days of gestation (Fig. 4B). Whereas activity was enhanced by as much as 5-fold in adults, UDP-*N*-acetylglucosamine activated fetal enzyme only by 30%. The data in Fig. 4 are for assays containing 1.6 mM UDP-*N*-acetylglucosamine. To exclude that the difference between adult and fetal enzymes in Fig. 4 was not due to differences in binding of UDP-*N*-acetylglucosamine by adult and fetal UDP-glucuronosyltransferases, activation was studied as a function of the concentration of UDP-*N*-acetylglucosamine. The data were normalized by plotting percent of maximum activation as a function of the concentration of UDP-*N*-acetylglucosamine. Data in Fig. 5 show that adult and fetal UDP-glucuronosyltransferases have essentially identical affinity for UDP-*N*-acetylglucosamine. Therefore, the response of these enzymes to the binding of this sugar nucleotide is what accounts for the differences depicted in Figs 4A and 4B.

Shown in Fig. 6 are the effects of UDP-*N*-acetylglucosamine on the activity of UDP-glucuronosyltransferase in fetal sheep just prior to parturition (141 days of gestation). It is seen that the fetal enzyme by this stage of development has become "adult-like" in regard to activation by UDP-*N*-acetylglucosamine.

DISCUSSION

The number of enzymes in intact liver catalyzing the glucuronidation of any aglycone remains to be established. It is not possible to state with certainty, therefore, the number of enzymes involved in the glucuronidation of acetaminophen. It is for this precise reason, however, that many aspects of the broad problem of detoxification via formation of glucuronides must be studied in intact microsomes rather than with single species of pure enzymes and by using the aglycone of immediate interest versus substrates of biochemical interest only.

It is well-known that the capacity of liver microsomes for glucuronidation of xenobiotic molecules is different in fetal as compared with adult animals [3–5]. It also is known that the development of glucuronidating activities in fetal and neonatal animals is complex [4–6]. Activity, depending on the substrate used, can be higher or lower in fetal versus adult animals. Moreover, depending on the substrate

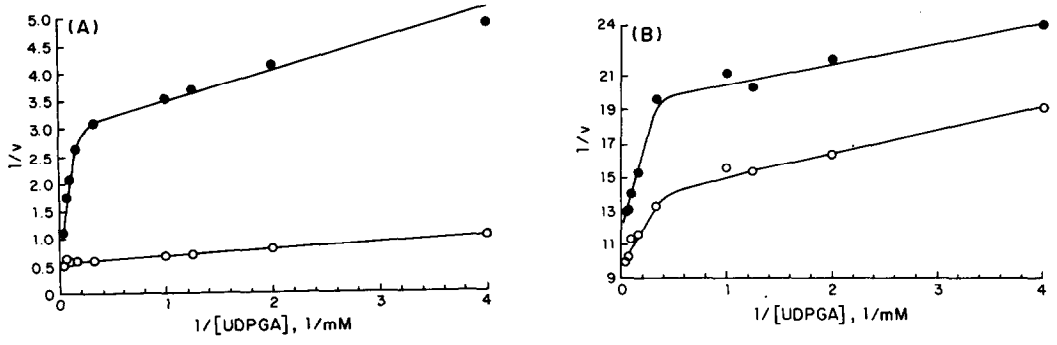

Fig. 4. Effect of UDP-*N*-acetylglucosamine on UDP-glucuronosyltransferase activity for hepatic microsomes from adult and fetal sheep. (A) Microsomal fraction from the liver of an adult sheep. (B) Microsomal fraction from the liver of a fetal lamb at 113 days of gestation. Initial rates of transferase activity were determined at 0.8 mM acetaminophen, 1 mM $MgCl_2$ in the absence (●) or presence (○) of 1.6 mM UDP-*N*-acetylglucosamine. The units of activity are μg of acetaminophen glucuronide formed/min/mg protein.

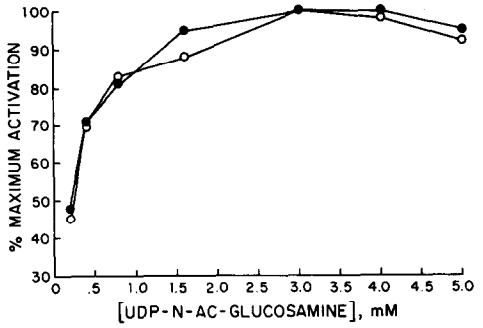

Fig. 5. Normalized extent of activation of hepatic microsomal UDP-glucuronosyltransferase by UDP-*N*-acetylglucosamine as a function of the concentration of UDP-*N*-acetylglucosamine for adult and fetal sheep. Initial rates of activity were measured at 1 mM $MgCl_2$, 0.8 mM acetaminophen, 0.8 mM UDP-glucuronic acid and 0–5 mM UDP-*N*-acetylglucosamine for microsomal fractions from the liver of an adult sheep (●) and the liver of a fetal lamb (○) at 113 days of gestation. The percent of maximum activation was calculated as:

$$\frac{(\text{activity with}) - (\text{activity without})}{(\text{maximum activity with}) - (\text{activity without})} \times 100\%$$

studied, activity may appear during fetal life; or appearance of activity may be delayed until after birth. The basis for this variation of activity as a function of gestational age is uncertain. It has been assumed tacitly, however, that differences in capacity to glucuronidate various compounds in animals of different fetal and perinatal ages reflect different levels of enzyme protein. The data presented above demonstrate that development of UDP-glucuronosyltransferases during fetal life is far more complex than has been appreciated because several kinetic properties of the UDP-glucuronosyltransferase(s) that catalyze(s) the glucuronidation of acetaminophen change prior to parturition, including affinity for substrates and responsiveness to allosteric effectors. It seems that there is a fetal type of UDP-glucuronosyltransferase in sheep liver and a distinct adult type. The fetal form as compared with the adult form is relatively unresponsive to activation by UDP-*N*-acetylglucosamine, has higher affinity for UDP-glucuronic acid, and has less affinity for acetaminophen. Based on the data in Table 1 and Fig. 5, it appears as if the fetal enzyme in sheep disappeared from the liver just prior to parturition and was replaced by the adult form. Maximum capacity for glucuronidation of acetaminophen, that is full expression of the adult form, occurs, however, only after birth.

It is known that phospholipid–protein interactions are important for regulating the activity and allosteric properties of UDP-glucuronosyltransferase [14, 17–20]. In fact, it can be shown with pure delipidated enzyme that affinity for substrates, activity at V_{max}, and responsiveness to activation by UDP-*N*-acetylglucosamine can be determined by the phospholipids used to reconstitute the function of delipidated enzyme [21–23]. It is possible, therefore, that maturation of the glucuronidation system involves modification of enzyme function by alteration of the phospholipids within the microsomal membrane. Of interest and perhaps of importance in this regard are observations of changes in the composition and content of hepatic microsomal lipids and in the membrane viscosity during the perinatal development of rat [24]. Whether this is in part the basis for the developmental pattern of the UDP-glucuronosyltransferase studied above or whether the developmental changes depend only on changes in the expression of different proteins will depend obviously on the study of pure enzymes from fetal and adult tissue. We believe that the present work in sheep, and similar but less extensive studies reported already for rat [9], emphasize the need for such studies.

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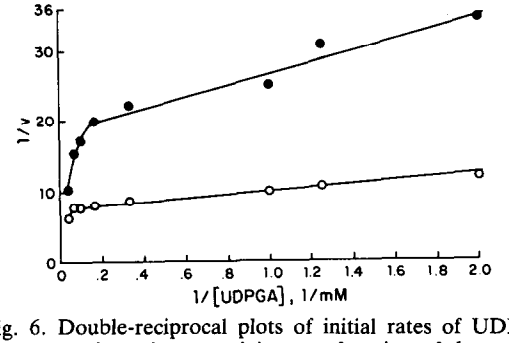

Fig. 6. Double-reciprocal plots of initial rates of UDP-glucuronosyltransferase activity as a function of the concentration of UDP-glucuronic acid in the absence or presence of UDP-*N*-acetylglucosamine for hepatic microsomes from one fetal lamb at 141 days of gestation. Initial rates of activity were measured at 0.8 mM acetaminophen, 1 mM $MgCl_2$ in the absence (●) or presence (○) of 1.6 mM UDP-*N*-acetylglucosamine. The units of activity are μg of acetaminophen glucuronide formed/min/mg protein.

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