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HEPATIC OXIDATIVE DRUG METABOLISM AND THE MICROSOMAL MILIEU IN A RAT MODEL OF CONGENITAL HYPERBILIRUBINEMIA*

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Abstract—The aims of this study were to evaluate the hypothesis that impaired glucuronidation of bilirubin and possibly of drug oxidation in the liver of homozygous (jj) Gunn rats may be due to an altered microsomal milieu. Accordingly, we investigated and compared *in vivo* and *in vitro* demethylation of aminopyrine, hepatic cytochrome P-450 levels, microsomal lipid composition, and microsomal membrane fluidity in icteric, homozygous (jj) Gunn rats and in their anicteric heterozygous (jJ) littermates. In both males and females, [^{14}C]aminopyrine demethylation *in vivo*, using the $^{14}CO_2$ breath test, was unimpaired in the icteric animals. Likewise, cytochrome P-450 levels in the icteric and nonicteric groups were similar, and aminopyrine kinetics *in vitro* in the females were comparable in icteric and nonicteric littermates. The main lipid classes were also similar in the homozygous and heterozygous female Gunn rats, whereas only minor changes were seen in the phospholipid fatty acyl composition with a small, but significant, increase in the unsaturated index in the icteric group. Despite this, there was no apparent effect on hepatic microsomal membrane fluidity as measured by the order parameter of I[12,3] and the rotational correlation time of I[1,14] in either female or male sets of homozygous and heterozygous Gunn rats. Our data, therefore, do not support an alteration of composition or fluidity of the microsomal milieu as a mechanism of impaired bilirubin glucuronidation and possibly of oxidation in these animals. They also absolve long-term unconjugated hyperbilirubinemia as a mechanism of hepatic microsomal dysfunction. Our study, therefore, indirectly suggests that abnormal glucuronidation of bilirubin and some other aglycones in homozygous Gunn rats is due to genetic abnormalities involving the enzyme(s) itself.

Gunn rats, mutants of the Wistar strain, in their homozygous phenotype (jj) lack hepatic UDP glucuronosyltransferase (EC 2.4.1.17) activity necessary to conjugate bilirubin [1]. These animals develop unconjugated hyperbilirubinemia and jaundice shortly after birth and usually exhibit bilirubin-induced central nervous system damage [2]. Their heterozygous counterparts (jJ) show a significant decrease in hepatic bilirubin conjugation (as compared to JJ controls), but are able to metabolize the pigment sufficiently so as to avoid jaundice and cerebral toxicity [1, 3, 4]. The homozygous, jaundiced Gunn rat serves as an animal model for the Crigler–Najjar type I human disorder which is also characterized by congenital inability to conjugate bilirubin, by deep jaundice, and by kernicterus [5, 6].

The UDP bilirubin glucuronosyltransferase deficiency in homozygous Gunn rats and Crigler–Najjar type I patients has been attributed to either a genetically determined deficiency in formation of the specific transferase enzyme or a genetically-induced alteration of the microsomal milieu containing the enzyme [7–9]. In support of the latter hypothesis are the reports that (1) changes in the lipid/protein microsomal micromilieu are known to alter the activity of glucuronosyltransferases [10–13], (2) decreased UDP glucuronosyltransferase activity for various aglycones in Gunn rat liver may be restored toward normal by various chemicals [14, 15], (3) drug oxidation (i.e. aminopyrine demethylation) and glucuronidation of some other substrates may also be impaired in microsomes of homozygous Gunn rats implying a more general metabolic defect than just for bilirubin [1, 3, 4, 9], and (4) microsomal membrane lipid composition and membrane fluidity (order parameter) may be altered in homozygous Gunn rats [16]. It has thus been suggested that physico-chemical changes in the microsomal membrane in homozygous Gunn rats may alter the conformation and catalytic properties of glucuronosyltransferase(s), and possibly other enzymes embedded therein [16]. In other mechanistic studies, impaired aminopyrine demethylation was not reproduced *in vitro* with short-term exposure to unconjugated bilirubin [9], but the conjugation of various aglycones

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could be suppressed by brief exposure to unconjugated bilirubin *in vitro* [17, 18] or in rat liver perfusions [19]. Studies of the effect of long-term unconjugated hyperbilirubinemia in Gunn rats on microsomal drug oxidation *in vivo* and *in vitro* do not appear to have been done. Hence, the possible effects of prolonged hyperbilirubinemia on microsomal structure and function in homozygous Gunn rats could not be dissociated from a primary "defect" in microsomal matrix [16].

The principal aim of this study was to investigate the postulated structural–functional abnormality of hepatic microsomes in homozygous Gunn rats by comparing their oxidative metabolic activity *in vitro* and *in vivo*, their lipid composition, and their fluidity with results obtained in their non-jaundiced heterozygous littermates. A corollary goal was to determine the effect of prolonged hyperbilirubinemia on microsomal function. A comparison of data in icteric and nonicteric littermates would also accomplish this. Such a composite experimental approach, apparently not adopted previously, would also serve to decrease any other within-specie variation that may confound the interpretation of the data. The results of these studies are the substance of this paper.

MATERIALS AND METHODS

Animals

Homozygous (jj) and heterozygous (jJ) female and male Gunn rats were obtained initially from the Skin and Cancer Hospital, Department of Dermatology, Philadelphia, PA, and later from the same stock at Northwestern University School of Medicine, Chicago, IL, through the courtesy of Dr. D. Ostrow. Homozygous Gunn rats were easily identified as icteric offspring of jj and jJ matings. Many of these animals showed some evidence of neurologic damage (i.e. unsteady gait), and all exhibited jaundice which was confirmed by elevated serum unconjugated bilirubin and bilirubin-stained tissues at sacrifice. Heterozygotes were nonjaundiced offspring of jj and jJ matings. They showed no evidence of kernicterus, tissues were not bilirubin-stained at sacrifice, and serum bilirubins were not elevated in a subset of these animals. Animals used were 130–180 g in weight; they were acclimated for 3 weeks to local conditions and fed *ad lib.* the Wayne Lab Blox rat diet which was previously autoclaved. Homozygous and heterozygous littermates were compared for any given study. Most of the data were obtained in female rats, as in males aminopyrine metabolism is under androgenic hormonal control. However, hepatic cytochrome P-450 concentration, the aminopyrine breath test, and fluidity data were also obtained in male rats.

Chemicals

[*Dimethyl*-^{14}C]Aminopyrine (sp. act. 99.5 mCi/mmole) was purchased from New England Nuclear, Boston, MA. The radiochemical purity was 98% determined by thin-layer and paper chromatography. Ethanolamine, methanol and aminopyrine were purchased from Fischer Scientific. Solvents used for analytical studies were HPLC grade and other reagents, analytical grade. Spin labels, 2-(3-carboxypropyl)-4,4-dimethy-12-tridecy-13-oxazolidinyloxyl (I[12,3]), a near aqueous-lipid interface hydrophobic probe, and 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethy-13-oxazolidinyloxyl (I[1,14]), a deep membrane interior hydrophobic probe, were purchased from the Syva Co., Palo Alto, CA. Aqueous Counting Solution (ACS) was purchased from Amersham, Arlington Heights, IL.

Aminopyrine breath test (ABT)

[*Dimethyl*-^{14}C]Aminopyrine (0.89 μCi/200 g body wt) was administered by intraperitoneal (i.p.) injection in 0.5 ml saline, and the animals were placed in separate glass containers. Air was drawn through the chamber by vacuum and water was extracted from the expired air by bubbling through concentrated sulfuric acid. Expired CO_2 was trapped in a scintillation vial containing 10 ml of a 2:1 (v/v) methanol–ethanolamine mixture. Ten consecutive samples of 15-min duration were collected starting immediately after the i.p. injection. Trapped $^{14}CO_2$ was determined by scintillation spectrometry, after addition of 10 ml ACS. An automatic external standardization procedure was used to determine quenching. The rate of elimination of expired $^{14}CO_2$ was calculated as the cumulative percent (% dpm) of the injected dose.

Aminopyrine N-demethylase activity in vitro

Animals were anesthetized with ether and exsanguinated by aortic puncture. The liver was perfused *in situ* with ice-cold 0.9% saline via the portal and hepatic veins, dissected free, and then homogenized in 5 vol. (vol./g) of 0.15 M KCl, using a Potter–Elvehjem homogenizer and Teflon pestle. The homogenate was centrifuged at 8,000 *g* for 15 min (4°), and the subsequent supernatant fraction was centrifuged at 18,000 *g* for 15 min (4°). The microsomal pellet was then isolated by ultracentrifugation at 105,000 *g* (4°) for 60 min. The pellet was resuspended in 0.15 M KCl using a Polytron blender to a protein concentration of 1–2 mg/ml. Protein concentrations were estimated by the method of Lowry *et al.* [20], using bovine serum albumin as standard.

Aminopyrine *N*-demethylase (APND) activity was determined according to the method of Litterst *et al.* [21]. The reaction mixture contained aminopyrine (0.67 to 25.0 mM), an NADPH-generating system (5 mM $MgCl_2$, 10 mM glucose-6-phosphate, 2 I.U. glucose-6-phosphate dehydrogenase and 1 mM $NADP^+$) and the microsomal suspension (500 μl), to a final volume of 3 ml in 100 mM Tris–HCl buffer (pH 7.4). This was incubated at 37° for 15 min, and the reaction was stopped with 1 ml each of $ZnSO_4$ (20%) and saturated $Ba(OH)_2$. The reaction was linear with time in our assay. The concentration of formaldehyde was determined by the method of Nash [22] as modified by Cochin and Axelrod [23]. Kinetic parameters, V_{max} and K_m, were determined from the Michaelis–Menten equation using a modified iterative nonlinear regression computer program with proportional standard deviation weighting [24].

Cytochrome P-450 estimation

A portion of the microsomal pellet prepared for

each of the APND *in vitro* studies was resuspended in 0.1 M phosphate buffer (pH 7.4). The concentration of cytochrome P-450 was determined by difference spectroscopy according to the method of Omura and Sato [25] using a Cary model 219 spectrophotometer and an extinction coefficient of 91 $mM^{-1} cm^{-1}$.

Microsomal membrane fluidity

Homozygous and heterozygous Gunn rats were killed by decapitation without anesthetic, and liver microsomes were prepared as described above. The microsomal pellets were resuspended by hand using a glass/Teflon homogenizer in 0.05 M Tris–HCl buffer (pH 7.4) to a protein concentration of 30–40 mg/ml and then stored at −70°. The samples were thawed on the study day, gently vortexed, and the suspension added to light-shielded tubes onto which aliquots of the spin label, I[12,3] or I[1,14], had been evaporated. Preliminary studies established unequivocally that freezing at −70° did not alter microsomal membrane fluidity. These tubes were vortexed, incubated in an agitating water bath at 37° for 15–30 min, and then stored on ice before use. The spin label to microsomal protein concentration range (μg/mg) was 0.5 to 3.5 for I[12,3] and 0.1 to 1.2 for I[1,14].

Electron spin resonance (ESR) spectra were obtained at 20 and 37° using a Varian E-109 or E-4 X-band spectrometer (Varian Associates, Palo Alto, CA) equipped with an E-238 (flat cell) cavity. Sample temperature was maintained to ±1° by a Varian E-257 variable temperature accessory or equivalent apparatus. The microwave power was 10 mW; the modulation amplitude, 1 gauss; the time constant, 0.3 sec; and the scan time, 8 min. Multiple scans of the less intense spectral features and expansion of narrow width features were made to increase accuracy.

The order parameter, S, was calculated for I[12, 3] as described by Gordon *et al.* [26], with the addition of 1.6 G to $2A_{\perp}$, as formulated by Hubbel and McConnell [27]. The rotational correlation time, τ, was calculated using the formula least sensitive to power saturation with the amplitude ratios of both the middle and high field resonances [28].

Microsomal lipid analysis

The lipid composition of the hepatic microsomes was determined in JJ, jJ and jj *female* rats. Cholesterol was measured by the method of Bobson *et al.* [29] and phospholipids by the method of Bartlett [30]. Fatty acid composition of the phospholipids was determined by gas–liquid chromatography using a Varian 2100 chromatograph. Six-foot glass columns were packed with 10% SP-2340 on 100/120 mesh Supelcopost (Supelco, Inc.). Carrier gas was nitrogen (60 ml/min) with column temperature programmed from 175 to 225° (6°/min). Fatty acids were detected by ionization level upon oxidation in a hydrogen flame. Peak areas were calculated by a Varian integrator (model 485).

Bilirubin estimation

Blood samples were collected into chilled heparinized glass tubes and centrifuged at 4°. Plasma was stored at −20°. Total (unconjugated + conjugated) and direct (conjugated) bilirubin concentrations were determined using a commercially available kit (Sigma Chemical Co., St. Louis, MO; Bulletin No. 605) and a Gilford 2400 spectrophotometer. The bilirubin concentration was calculated using a molar extinction coefficient of alkaline azobilirubin of 73,000 $M^{-1} cm^{-1}$ at 600 nm.

Statistical analysis

Values are expressed as either the mean ± standard error of the mean (S.E.M.) or as the mean ± the standard deviation (S.D.). Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) [31]. Multivariate analysis of repeated measure design was used to compare statistically the Michaelis–Menten kinetic curves. Student's t-test for unpaired data was used to analyze the difference in the means. A $P < 0.05$ (two-tailed) was accepted as significant.

RESULTS

Bilirubin estimations

The concentration of unconjugated bilirubin was low or at the limits of detectability in the heterozygous, anicteric, female and male rats, but was increased markedly in the homozygous, icteric (jj) phenotype. The values were 10.82 ± 0.92 mg/dl (mean ± S.E.) in the female homozygotes and 9.99 ± 2.20 mg/dl in the male homozygotes (Table 1). The concentration of conjugated bilirubin was low or essentially unreadable in all groups (Table 1).

Aminopyrine metabolism

Breath tests. The rate of elimination of [^{14}C]aminopyrine, expressed as the cumulative percent of injected dose exhaled as $^{14}CO_2$ over 150 min following drug administration, for male and female homozygous and heterozygous Gunn rats is shown in Fig. 1, A and B respectively. The elimination rates in male homozygous and heterozygous Gunn rats were similar (25.50 ± 2.20 and 25.50 ± 6.50% respectively, $P > 0.05$). The elimination rate over 150 min in the homozygous (jj) females was actually slightly *greater* than in the female heterozygotes (20.19 ± 0.81% for jj vs 18.12 ± 0.52% for jJ rats, $P = 0.038$). As shown previously, aminopyrine metabolism was more rapid in the male rats, although the female and male groups are not strictly comparable since the studies were done at different times and with different lots of aminopyrine.

Aminopyrine N-demethylase activity in vitro. As shown in Table 1, the liver/body weight ratios in the heterozygous and homozygous females and males were comparable for each sex. Likewise, the total cytochrome P-450 concentration did not differ in the icteric and nonicteric females and males. In the females, wherein kinetic studies were done, the V_{max} for aminopyrine expressed either as activity of the enzyme per nmole cytochrome P-450 or per mg microsomal protein was slightly lower in the icteric group as compared to the heterozygotes, but this was not statistically significant ($P > 0.05$). Likewise, the K_m values for aminopyrine in the two groups of female Gunn rats were similar (Table 1).

Table 1. Cytochrome P-450 concentration and aminopyrine *N*-demethylase activity in Gunn rat microsomes

	Gunn rats	
	Icteric (jj)	Anicteric (jJ)
Liver/100 g body weight		
Male	4.13 ± 0.17 (4)*	3.83 ± 0.09 (4)
Female	4.70 ± 0.32 (6)	4.71 ± 0.23 (9)
Serum bilirubin (mg/dl)		
Male—Direct	0.27 ± 0.10 (6)	0.01 ± 0.001 (4)
—Indirect	9.99 ± 2.20†	0.25 ± 0.097
Female—Direct	0.09 ± 0.02 (14)	0.02 ± 0.002 (10)
—Indirect	10.82 ± 0.92†	0.02 ± 0.013
Cytochrome P-450 (nmoles/mg protein)		
Male	0.930 ± 0.14 (4)	0.890 ± 0.08 (4)
Female	0.630 ± 0.07 (6)	0.657 ± 0.05 (6)
Aminopyrine kinetics		
V_{max} (nmoles formaldehyde/hr/nmole P-450)		
Female	341.3 ± 54.2 (7)	415.7 ± 25.9 (9)
V_{max} (nmoles formaldehyde/hr/mg protein)		
Female	238.8 ± 31.5 (7)	313.2 ± 36.5 (9)
K_m (mM)		
Female	2.55 ± 0.96 (7)	2.30 ± 0.58 (9)

* Number of animals per group, mean ± S.E.M.
† $P < 0.05$ compared to jJ rats.

Hepatic microsomal studies

Lipid composition. In the female homozygous and heterozygous rats, the cholesterol/protein, phospholipid/protein and cholesterol/phospholipid ratios were similar ($P > 0.05$) (Table 2). The various classes of phospholipids were not studied but the fatty acyl distribution of the phospholipids was examined (Table 3). There was a small decrease in 18:1 and 18:2 (linoleic acid) and slight increases in 18:1 (oleic acid) and fatty acids with more than twenty acyl groups in the icteric animals ($P < 0.05$), but these changes were minor. The percentage of saturated fatty acids was similar in both groups, but the unsaturated index, determined by multiplying the number of double bonds by the fractional distribution of the fatty acids, was increased significantly in the jj group ($P < 0.05$).

Microsomal membrane fluidity. As shown in Table 4, the order parameter, *S*, determined using the surface probe (I[12,3]), was not significantly different in the icteric and nonicteric groups, either in females or males when measured at 20 and 37°. Likewise, the rotational correlation time coefficient, τ, which is calculated using the deep probe (I[1,14]), was similar in the homozygous and heterozygous animals of each sex. In other studies, not shown, in normal (JJ) male and female Wister rats, hepatic microsomal fluidity measurements (*S* and τ) at both 25 and 37° were comparable to values obtained for each sex in these anicteric heterozygotes. These JJ data are not shown as they were not obtained concurrently with the heterozygote results.

DISCUSSION

The physico-chemical environment of glucuronosyltransferases for various substrates may influence the activity of these enzymes [10–13]. This has been attributed primarily to an alteration in the lipid/protein composition and interaction within microsomes. Accordingly, the genetically determined impairment of bilirubin glucuronidation in homozygous Gunn rats, and possibly in the Crigler–Najjar type I hyperbilirubinemia of children, may be due to such an abnormality in the micromilieu of this enzyme [9, 16]. Furthermore, since the components of the cytochrome P-450 oxidizing systems are likewise embedded within the microsomal membrane, such oxidative processes may also be inhibited in the hepatic microsomes of homozygous Gunn rats [8, 9]. In addition to a probable genetically-mediated alteration in the composition of the microsomes, the possibility of an effect of prolonged unconjugated hyperbilirubinemia on microsomal function was raised [16].

The principal aims of this study were to test these hypotheses. First, we assessed both *in vivo* and *in vitro* the metabolism of aminopyrine, a probe drug demethylated by the cytochrome P-450 system primarily in liver microsomes. This drug was chosen since (1) it was used previously, albeit in only limited *in vitro* studies of male Gunn rats, to advance the altered micromilieu hypothesis, (2) it is a commonly used probe to examine hepatic oxidative metabolism, and (3) it is available as a substance labeled in the methyl groups for $^{14}CO_2$ breath analysis of drug demethylation *in vivo*. Our initial studies were carried out in male rats, since *in vitro* impairment of aminopyrine metabolism has been described previously for this group [9]. In comparing labeled aminopyrine demethylation by breath test analysis, however, we found no differences in the icteric homozygous (jj) male Gunn rats as compared to nonicteric heterozygous (jJ) littermates (Fig. 1A). Since aminopyrine metabolism is enhanced by male hormones, we studied in greater detail comparable

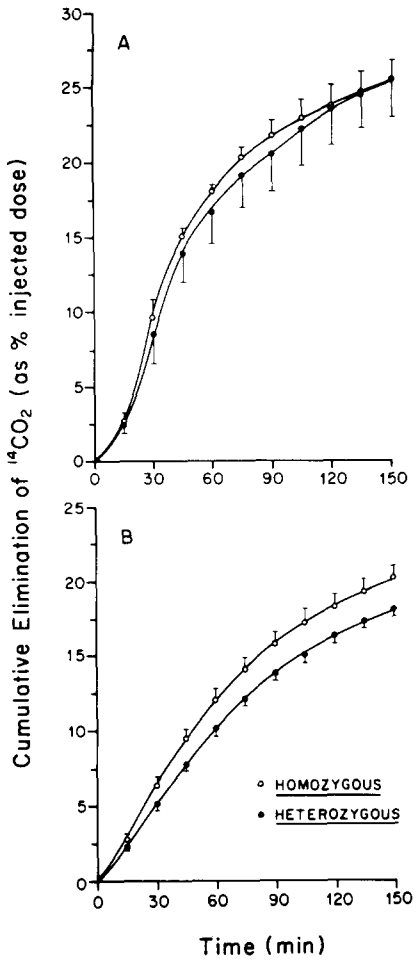

Fig. 1. Aminopyrine breath test. In *male* rats (A) the elimination of $^{14}CO_2$ was comparable in icteric jj rats (N = 3) and the anicteric (jJ) heterozygotes (N = 7). The cumulative elimination of $^{14}CO_2$ (mean ± S.E.M.) was slightly but significantly ($P < 0.05$) increased in *female* homozygous (jj) icteric Gunn rats (N = 12) compared to heterozygous (jJ) anicteric (N = 13) Gunn rats (B).

groups of female Gunn rats. Again, there was no evidence of decreased aminopyrine demethylation by the breath test in the icteric homozygotes as compared to the heterozygotes (Fig. 1B). In fact, the icteric animals had a slightly enhanced aminopyrine demethylation. In addition, the hepatic cytochrome P-450 levels were also comparable for each sex in the icteric and nonicteric groups (Table 1).

Since the aminopyrine breath test may reflect not only aminopyrine metabolism but also its distribution [32], we next assessed aminopyrine kinetics *in vitro* in liver microsomes in the female Gunn rat groups. As is evident in Table 1, the aminopyrine kinetics were comparable in the icteric and nonicteric groups. Thus, contrary to prior more limited *in vitro* studies in male homozygous Gunn rats and normal Wistar rats at only one aminopyrine concentration [9], we found no alteration in aminopyrine metabolism in the female homozygous Gunn rats as compared to their nonicteric littermates. Since termination of these studies, similar conclusions have been reported in liver of male homozygous Gunn rats and normal Wistar rats [33]. In such livers perfused with sodium carbonate or bilirubin for 60 min, cytochrome P-450 levels, aminopyrine *N*-demethylase activity and aniline hydroxylase activity were similar in jaundiced Gunn rats and normal Wistar rats, and the even higher liver bilirubin, after its infusion, did not alter these measurements [33]. Interestingly, changes in liver bilirubin with dietary and chemical manipulations also did not influence hepatic glucuronidation of 4-nitrophenol [33]. Also, very recently no difference in the activity of glucose-6-phosphatase at various temperatures was found in the microsomes of homozygous icteric Gunn rats and control Wistar rats [34], suggesting similarity in the microsomal lipid domain in these two groups.

Microsomal composition was assessed next. The main lipid classes were similar in the homozygous and heterozygous female Gunn rats (Table 2),

Table 3. Fatty acid distribution of microsomal membrane phospholipids obtained from female homozygous (icteric) and heterozygous (anicteric) Gunn rats

Fatty acid	Gunn rats Icteric (jj)	Anicteric (jJ)
Number per group	7	7
Pre-16:0	0	0
16:0	17.87 ± 0.36*	17.54 ± 0.88*
16:1	0.31 ± 0.12	0.33 ± 0.10
18:0	35.19 ± 1.03†	38.64 ± 1.16
18:1	6.76 ± 0.54†	5.14 ± 0.13
18:2	6.39 ± 0.68†	9.56 ± 0.44
Pre-20:3	0	0.029 ± 0.018
20:3w9	0	0.029 ± 0.018
20:3w6	0.11 ± 0.06	0.044 ± 0.03
20:4	27.03 ± 1.03	23.47 ± 1.78
HFA‡	6.31 ± 0.23†	5.20 ± 0.42
Total	99.97%	99.98%
Percent saturation	53.06 ± 1.01	56.19 ± 1.60
Unsaturated index	1.54 ± 0.04†	1.39 ± 0.07

* Mean ± S.E.M.
† $P < 0.05$ compared with jJ rats.
‡ Fatty acyl groups > 20.

Table 2. Cholesterol and phospholipid concentration in female hepatic microsomal membranes

Gunn rats	No. per group	Cholesterol/Protein (mg/mg)	Phospholipid/Protein (mg/mg)	Cholesterol/Phospholipid (mg/mg)
Icteric (jj)	19	0.0368 ± 0.0016	0.2360 ± 0.0123	0.1591 ± 0.0054
Anicteric (jJ)	11	0.0331 ± 0.0037	0.1888 ± 0.0117	0.1748 ± 0.0156

Values are mean ± S.E.M.

Table 4. Electron spin resonance of hepatic microsomal membranes from male and female homozygous (icteric) and heterozygous (anicteric) rats

Temperature (°C)	Parameter	Gunn rats Homozygous (jj)	Heterozygous (jJ)	P value
A. Male rats				
20	S*	0.663 ± 0.011† (N = 4)	0.654 ± 0.006 (N = 5)	0.15
	τ‡	1.91 ± 0.040 (N = 4)	1.83 ± 0.110 (N = 5)	0.21
37	S	0.564 ± 0.004 (N = 4)	0.559 ± 0.004 (N = 5)	0.12
	τ	1.29 ± 0.010 (N = 4)	1.26 ± 0.040 (N = 5)	0.12
B. Female rats				
20	S	0.646 ± 0.031 (N = 6)	0.650 ± 0.015 (N = 6)	0.81
	τ	1.93 ± 0.120 (N = 5)	1.86 ± 0.140 (N = 5)	0.40
37	S	0.554 ± 0.020 (N = 6)	0.549 ± 0.018 (N = 6)	0.66
	τ	1.27 ± 0.050 (N = 5)	1.21 ± 0.060 (N = 5)	0.13

* S = order parameter.
† Mean ± S.D.
‡ τ = rotational correlation time (sec × 10^{-9}).

whereas minor changes only were seen in the phospholipid fatty acyl composition, with a small but significant increase in the unsaturated index in the jj as compared to jJ Gunn rats (Table 3). Similar differences in the distribution of palmitic acid (decrease) and arachidonic acid (increase) in the jaundiced jj male rats as compared to normal Wistar (JJ) rats have been reported in one study [16], but not by others [34]. Specific phospholipids were not measured by us but no differences in these have been reported previously in male jj Gunn rats vs normal Wistar rats [16]. Despite the small differences in fatty acids seen in the homozygous Gunn rats, there was no apparent effect on hepatic microsomal membrane fluidity as measured by the order parameter of I[12,3] and the rotational correlation time of I[1,14] in either the female or male sets of homozygous and heterozygous Gunn rats (Table 4). These data are consistent with other recent fluidity studies in male homozygous vs heterozygous Gunn and normal Wistar rats using both electromagnetic resonance spectroscopy and fluorescence polarization techniques [34], but Gourley *et al.* [16] did observe a significant decrease in hepatic microsomal membrane fluidity measured by fluorescence polarization in jj vs JJ (normal Wistar) male rats. In the latter study, rats were fed a "standard high fat diet" compared to the low fat (Wayne Lab-Blox) diet used by us. This may explain the apparent inconsistency of these data, as dietary fat is known to affect both the phospholipid content and fluidity of the microsomal membrane [13, 35].

Our data, therefore, employing both *in vivo* and *in vitro* techniques did not reveal any abnormality in hepatic aminopyrine metabolism in the icteric, homozygous Gunn rats. In addition, we did not demonstrate important changes in lipid composition of hepatic microsomes in these animals, and microsomal membrane fluidity was unaltered. Clearly, our study does not support the concept of altered composition or fluidity of the microsomal milieu as a mechanism of impaired bilirubin glucuronidation in these animals, and absolves long-term unconjugated hyperbilirubinemia as a mechanism of hepatic microsomal dysfunction. In support of this, no consistent abnormality in hepatic drug oxidation has been demonstrated in patients with the milder long-term unconjugated hyperbilirubinemia of Gilbert's syndrome (R. K. Roberts Personal Communication). However, even unaltered composition and fluidity of the microsomal milieu do not preclude genetically determined spatial/structural abnormalities of the bilayer.

In our studies we elected to compare homozygous icteric animals with their nonicteric heterozygous littermates. We believe that this promotes a tight experimental design and essentially eliminates possible differences in specie variations that we have observed in preliminary studies among various normal Wistar rat strains. It could be argued that even the minor impairment of hepatic bilirubin conjugation in our heterozygous anicteric controls could be accompanied by altered microsomal structure and function, invalidating it as a proper control. We do not believe that such an argument is tenable since it would not explain the major difference in bilirubin conjugation in the homozygous vs heterozygous Gunn rats, which is the crux of the altered microsomal milieu concept. Another possibility, selective deficiency of UDP-glucuronic acid in the homozygote Gunn rat, has been ruled out recently [36]. Our studies thus favor the view that the abnormal bilirubin (and other aglycone) glucuronidation in homozygous Gunn rats is due to genetic abnormalities involving the enzyme(s) itself.

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