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Evidence against an abnormal hepatic microsomal lipid matrix as the primary genetic defect in the jaundiced Gunn rat

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The congenitally jaundiced Gunn rat does not conjugate bilirubin but does conjugate bilirubin dimethyl diester. Partial defects in conjugating *p*-nitrophenol and demethylating aminopyrine are also evident. A proposed mechanism to explain this combination of findings is a defective microsomal membrane. To examine the 'matrix' of Gunn microsomal membranes, hepatic microsomes were isolated from Gunn (*jj*) and outbred Wistar (*JJ*) rats and were studied by electron paramagnetic resonance spectroscopy of 7-doxylstearic and 12-doxylstearic acid probes, fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene, glucose-6-phosphatase activity vs. temperature, and lipid analysis. The data indicate several factors related to lipid bilayer order do not differ in microsomes from *jj* and *JJ*.

The homozygous Gunn rat has non-hemolytic hyperbilirubinemia due to a genetically determined total absence of hepatic microsomal UDPglucuronate bilirubinglucoronoside glucuronosyltransferase (EC 2.4.1.77) (B-trans) activity [1,2]. The biochemical abnormality which results in the absence of B-trans activity has not been determined, but there are reasons to postulate a general microsomal membrane abnormality rather than a specific enzyme defect. In addition to the B-trans defect, the Gunn rat has partial defects in demethylation of aminopyrene and conjugation of *p*-nitrophenol [3–6]. In addition, it is able to convert bilirubin dimethyl diester to bilirubin glucuronide [7], which presumably requires B-trans [8], and B-trans has been found immunochemically in Gunn rat hepatic microsomes [9]. Since the character of the membrane can affect the activity of tightly bound enzymes, the Gunn rat's hepatic microsomal membrane could be defective [10].

The extent of abnormality of the lipid membrane required to alter the function of B-trans is not known. A possible mechanism by which an alteration in lipid could alter enzyme activity is the way the substrates of enzymes interact or diffuse within the bilayer domain. Such an abnormality might explain the unmasking of B-trans when bilirubin dimethyl diester is used as a substrate since the chemical alteration of the bilirubin molecule alters its solubility characteristics [7]. Changes in the lipid composition of the microsomal mem-

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TABLE I

CHOLESTEROL AND PHOSPHOLIPID COMPOSITION OF MICROSOMAL MEMBRANES

Figures are presented as means ± S.D.

	Cholesterol		Phospholipid		Cholesterol/phospholipid ratio
	mg/mg protein	% [a]	mg/mg protein	% [a]	
jj ($n = 5$)	0.028 ± 0.005	5.2 ± 0.8	0.513 ± 0.093	94.8 ± 0.8	0.055 ± 0.009
JJ ($n = 5$)	0.028 ± 0.008	4.9 ± 1.1	0.515 ± 0.118	95.1 ± 1.1	0.055 ± 0.007

[a] % of total cholesterol plus phospholipid.

brane occurring during early neonatal development of the rat have been related to changes in activity of microsomal enzymes including B-trans [11]. Altering the lipid composition of the membrane through dietary manipulation alters certain parameters of microsomal enzyme function as well [12]. However, such changes in lipid structure, no matter how dramatic, have not resulted in total absence of enzyme activity.

Other investigators have looked for a possible membrane defect in the Gunn rat with contradictory results. Gourley et al. [13] found subtle differences in membrane order and fatty acid composition between Gunn and Wistar liver microsomes. Conversely, Schenker's laboratory has been unable to detect any differences between them [4].

To resolve this issue, we studied the lipid membrane of hepatic microsomes isolated from homozygous male Gunn rats (*jj*) and outbred male Wistar litter mates (*JJ*) which weighed 100–150 g when killed. Microsomes were isolated in 0.25 M sucrose, 0.001 M EDTA and were chosen for further study according to B-trans activity determined by the method of Strebel and Odell [2]: *jj*($n = 6$) = 0 μmol conjugated/g liver per 10 min and *JJ*($n = 6$) = 117.7 ± 10.9 (S.D.) (for reference *Jj* (heterozygotes, $n = 7$) = 66.0 ± 9.4). Four independent methods were used to examine the membrane lipid.

First, the lipid composition was analysed according to published procedures [14–17]. Table I summarizes the cholesterol and phospholipid content of the microsomal fractions from *jj* and *JJ*, and Table II gives their fatty acid compositions. No gross abnormality in the lipid composition of the microsomal membrane from *jj* is evident from these analyses.

Second, the activity of glucose-6-phosphatase of fresh microsomal preparations was determined by the method of Zakim and Vessey [18] at temperatures varying from 11 to 38°C (measured to ±0.1°C), and Arrhenius plots (log activity vs. $1/T$) were constructed from the resulting data [19]. Fig. 1 illustrates the results, which are identical in most respects for *jj* and *JJ*. Both demonstrate a slight inflection at 34.7°C, and the mean activity for each at 37.0°C is the same (2.45–2.51 μmol hydrolyzed/mg protein per 10 min). The activation energies are an identical 12.8 kcal/mol. These findings indicate no apparent differences in

TABLE II

FATTY ACID COMPOSITION OF MICROSOMAL MEMBRANES

Figures are presented as means ± S.D.

	Fatty acid series (% of total)				
	16:0	18:0	18:1	18:2	20:4
jj ($n = 5$)	24.5 ± 1.0	28.7 ± 3.1	9.4 ± 2.2	12.3 ± 1.1	16.6 ± 3.0
JJ ($n = 5$)	22.2 ± 2.4	29.9 ± 3.2	8.5 ± 0.7	13.2 ± 2.3	17.4 ± 1.1

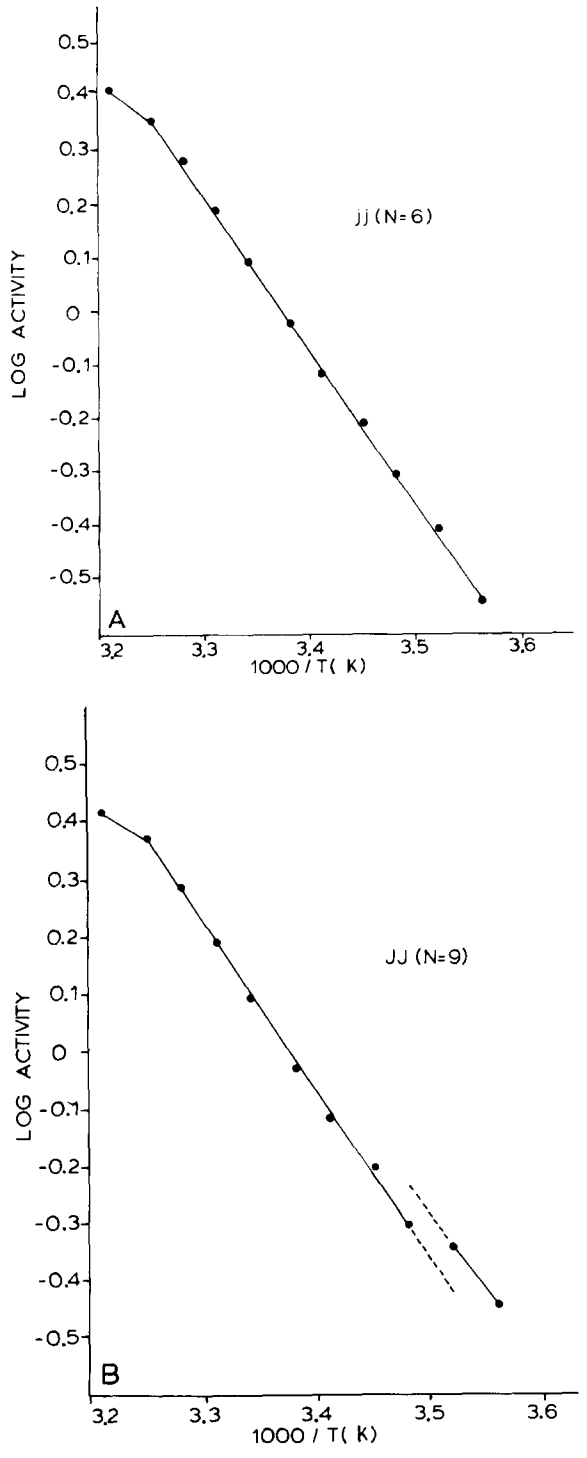

Fig. 1. Arrhenius plots of hepatic microsomal membrane glucose-6-phosphatase activity for *jj* and *JJ*. Log activity (μmol hydrolyzed/mg microsomal protein per 10 min) is plotted vs. $1/T$. In the range of temperatures in which membrane lipid phase changes would be expected (15–37°C), no difference was observed. (On the scale of $1000/T$(K): 3.2 ≡ 39.5°C; 3.3 ≡ 30.0°C; 3.4 ≡ 21.1°C; 3.5 ≡ 12.7°C; and 3.6 ≡ 4.8°C.)

the lipid domain around the glucose-6-phosphatase membrane attachment site since the function of this enzyme has marked lipid dependence [20]. Below 14.3°C the plots differ slightly. The meaning of this is unknown, but probably does not reflect a difference in the lipid domain because the phase transition temperature of microsomal membranes is well above 14.3°C.

Third, the order of the membrane lipid was investigated by electron paramagnetic resonance of 7- and 12-doxylstearic acid incorporated into the microsomes at a concentration of one mole percent of phospholipid content. The labels in 95% ethanol were pipetted into small glass tubes and dried under N_2. The microsomes were added, the tubes were vigorously agitated, and the labeled microsomes were taken for analysis. Spectra were determined at 6, 10, 20, 25, 30, and 38°C using a Varian 4502 spectrometer. Order parameters were calculated from the 7-doxylstearic acid spectra at each temperature by the method of Gaffney [21] according to:

$$S = \frac{1.748(O - I) - 2.412}{0.894(O) + 2.106(I) + 2.906}$$

where I is one half the inner extrema of the spectra in Gauss, O is one half the outer extrema of the spectra in Gauss, and S is the order parameter. In the case of the 12-doxylstearic acid, the outer extrema disappered due to the increased freedom of rotation of the spin label in this position, and order parameters could not be calculated. Thus, only the inner extrema, which are a linear function of S, were measured in Gauss for this probe at each temperature [21]. One animal from *jj* and *JJ* were studied. The data are given in Table III. The spectra determined at each temperature and with each probe were superimposable for both animals. In addition, Arrhenius plots of the order parameters calculated from the spectra of the 7-doxylstearic acid were identical (data not shown). These data indicate no differences in membrane order within the limitations of the probe technique.

Finally, the fluorescence anisotropy of 1,6-di-

TABLE III

ELECTRON PARAMAGNETIC RESONANACE PARAMETERS OF MOTIONAL FREEDOM OF PROBES IN MICROSOMAL MEMBRANES

T (°C)	*jj*		*JJ*	
	S [a]	A [b]	S	A
6.0	0.696 [c]	13.41	0.698	13.41
10.0	0.671	13.64	0.672	13.64
20.0	0.565	14.50	0.582	14.34
25.0	0.523	14.62	0.528	14.64
30.0	0.469	15.14	0.485	15.40
38.0	0.377	15.68	0.385	15.86

[a] Order parameter determined using 7-doxylstearic acid.
[b] Inner extrema measured using 12-doxylstearic acid (gauss).
[c] $n = 1$, no S.D. given.

phenyl-1,3,5-hexatriene (DPH) in the membrane lipid was determined as a function of temperature from 8 to 38°C in a Perkin-Elmer MPF-44B spectrofluorometer. Microsomal membranes were labeled by the addition of 1.0 mg membrane protein to 40 ml of 2.0 μM DPH in phosphate buffered saline [22]. Steady-state anisotropy values (A) were calculated by the formula;

$$A = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

where $I_{\parallel}$ is the emission intensity of the DPH fluorescence measure in a plane parallel to that of the exciting light, and $I_{\perp}$ is the emission intensity measured perpendicular to that of the exciting beam. Arrhenius plots of the steady-state anisotropy values were constructed for one each of *JJ* and *jj*. No clear phase transition was demonstrated, and the plots of the two preparations did not differ significantly (data not shown). A representative high (37.0°C) and low temperature (10.0°C) were chosen at which to study five each of *JJ* and *jj*. Table IV summarizes these data which illustrate *jj* to be indistinguishable from *JJ* with regard to the rotational freedom of DPH in the microsomal lipid.

The summation of these data provides strong evidence against a primary, genetic difference in the membrane lipid of the hepatic microsome as the cause of the conjugating defect of the Gunn rat. The minor differences found by Gourley et al.

TABLE IV

ANISOTROPY VALUES OF DPH IN MICROSOMAL MEMBRANES

Figures are presented as means ± S.D.

	A, 37°C	A, 10°C
jj ($n = 5$)	0.118 ± 0.018	0.192 ± 0.013
JJ ($n = 5$)	0.123 ± 0.017	0.211 ± 0.020

[13] cannot possibly explain the absence of B-trans activity in *jj*. Our data and those of Schenker et al. [4] indicate the lipid membrane of the hepatic microsome from *jj* to be indistinguishable from *JJ*, within the limitations of the techniques used. A lipid abnormality probably is excluded as an explanation of the microsomal defects of the Gunn rat.

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