enzymes and perhaps reduce neurotransmitter release, and (2) increased binding of calcium to the cytoplasmic surface of the synaptic plasma membrane could change the physical properties (fluidity, lateral phase separations) of the inner half of the membrane bilayer, and these membrane property changes could alter membrane-bound enzymes and membrane transport [18–22]. Thus, in addition to its direct effects on membrane physical properties [13, 23], ethanol may indirectly alter synaptic membrane properties by changing calcium binding.

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Microsomal enzyme deficiencies in the Gunn rat

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Gunn rats, in their jaundiced homozygous phenotype (jj or −/−), lack liver UDP glucuronyl transferase (EC 2.4.1.17) activity for bilirubin. Heterozygous animals (Jj or +/−) have demonstrable transferase activity for bilirubin which, however, is significantly less than the activity present in outbred normal rats (JJ, +/+) [1–3]. *In vitro* glucuronidation activities for other aglycones in the jj animal vary from markedly reduced to normal levels [1–7]. The deficiency in the Gunn rat is an animal model of the human disorder known as the Crigler–Najjar syndrome, in which bilirubin glucuronyl transferase (BGT) activity is also lacking and is transmitted as an autosomal recessive trait [8]. This transferase deficiency in both rat and man has been presumed to reflect a single gene defect in the synthesis of this enzyme protein. An alternative hypothesis that might explain these enzymatic abnormalities is a genetically controlled alteration of the membrane in which glucuronyl transferase is compartmented. Such an abnormality could either alter enzyme conformation or decrease substrate penetration through the microsomal membrane, thereby decreasing enzyme activity. If a membrane defect is the primary abnormality, other enzymes also located in microsomes might exhibit deficiencies which would segregate like that of the glucuronyl transferase in the Gunn rat. With this in mind, the activity of aminopyrine demethylase, an hepatic microsomal cytochrome P-450, mixed-function oxidase (EC 1.14.14.1), was assayed in male jj, Jj and JJ Gunn rats.

Materials and methods

The aminopyrine demethylase assay was modified from Matsubara *et al.* [9]. Incubation mixtures contained 10 mM aminopyrine, 0.4 mM NADP, 5 mM glucose-6-phosphate,

4 units of glucose-6-phosphate dehydrogenase, 5 mM $MgCl_2$, 50 mM Tris–HCl buffer (pH 7.6) and about 1 mg microsomal protein. Final volume was adjusted to 4 ml with 0.15 M KCl. After thermal equilibration of the assay medium for 3 min, the reaction was initiated by adding the microsomes and incubated at 37° in a Dubnoff shaker (112 cpm). Samples of 0.5 ml were removed at 0, 3 and 6 min and were added immediately to 0.3 ml of iced 20% (w/v) trichloroacetic acid. After centrifugation, a 0.5-ml aliquot of the protein-free supernatant fraction was treated with 0.25 ml of Nash reagent [10] and incubated in a water bath at 60° for 10 min. The absorbance of the resulting solution was measured at 412 nm. Saturation kinetics were confirmed by a linear correlation coefficient greater than 0.9 (product formed vs incubation time).

Male Gunn rats (300–500 g) were maintained on a standard high-fat diet and water *ad lib*. "Known Jj" rats were the non-jaundiced offspring of Jj × jj matings. Other Jj or JJ rats were the non-jaundiced offspring of Jj × Jj matings and were distinguished by their microsomal bilirubin glucuronyl transferase activity, using the assay of Strebel and Odell [11]; Jj ≤ 35 and JJ ≥ 45 μg bilirubin conjugated per 30 min per mg protein N. All assays were performed with fresh liver microsomes which had been prepared as previously described [11]. Microsomal protein was determined by the method of Sutherland *et al.* [12], using bovine serum albumin as standard and correlated with total nitrogen measured by a micro-Kjeldahl procedure [13].

To test whether any of the observations could be explained by the bilirubin present in jj microsomes but lacking in microsomes from all non-jaundiced groups, bilirubin-treated microsomes from five male Jj rats were included in the standard assay for aminopyrine demethylation. This was done by using microsomal pellets prepared from a 1:1 or 1:2 mixture of 10,000 *g* supernatant and bilirubin–albumin solutions (40.2 mg/100 ml bilirubin in a 1.5:1 molar ratio with albumin). These bilirubin-containing microsomes were assayed for aminopyrine demethylation, as were control microsomes and microsomes containing only the extra albumin.

Results and discussion

The bilirubin glucuronyl transferase and corresponding aminopyrine demethylase activities are shown in Table 1. Bilirubin glucuronyl transferase activities found in the "known Jj" rats were similar to those previously reported [11]. Non-jaundiced offspring of Jj × Jj matings were classified, as described in Materials and Methods. The Jj thus classified exhibited transferase activities similar to the "known Jj" rats. The JJ rats had activities greater than both groups of Jj rats [one-way analysis of variance (ANOVA): $F(2,35) = 91.88$, $P < 0.001$]. Homozygous jaundiced, jj, animals were identified by the development of icterus at several days of age.

Table 1. Bilirubin glucuronyl transferase (BGT) and aminopyrine demethylase (AD) activities in various male Gunn rats*

Rats	Enzyme activities BGT†	AD‡
jj		12.58 ± 2.02 (12)
Jj§	28.3 ± 7.1 (15)	14.75 ± 3.82 (15)
Jj¶	28.0 ± 5.2 (13)	14.52 ± 3.09 (13)
JJ¶	66.7 ± 10.8 (10)	15.61 ± 1.80 (10)

* Results are means ± S.D. See text for statistical analysis. N = the number in parentheses.

† Expressed as μg bilirubin conjugated·(30 min)$^{-1}$·(mg protein N)$^{-1}$.

‡ Expressed as nmoles formaldehyde produced·min^{-1}·(mg protein)$^{-1}$.

§ Non-jaundiced offspring of Jj × jj mating.

¶ Non-jaundiced offspring of Jj × Jj mating.

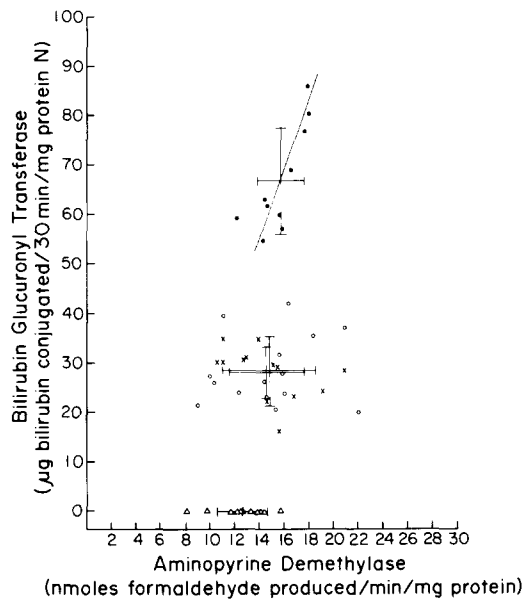

Fig. 1. Bilirubin glucuronyl transferase and aminopyrine demethylase activities in Gunn rats. Key; jj (△), "known Jj" (○), outbred Jj (×) and JJ (●). The vertical and horizontal lines with crossbars are the mean ± 1 S.D. of the bilirubin glucuronyl transferase and aminopyrine demethylase activities, respectively. The regression line drawn for the results in JJ animals was determined by the method of least squares and has a correlation coefficient of 0.81, $P < 0.01$.

Comparing jj, known Jj and JJ rat aminopyrine demethylase activities using the one-way analysis of variance yielded $F(2,34) = 3.37$, $P < 0.05$. We observed that jj < Jj < JJ in aminopyrine demethylase activity. If the Jj "unknown" group was included in the ANOVA, we found $F(3,44) = 2.23$, which corresponded to P of approximately 0.1. This is suggestive, although not statistically significant. The F test is an omnibus test, which is not particularly sensitive to a specific pattern. Therefore, because we hypothesize an ordering of aminopyrine demethylase similar to BGT, these data provide further support.

A comparison of the variation about the mean (variance ratio F-test) showed significantly more variance in the heterozygous groups than in both JJ and jj animals ($P < 0.05$). A further distinction between the Jj and JJ animals is illustrated in Fig. 1. A highly significant correlation ($r = 0.81$, $P < 0.01$) was seen between the aminopyrine demethylase and bilirubin glucuronyl transferase activities in JJ rats which was not present in Jj animals.

The effect of bilirubin on aminopyrine demethylation is demonstrated in Table 2. Analysis of variance implies no difference in aminopyrine demethylase activity between microsomes treated with or without bilirubin. Hence, the presence of bilirubin cannot be used as an explanation for the decreased aminopyrine demethylase activity measured in jj rats. Indeed, previous data have shown that bilirubin actually stimulates the glucuronidation of morphine and *p*-nitrophenol [14] in rat liver microsomal preparations. Bilirubin cannot be postulated as the cause of the observed differences between Jj and JJ rats since bilirubin levels are normal in both groups.

The microenvironment of an enzyme is clearly important in determining enzyme function [15]. In the case of membrane-bound enzymes, this environment is determined

Table 2. Aminopyrine demethylase activity in liver microsomes of five male Jj Gunn rats (BGT = 30.7 ± 6.4) pre-exposed to either albumin or bilirubin–albumin*

Microsomes used in assay	Aminopyrine demethylase activity (mean ± S.D.) [nmoles formaldehyde produced·min^{-1}·(mg protein)$^{-1}$]
Microsomes alone	13.48 ± 3.51
1:1 (microsomes:albumin)	9.35 ± 0.95
1:1 (microsomes:bilirubin–albumin)	11.20 ± 3.96
2:1 (microsomes:albumin)	10.93 ± 4.81
2:1 (microsomes:bilirubin–albumin)	12.71 ± 3.61

* See Materials and Methods for details. ANOVA for the five groups: F(4,20) = 1.00 implies that there is no statistically significant difference between these groups.

by the matrix in which the enzyme is embedded. The function of enzymes embedded in the endoplasmic reticulum, like enzymes of many other biological membranes, is intimately related to the microenvironment provided by membrane lipids [16]. An alteration of the lipids or structural proteins of this microenvironment might explain many observations regarding glucuronyl transferase.

Glucuronyl transferase is situated in the lipid bilayer of the microsomal membrane [17]. While the activity of bilirubin glucuronyl transferase is negligible in jaundiced Gunn rats, the glucuronidation of a number of other substrates by glucuronyl transferase is either normal [4, 5] or partially defective [1–3, 6, 7]. The defective *in vitro* conjugation of some substrates can be increased to normal rates by the addition of both detergents and diethylnitrosamine, suggesting abnormal enzyme–phospholipid interactions, i.e. an abnormal constraint of the membrane-bound enzyme [18, 19].

The interpretation that the deficiency in Gunn rats is due to a defective microsomal enzyme responsible for glucuronidation of bilirubin does not explain the reduced glucuronide formation using other substrates. Nakata *et al.* [20] have proposed the existence of multiple glucuronyl transferases which may have a common sub-unit essential for binding UDP-glucuronic acid. A mutation in this subunit might account for the abnormal glucuronyl transferase activities in the Gunn rat. Weatherill and Burchell [21] dispute this theory with data obtained in their purification of Wistar and Gunn rat glucuronyl transferase. Using affinity chromatography, a method dependent on the UDP-glucuronic acid binding site, the yield of purified glucuronyl transferase is similar for both strains of rats. Furthermore, the purified enzymes from these two strains are indistinguishable, both electrophoretically and immunologically.

A genetically controlled alteration of the membrane matrix in which the enzyme is located might account for the multiple transferase deficiencies. The importance of the membrane composition is exemplified by the decreased activity of Rotenone-insensitive NADPH–cytochrome *c* reductase when this enzyme is assayed in microsomes prepared from Morris 7777 hepatoma cells. These tumor cells have a 45% lower than normal content of microsomal phospholipids [22]. Density distributions of submicrosomal components from Gunn rats show abnormalities in the properties of the plasma membrane and mitochondrial outer membrane. However, when Gunn rat microsomes were treated with digitonin, a membrane-perturbing agent, density profiles similar to control rats were found [23]. Abnormal phospholipid–enzyme interactions have also been suggested by Bock *et al.* [18]. Their proposal is supported by the observation that glucuronyl transferase in microsomal fractions requires phospholipids to exhibit maximum activity [24–26]. Furthermore, the dependence of glucuronyl transferase on the fluidity of the lipids in guinea pig liver microsomal membranes has been demonstrated by electron paramagnetic resonance [27]. Similarly, a dramatic rise in glucuronyl transferase activity occurs after birth [11] in rat microsomes at a time when the membrane in which it is isolated undergoes a marked increase in phospholipids [28]. A similar dependence on lipids is seen in the microsomal cytochrome P-450 enzymes [29]. Aminopyrine demethylase is such an enzyme.

Further evidence that a membrane defect may represent the primary abnormality in the jaundiced Gunn rat and the Crigler–Najjar syndrome of humans was recently reported from this laboratory [30]. Hepatic microsomes from jj rats and a Crigler–Najjar patient form bilirubin mono- and diglucuronides enzymatically if the dimethyl diester of bilirubin is used as the substrate. It is postulated that the configuration change induced by the esterification of bilirubin promotes better lipid penetration and allows substrate access to the membrane-imbedded glucuronyl transferase.

The differences in aminopyrine demethylase activity seen among jj, Jj and JJ Gunn rats are not as impressive as that of the glucuronyl transferase activity for bilirubin, but are similar to the glucuronyl transferase activity when *p*-nitrophenol is used as substrate [7].

The present study documents yet another heritable enzyme deficiency of microsomes in the Gunn rat which segregates like the glucuronyl transferase deficiency. The coexistence of these two unrelated enzyme deficiencies in the Gunn strain of rat might be better reconciled by an hypothesis that a microsomal membrane defect in enzyme compartmentation may be the primary abnormality in the Gunn rat and possibly in the Crigler–Najjar syndrome in humans.

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Inhibitors of hepatic mixed function oxidases—V*. Inhibition of aminopyrine *N*-demethylation and enhancement of aniline hydroxylation by benzoxazole derivatives

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Many nitrogen heterocycles are potent inhibitors of microsomal mixed function oxidases (MFO) [1–12]. Studies with imidazole derivatives (including benzimidazoles) have described the dependence of inhibitory activity on hydrophobicity [2, 7] and the influence of steric factors on inhibitory activity and binding affinity to cytochrome P-450 [3]. In these investigations of potential inhibitors, some compounds have been found to enhance microsomal MFO activity *in vitro*. In an investigation of oxazoles and thiazoles, Smith and Wilkinson [9] found that benzoxazole stimulated the *N*-demethylation of *p*-chloro-*N*-methylaniline in rat liver microsomes. However, Holder *et al.* [7] had previously reported that two benzoxazole derivatives inhibited the *N*-demethylation of aminopyrine and stimulated the hydroxylation of aniline. To investigate more fully the interaction of benzoxazoles with microsomal oxidases we have studied the effects of a series of benzoxazoles on aminopyrine *N*-demethylase (APDM) and aniline *p*-hydroxylase (AH) activities in hepatic microsomes from phenobarbitone (PB)-treated rats. These enzyme substrates were chosen as compounds which afford cytochrome P-450-difference spectra representative of the two principal types, namely type I (aminopyrine) and type II (aniline) [13, 14].

Materials and methods

Alkylbenzoxazole derivatives (compounds I, III–VII and IX, Table 1) were synthesised by refluxing equimolar quantities of *o*-aminophenol or 2-amino-4-methylphenol with the appropriate organic acid [15]. Compound VII was purified by recrystallisation from petroleum ether/toluene and the other benzoxazoles, which were liquids at ambient temperature, were purified by distillation. All synthesised compounds had physical properties similar to reported values and gave satisfactory elemental analyses (Australian Microanalytical Service, C.S.I.R.O., Melbourne, Vic.). Compounds II, VIII and XI were purchased from Eastman Kodak Co., Inc. (Milwaukee, WI); VIII and XI were recrystallised from aqueous ethanol before use. 2-Amino-5-chlorobenzoxazole (compound XII, zoxazolamine) was obtained from K and K Labs, Inc., (Plainview, NY) and recrystallised from methanol/benzene. Biochemicals and cofactors were from Sigma Chemical Co., (St. Louis, MO) and all other chemicals were analytical grade.

Microsomes were prepared from the livers of male Wister rats (100–150 g) that had been treated with phenobarbitone. APDM and AH activities were followed by the formation of formaldehyde and *p*-aminophenol respectively, as previously described [7]. Benzoxazole derivatives did not interfere with the assays for either formaldehyde or *p*-aminophenol. I_{50} values were determined in duplicate from incubation with at least five concentrations of inhibitor. Cytochrome P-450-difference spectra were recorded with an Aminco DW-2 Spectrophotometer (American Inst. Co., Silver Springs, MD) at a microsomal protein concentration of 1 mg/ml. Wavelengths were determined with respect to characteristic absorbance peaks of holmium oxide.

Structure–activity relationships were examined by multiple linear regression analysis, using the Cyber 72 computer at the Sydney University Computer Centre. Partition coefficients (log $P_{octanol/water}$) were derived or calculated from the literature [16, 17] by addition of the summed hydrophobic

* The first four papers in this series were A. Bobik *et al.*, *Xenobiotica* **5**, 65 (1975), reference 7, A Bobik *et al.*, *J. Med. Chem.* **20**, 1194 (1977) and P. J. Little and A. J. Ryan, *J. Med. Chem.* (in press).