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DIFFERENCES IN UDP-GLUCURONOSYLTRANSFERASE ACTIVITIES IN CONGENIC INBRED RATS HOMOZYGOUS AND HETEROZYGOUS FOR THE JAUNDICE LOCUS

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Abstract—The genic transfer of the jaundice locus (jj) from the Gunn rat into the inbred RHA/++ rat produced congenic inbred homozygous RHA/jj rats which lacked detectable bilirubin UDP-glucuronosyltransferase activity. Congenic inbred RHA/j+ rats contained half the activity for bilirubin of the RHA/++ strain. Constitutive activities for glucuronidation of sixteen substrates of twenty-one tested were inherited additively. Approximately seven groups were discernible based on the defect in activity for these substrates in the RHA/jj strain. Activity for 1-hydroxybenzo[*a*]pyrene was, after that for bilirubin, the most severely reduced (188-fold), while no differences in the glucuronidation of three androgens and of the 6-hydroxy-, 10-hydroxy-, and 11-hydroxybenzo[*a*]pyrenes were observed. The conjugation of other substrates was affected to an intermediate extent. Most of the twenty-one glucuronidating activities were induced by phenobarbital in the RHA/jj strain as well as in the RHA/++ and RHA/j+ strains. Activities for 9-hydroxybenzo[*a*]pyrene and for the 2-hydroxy- and 4-hydroxybiphenyls were induced such that the defect was overcome, and the RHA/jj had the same level of activity as the RHA/++ strain. Cytochrome p-450 content and cytochrome *c* reductase and aminopyrine demethylase activities were unaffected in the congenic strains. Cytochrome p-450 content and cytochrome *c* reductase activity were induced ~2.5- and 2.0-fold, respectively, by phenobarbital while aminopyrine demethylase activity was induced about 30% in each strain. The congenic inbred rats should provide a stable and reproducible genetic model for studying defective UDP-glucuronosyltransferase specified by the jaundice (jj) locus.

The jaundice locus (jj) in the Gunn rat, a mutant of the Wistar strain, is associated with essentially totally defective *in vivo* and *in vitro* bilirubin UDP-glucuronosyltransferase activity. Certain substrates, such as 2-aminophenol and 2-aminobenzoate [1], are poorly glucuronidated by the Gunn rat while other substrates, such as tetrahydrocortisone and morphine [1], are glucuronidated at normal rates. Controversy surrounds certain activities in the Gunn rat. The activity for *p*-nitrophenol, for example, is reported to be unaffected [2, 3] whereas other studies indicate that activity for *p*-nitrophenol is markedly reduced [4, 5]. Although differences in assay conditions could account for these contradictory results, it is also considered possible that the Gunn rats are genetically impure [6]. Hence, in this study a survey of a number of transferase† substrate activities, cytochrome P-450-content, a cytochrome p-450-dependent monoxygenase activity, and cytochrome *c* reductase activity was made on microsomal fractions from two congenic inbred strains of rats with the jaundice locus, heterozygous (RHA/j+) or homozygous (RHA/jj). The normal inbred strain, RHA/++, was also studied.

Several advantages are derived from the use of these congenic strains. The animals are inbred and should provide a reproducible model for future studies. In addition, the effect of the jaundice locus including a few other linked loci on defective activity can be studied under conditions where the mutant gene(s) has been transferred to a new genetic background. If that is so, one can assess from the pattern of defective enzymes whether a small segment of chromosomal material transferred to the congenics [7] is responsible or whether the genetic background of the Gunn rat is crucial to the jaundice phenotype.

MATERIALS AND METHODS

Chemicals. Androsterone, testosterone, dihydrotestosterone, phenolphthalein, *p*-nitrophenol, naphthol, bilirubin, 4-methylumbelliferyl-β-D-glucuronide, cytochrome *c*, and UDP-glucuronic acid were from the Sigma Chemical Co., St. Louis, MO. [1,2-^{3}H(N)]Androsterone (40.8 Ci/mmole), [1,2,6,7-^{3}H(N)]testosterone (93.9 Ci/mmole), and [1,2-^{3}H(N)]dihydrotestosterone (40.0 Ci/mmole) were from the New England Nuclear Corp., Boston, MA. 4-Methylumbelliferone was from the Aldrich Chemical Co., Milwaukee, WI. Aminopyrine was

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† Abbreviations and short terms used: transferase, UDP-glucuronosyltransferase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; PB, phenobarbital; and 4-MU, 4-methylumbelliferone.

obtained from Merck, Rahway, NJ. The 2-hydroxy- and 4-hydroxybiphenyls were from the Eastman Kodak Co., Rochester, NY, and were recrystallized from *n*-hexane and methanol respectively. All benzo[*a*]pyrene phenols were obtained from the National Cancer Institute Chemical Repository. CHAPS was from Calbiochem, La Jolla, CA. All other chemicals were reagent grade.

Source of rats. All male rats were obtained from Dr. Carl T. Hansen of the Veterinary Resources Branch of the National Institutes of Health. The normal inbred rat designated RHA/++ (*r*oman *h*igh *a*voidance selected from Wistar stock in a behavioral study for high avoidance to electric shock [8]) was crossed with the Gunn rat carrying the jaundice locus (jj) to generate F_1 hybrids. F_1 hybrids were intercrossed and finally incrossed through twelve cycles while selecting for the jaundice locus to generate a congenic inbred strain, RHA/jj, homozygous at the jaundice locus on the RHA/++ background. Inbred RHA/j+ but heterozygous at the jaundice locus were generated by crossing the inbred RHA/jj and RHA/++ strains. The RHA/j+ or RHA/jj should be genetically similar to the RHA/++ except heterozygous and homozygous, respectively, at the jaundice locus.

Treatment of rats and preparation of microsomes. Treated rats received sodium phenobarbital (0.75 g/l) in drinking water for 10 days prior to being killed. Control rats received only water. After starvation for 24 hr and exsanguination of each rat, the liver was excised (kept at 0–4° for all further manipulations), homogenized in 5 vol. of 0.25 M sucrose with a LKB Polytron, and spun at 15,000 *g* for 15 min. The microsomes were spun down at 100,000 *g* for 1 hr and then washed by resuspension in 0.15 M KCl containing 10 mM EDTA (pH 7.2) before a final 1 hr centrifugation at 100,000 *g*. The microsomal pellets were resuspended in 7.8 ml of 0.1 M potassium phosphate buffer (pH 7.6) containing 20% glycerol per g of liver weight. Microsomes were stored at −80° until used.

Assay of cytochrome P-450 content. Cytochrome P-450 content was measured according to Omura and Sato [9].

Assay of aminopyrine demethylase activity. Aminopyrine demethylase activity was determined [10] with 2.0 mM aminopyrine in the reaction mixture.

Assay for cytochrome c reductase activity. Cytochrome *c* reductase activity was determined using cytochrome *c* as substrate in 0.1 M potassium phosphate buffer (pH 7.4) as described [11].

Activation of UDP-glucuronosyltransferase and assay for activity. For every substrate, microsomes were maximally activated with 0.5 mg CHAPS/mg protein and left on ice for at least 20 min. The protein concentration was 1.0 mg/ml, except for the bilirubin glucuronidation assay in which the activated protein concentration was 5 mg/ml. Aliquots of activated microsomes were then added to the appropriate reaction tube for assay.

Bilirubin transferase activity was determined by the method of Heirweigh *et al.* [12, 13] as modified in Ref. 14. Glucuronidation of the twelve phenols of benzo[*a*]pyrene was measured as described in Ref. 15. 4-Methylumbelliferone transferase activity was determined with 1.0 mM substrate in a reaction mixture as previously described [16, 17] with the following modifications. The reaction was stopped by adding water-saturated ethyl acetate (a final volume of 10 ml), and the reaction volume was brought to 1.0 ml with water. Unconjugated 4-methylumbelliferone was twice extracted into the ethyl acetate phase and drawn off. Fluorescence (measured on an Aminco–Bowman spectrophotofluorometer) of the product, 4-methylumbelliferyl-β-D-glucuronide, was measured using an aliquot of the extracted reaction mixture added to 0.25 M glycine–HCl buffer (pH 10.25) with wavelength maxima determined to be 320 nm for activation and 380 nm for emission. The amount of product formed was estimated by comparing its fluorescence with that of standard 4-methylumbelliferyl-β-D-glucuronide under the same conditions.

Phenolphthalein transferase activity was measured according to modifications of published procedures [18, 19]. A 0.2-ml reaction volume contained 90 mM Tris–maleate buffer (pH 7.6), 5 mM $MgCl_2$, 0.35 mM phenolphthalein, and 5 mM UDP-glucuronic acid to start the reaction. The reaction was stopped with 0.25 M glycine–HCl buffer (pH 10.25), and the optical density was read at 555 nm. 1-Naphthol transferase activity was determined by the method of Mackenzie and Hänninen [20]. 4-Nitrophenol transferase activity was determined with 0.35 M substrate according to Hänninen [21] except that the buffer was 50 mM tris–maleate (pH 7.4). Activity towards 4-hydroxybiphenyl was determined with 0.5 mM substrate, and product was standardized according to Bock *et al.* [22]. The fluorescence of the glucuronide was determined at wavelength maxima of 295 nm for excitation and 335 nm for emission. Activity towards 2-hydroxybiphenyl was determined under the same incubation conditions, substrate concentration, and standardization of product as used for 4-hydroxybiphenyl. The 2-hydroxybiphenyl-glucuronide wavelength maxima were determined to be 293 nm for excitation and 338 nm for emission. Activity for testosterone was measured using 0.6 mM substrate as described [23]. Transferase activities, using 0.2 mM androsterone and 0.2 mM dihydrotestosterone, were measured using the same conditions as for testosterone.

Protein was determined according to the method of Lowry *et al.* [24] using bovine serum albumin as standard.

RESULTS

Liver microsomes, either control or phenobarbital-induced, from one inbred and two congenic inbred strains of RHA rats were assayed for cytochrome P-450 content, a monooxygenase activity, cytochrome *c* reductase activity, and twenty-two different transferase activities. Activities in inbred congenic RHA/j+ and RHA/jj heterozygous and homozygous rats, respectively, at the jaundice locus were compared to the normal inbred RHA/++ rat. Data in Table 1 show that the cytochrome p-450 contents in all control rats were the same and that phenobarbital treatment caused more than a 2-fold increase in all three strains. The levels

Table 1. Cytochrome P-450 content, and aminopyrine demethylase and cytochrome *c* reductase activities, in control and phenobarbital-treated inbred and congenic inbred rats heterozygous and homozygous at the jaundice (jj) locus*

Treatment	Rat strain	Cytochrome P-450 content (nmoles/mg protein)	Aminopyrine demethylase activity (nmoles/min/mg protein)	Cytochrome *c* reductase activity (μmoles/min/mg protein)
Control	RHA/++	0.56	12.0	0.22
Control	RHA/j+	0.64	13.1	0.23
Control	RHA/jj	0.65	14.0	0.19
PB-treated	RHA/++	1.66	16.7	0.46
PB-treated	RHA/j+	1.67	17.0	0.41
PB-treated	RHA/jj	1.68	18.0	0.39

* For each assay, the results are averaged for two different determinations.

of aminopyrine demethylase activity were approximately the same in all controls and were induced about 30%, while cytochrome *c* reductase activities were induced 2-fold, in each strain by phenobarbital.

A survey of activities for twenty-two different transferase substrates indicated that approximately six different levels of deficiencies existed for the transferase enzymes (Table 2) in the RHA/jj strain. Bilirubin transferase activity, similar to results observed in the Gunn rat [1], was the only activity that was nondetectable in the control or phenobarbital-treated congenic RHA/jj strain. Constitutive activity for 1-hydroxybenzo[*a*]pyrene was, after activity for bilirubin, the most drastically reduced (188-fold) in the RHA/jj strain. Activities towards the 2-hydroxy- and 3-hydroxybenzo[*a*]pyrenes was reduced 11-fold while those for 4-methylumbelliferone and the 2-hydroxy-and 4-hydroxybiphenyls were reduced 6.3- to 8.5-fold. On the other hand, activities for most of the benzo[*a*]pyrene phenols, such as the 5-hydroxy-, 7-hydroxy-, 8-hydroxy-, 9-hydroxy-, and 12-hydroxybenzo[*a*]pyrenes, were only reduced 2- to 3-fold, as were activities for 1-naphthol, *p*-nitrophenol and phenolphthalein. Finally, activities for the three androgens and the 6-hydroxy-, 10-hydroxy-, and 11-hydroxybenzo[*a*]pyrenes were only slightly, or not, affected by the recessive jj genotype inbred into the RHA rat.

Phenotypically, the constitutive activities for bilirubin and the other first sixteen transferase substrates, listed in Table 2, were expressed according to additive inheritance in these three strains of rats as seen in an earlier study with Gunn rats [25] using bilirubin. Phenobarbital treatment of rats (Table 2) appeared to enhance groups of substrate activities differently and not in accordance with the six groups that showed deficient constitutive activities in the RHA/jj rat.

As activity for 1-hydroxybenzo[*a*]pyrene was severely reduced in the RHA/jj rat, we saw the greatest induction (162-fold) by phenobaribtal for this substrate activity followed by inductions of 11- to 17-fold for 2-hydroxybiphenyl, 4-hydroxybiphenyl and 2-hydroxybenzo[*a*]pyrene. Activities for the substrates, 4-methylumbelliferone and the 3-hydroxy-, 4-hydroxy-, 8-hydroxy-, and 9-hydroxybenzo-[*a*]pyrenes were induced to about the same extent (3- to 5-fold). Finally, low levels (1.3- to 1.9-fold) of induction of activities for 1-naphthol, *p*-nitrophenol, testosterone, dihydrotestosterone, and the 5-hydroxy-, 6-hydroxy-, and 11-hydroxybenzo[*a*]pyrenes were observed.

The substrates, bilirubin, phenolphthalein, androsterone, and the 7-hydroxy-, 10-hydroxy-, and 12-hydroxybenzo[*a*]pyrenes, were not affected by phenobarbital treatment of the RHA/jj rat. Although the substrate groups with different levels of deficiency for transferase activity (Table 2, column 4) did not correspond entirely with the groups for levels of phenobarbital induction (Table 2, column 8), generally the greatest level of induction occurred for the most severely deficient activity. Furthermore, it appeared that phenobarbital pretreatment increased the activities for testosterone, the 9-hydroxy- and 11-hydroxybenzo[*a*]pyrenes, and the 2-hydroxy- and 4-hydroxybiphenyls to the maximum level established in the RHA/++ rat.

DISCUSSION

A survey of activities towards twenty-two different transferase substrates in the congenic inbred strain, RHA/jj, has indicated that only four activities were reduced by at least one order of magnitude or greater, while approximately one-half of the activities were less than 10-fold reduced and six substrate activities were essentially unaffected by the jj locus. Consistent with many earlier studies [1], bilirubin transferase was nondetectable in the jaundice strain while there was at least detectable activity towards all other substrates. All of the affected activities appeared to be inherited additively. In a previous study with Wistar rat and heterozygous and homozygous Gunn rat microsomes that had been digitonin-activated, the constitutive activities towards 4-methylumbelliferone were approximately the same in the three strains, whereas the activities towards bilirubin [26] and *p*-nitrophenol [26, 27] were additive.

The use of the twelve benzo[*a*]pyrene phenols as transferase substrates indicated that this class of compounds distributed into each group (described in Table 2) from severely reduced (1-hydroxybenzo[*a*]pyrene) to those that were

Table 2. UDP-glucuronosyltransferase activities in control and phenobarbital-treated inbred and congenic inbred rats heterozygous and homozygous at the jaundice (jj) locus*

Groups	Substrate	Control activity				PB-treated activity			
		RHA/++	RHA/j+	RHA/jj	Fold-reduction in constitutive activity in RHA/jj	RHA/++	RHA/j+	RHA/jj	Fold induction in RHA/jj
1	Bilirubin	2.18	1.03	ND†		3.29	1.30	ND	
2	1-OH-BP	32.01	16.87	0.17	>188	47.76	36.40	27.45	162
3	2-OH-BP	3.05	1.69	0.27	11	5.70	4.45	3.26	12
	3-OH-BP	6.89	4.72	0.63	11	10.20	6.21	3.00	4.8
4	4-MU	103.93	52.16	16.07	6.5	151.73	119.36	56.03	3.5
	2-OH-Biphenyl	148.93	119.10	23.82	6.3	233.08	225.97	267.96	11.0
	4-OH-Biphenyl	428.54	276.62	51.32	8.4	1010.30	1000.24	883.95	17.0
5	4-OH-BP	19.32	14.86	4.35	4.4	25.25	19.61	14.06	3.2
6	5-OH-BP	12.07	9.00	5.92	2.0	15.76	12.93	9.61	1.6
	7-OH-BP	13.87	8.44	5.20	2.7	16.87	10.51	5.21	1.0
	8-OH-BP	6.99	3.90	2.42	3.0	10.91	8.45	5.80	2.4
	9-OH-BP	13.33	8.86	6.02	2.2	18.90	18.08	17.89	3.0
	12-OH-BP	19.90	14.06	9.58	2.1	22.79	14.99	7.89	1.0
	Phenolphthalein	10.21	9.28	3.96	2.6	9.98	7.24	3.30	1.0
	1-Naphthol	30.02	20.41	9.57	3.0	43.17	29.04	13.89	1.5
	p-Nitrophenol	49.34	31.01	15.70	3.0	76.60	53.78	30.50	1.9
7	6-OH-BP	38.96	43.66	35.30	1.1	52.47	50.89	44.17	1.25
	10-OH-BP	18.69	17.80	14.07	1.3	19.03	17.95	13.83	1.0
	11-OH-BP	16.10	16.07	12.27	1.3	19.79	19.88	17.10	1.4
	Androsterone	2.94	2.92	2.91	1.0	2.83	2.58	2.93	1.0
	Testosterone	1.90	1.76	1.60	1.2	3.07	2.73	2.72	1.7
	Dihydrotestosterone	2.34	2.16	2.18	1.1	3.47	2.94	2.85	1.3

* Activity for each substrate is expressed as nmoles/(min·mg protein).
† Not detectable.

unaffected (6-hydroxy-, 10-hydroxy-, and 11-hydroxybenzo[*a*]pyrenes). These differences suggest that more than one transferase in the rat was responsible for conjugating this series of phenols. A recent study [15] using microsomes from C57BL/6N mice indicated that at least two classes of enzymes separated by charge heterogeneity are responsible for conjugating the phenols.

Activity for each of the androgens examined in this study was unaffected by the jj locus. The glucuronidation of one other steroid, tetrohydrocortisone [1], was shown to be at normal levels in the homozygous Gunn rat.

Induction of transferase activities by phenobarbital in the RHA/jj strain, generally, was greatest for those substrates (Table 2, column 8) which were most severely reduced by the jj locus. Activities for 9-hydroxybenzo[*a*]pyrene and the 2-hydroxy- and 4-hydroxybiphenyls were induced in the homozygous and heterozygous strains such that the final absolute levels of activities were about the same in all three strains. The induction of *p*-nitrophenol transferase activity in the RHA/jj strain is consistent with two earlier studies [28, 29] with the homozygous Gunn rat. Thus, it appears that these various activities are affected differently by the recessive locus suggesting more than one defect or that one defect has an unequal effect on activities.

Other membrane properties such as cytochrome P-450 content, and aminopyrine demethylase and cytoshrome *c* reductase activities were unaffected in the congenic RHA/jj strain. A study by Gourley *et al.* [30] showed a small diminution (20%) in aminopyrine demethylase activity in homozygous Gunn rat microsomes. Our results suggest, however, that a defective membrane is not the primary lesion in the congenic rat and that the microenvironment is most likely the same for the three strains. The report that purified transferase from the Gunn rat [31], unlike the purified enzyme from the Wistar rat, was subject to activation by diethylnitrosamine suggests that at least one enzyme specified by the jj locus is structurally defective. A structural defect in transferase could cause aberrant conformations and, thus, affect enzyme insertion into the membrane and microenvironment.

Since most substrate activities in this study were decreased in the jaundice strain, RHA/jj, any model concerned with the primary lesion must account for the pleiotropic effect on activities. If more than one defect exists, e.g. a membrane component as well as a structural enzyme, and the defects are not closely linked, one should be able to generate recombinant inbred lines, using the Wistar and the congenic RHA/jj strains, with separate defects. The congenic inbred RHA/jj strain described in this study should provide a genetic model for inheritable jaundice that is stable and reproducible.

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