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DECREASED CONJUGATION OF *p*-AMINOBENZOIC ACID IN THE ICTERIC NEWBORN GUNN RAT*

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Abstract—The metabolism of *p*-aminobenzoic acid in the icteric and non-icteric Gunn rat was studied. The urinary metabolites of *p*-aminobenzoic acid identified were the parent compound, *p*-aminohippuric acid and *p*-aminobenzoic acid glucuronide. The urinary data indicated that the icteric Gunn rat not only excreted significantly less of the *p*-aminobenzoic acid within 24 hr but also excreted significantly less of the compound as the glucuronide. In the newborn icteric rat, the major excretory product was the unchanged *p*-aminobenzoic acid, whereas in the adult most of the drug was excreted as the glycine conjugate. The data *in vivo* and *in vitro* indicated that the icteric newborn rat converts significantly less *p*-aminobenzoic acid to *p*-aminohippuric acid than does the non-icteric rat. The synthesis of *p*-aminohippuric acid is quite low in both genotypes at birth, but rapidly approaches adult levels at 30 days of age. In view of the data presented, the increased toxicity of *p*-aminobenzoic acid in the 2-day-old icteric Gunn rat may be the result of a decreased *p*-aminobenzoic acid metabolism in these rats due to a deficiency in the activity of the enzyme UDP-glucuronyltransferase and possibly glycine-*N*-acyltransferase.

The major pathways for the metabolism of *p*-aminobenzoic acid (PAB) in the rat are acetylation, glycine conjugation and glucuronidation. At low doses *p*-aminobenzoic acid is excreted primarily as *p*-acetamido-benzoic acid; however, as the dosage was increased, the percentage of dose excreted as the acetylated compound decreased while the excretion of the conjugated metabolites increased [1, 2].

The conjugation of *p*-aminobenzoic acid with glycine to form *p*-aminohippuric acid (PAH) is catalyzed by the enzyme glycine-*N*-acyltransferase (EC 2.3.1.13) [3]. There is general agreement that in the newborn mouse [4], rat [5] and human [6] the activity of this enzyme is quite low; however, in the mouse and rat a subsequent rapid increase in activity to adult levels occurs within a few weeks after birth.

The formation of *p*-aminobenzoic acid glucuronide (PABG) is catalyzed by UDP glucuronyltransferase (EC 2.4.1.17) [3]. The deficiency of this enzyme in the Gunn rat for the substrate bilirubin results in hyperbilirubinemia. Schmid *et al.* [7] reported that the jaundiced Gunn rat excreted significantly less glucuronic acid after the administration of *o*-aminobenzoic acid than non-jaundiced littermates. However, they reported no deficiency in the conjugation of *o*-aminobenzoic acid with glycine in the adult rat.

In view of a previous report [8] from this laboratory which indicated that *p*-aminobenzoic acid was significantly more toxic to the 2-day-old icteric Gunn rat than to its non-icteric littermate, this investigation was conducted to study the conjugation pathways of *p*-aminobenzoic acid for glycine and glucuronic acid in the icteric homozygous and non-icteric heterozygous Gunn rat.

MATERIALS AND METHODS

Animals. Male and female rats ranging in age from 2 to 137 days of age were employed. Heterozygous (Jj) and homozygous (jj) Gunn rats were selected from our colony, and Wistar rats were obtained from Carworth Farms.

Thin-layer chromatography of urinary metabolites. Female homozygous and heterozygous rats ranging in age from 6 to 137 days of age were given *p*-aminobenzoic acid (potassium salt) intraperitoneally. The rats were placed in metabolic cages after the injection and urine was collected 0–3, and in some cases up to 24, hr after injection. Urine was collected from the 6-day-old rats by stimulation of the perineum. Urinary metabolites were isolated using Eastman Silica gel chromatogram sheets with fluorescent indicator, in an *n*-butanol–acetic acid–water (4:1:1, v/v) solvent system [9]. The R_f values were 0.80, 0.62 and 0.28 for PAB, PAH and PABG respectively. Metabolites were identified by reference to standard compounds observed under ultraviolet light. Proof of identity of the glucuronide was made by incubating the urine with beta-glucuronidase and demonstrating the disappearance of the metabolite at the R_f tentatively identified as the glucuronide. Individual chromatogram spots were removed into stoppered test tubes and extracted with 0.1 N NaOH. Total diazotizable material was determined by the method of Bratton and Marshall [10].

Acetylation of PAB and PAH. The level of acetylated compounds in the urine was determined by the difference in total diazo reactants, measured by the methods of Bratton and Marshall, before and after the hydrolysis of the sample for 30 min in a boiling

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water bath with an equal volume of 1.2 N hydrochloric acid.

Plasma half-life of administered PAH. To compare renal clearance of PAH between genotypes, four groups of rats were given PAH at a dose of 200 mg/kg of body weight. The two older groups (31 ± 2- and 100 ± 10-day-old females) were given the drug intravenously, while the 2- and 7-day-old groups were given the drug intraperitoneally. In the two younger groups, consisting of both male and females, six to eight rats were sacrificed at each time period (0.5, 1.0, 1.5 and 2.0 hr after injection). In the two older groups, the 31-day-old rats were sacrificed at each time period, whereas in the adult, repeated blood samples were taken from the infra-orbital sinus at 10, 20, 30 and 40 min after injection.

Glycine conjugation in vivo. Four groups of heterozygous and homozygous Gunn rats of age 2, 7, 32 ± 3 and 100 ± 10 days were used in this part of the study. In order to obtain sufficient numbers of 2- and 7-day-old rats, both male and females were included in these groups, with female rats being used in the other groups. All rats were injected with PAB at a dosage of 200 mg/kg of body weight. Blood samples were taken at designated times after the administration of PAB after sacrificing the rats by decapitation. In the adult group, repeated blood samples were taken from the infra-orbital sinus.

An additional group of 7- and 100-day-old rats was given glycine intraperitoneally at a dosage of 500 mg/kg of body weight 30 min prior to the injection of PAB to determine if glycine was rate-limiting.

The isolation of plasma PAH was made by the method of Cohen and McGilvery [11] using a buffer of 0.4 M disodium phosphate adjusted to a pH of 3.95 with 0.2 M citric acid. The quantitation of PAH and total diazotizable compounds in plasma was measured by the method of Bratton and Marshall [10].

Liver glycine conjugation in vitro. Rats representing four age groups (2, 7, 32 ± 2 and 107 ± 5 days) were used in this phase of the study to determine the development of the PAH-synthesizing system. Male and female heterozygous and homozygous Gunn rats and adult male Wistar rats were studied. The two older groups of rats were fasted for 16–18 hr before sacrifice and were given 5% dextrose in their drinking water during this time. These rats were killed by decapitation and the livers perfused with cold 0.25 M sucrose and removed. The 2- and 7-day-old rats were killed by decapitation and the livers pooled in order to obtain sufficient tissue. The livers were homogenized in cold 0.25 M sucrose using a motor-driven Potter–Elvehjem homogenizer with a Teflon pestle. A 10% (w/v) homogenate was used with the exception that for the 2- and 7-day-old groups a 20% (w/v) homogenate was prepared because of the low enzyme activity at this age.

The incubation procedure used was a modification of the method reported by Gorodischer *et al.* [4]. The incubation mixture contained: potassium phosphate buffer (pH 7.56), 25 μmoles; $MgCl_2$, 5 μmoles; glycine, 100 μmoles; furmarate, 2.5 μmoles; ATP, 2.5 μmoles; PAB, 3.0 μmoles; and 0.3 ml of a 10 or 20% liver homogenate. The final volume of 1 ml was incubated for 30 min at 37° in a Dubnoff metabolic shaker. The reactions were stopped by the addition of 4 ml of ice-cold 0.2 N trichloro-acetic acid to each flask. The isolation and quantitation of PAH from the incubation medium were by the method of Cohen and McGilvery [11] and Bratton and Marshall [10] respectively.

Protein levels were determined by the method of Lowry *et al.* [12] with bovine serum albumin as the standard.

Statistical procedures. Statistical comparisons were made by Student's *t*-test with the level of significance set at $P < 0.05$. Plasma $T_{\frac{1}{2}}$ values for PAH were calculated from a least squares exponential curve fit program using a Wang computer. Differences in the $T_{\frac{1}{2}}$ values of Table 2 were determined by first calculating the slopes by least squares linear regression, and then comparing slopes by the method described by Ostle [13]. The $T_{\frac{1}{2}}$ values were calculated by a method described by Notari [14] where $T_{\frac{1}{2}} = 0.5\ X_0/K_0$, where X_0 is the initial concentration. Initial drug concentrations were similar for both genotypes within age groups.

RESULTS

The recovery of total diazo compounds excreted in urine in a 24-hr period after PAB administration is presented in Fig. 1. The homozygous Gunn rat excreted significantly less of the administered drug during the 24-hr period after treatment in the 18-, 89- and 137-day age groups. The percentage of the dose recovered in the 24-hr period for both genotypes increased as the rats matured.

The urinary metabolites measured were the parent compound, PAB; the glycine conjugate, PAH; and the glucuronic acid conjugate, PABG. These three compounds were the only significant urinary metabolites observed after PAB administration. The distribution of these metabolites in the urine collected 0–3 hr after injection is presented in Fig. 2. With the exception of the PAH values for the 6-day-old rats, there were significant differences between genotypes for each metabolite and age group. As the heterozygous rat matured, there appeared to be a decrease in the

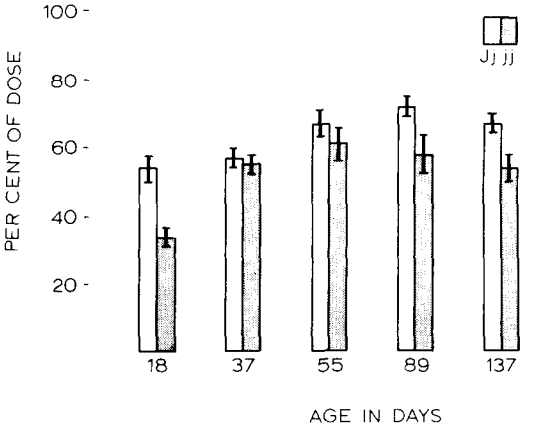

Fig. 1. Twenty-four-hr urinary excretion of PAB and diazotizable metabolites in the Gunn rat. Female rats were injected i.p. with PAB at a dose of 400 mg/kg of body weight. Each column represents the mean ± S.E.M. from a minimum of five rats.

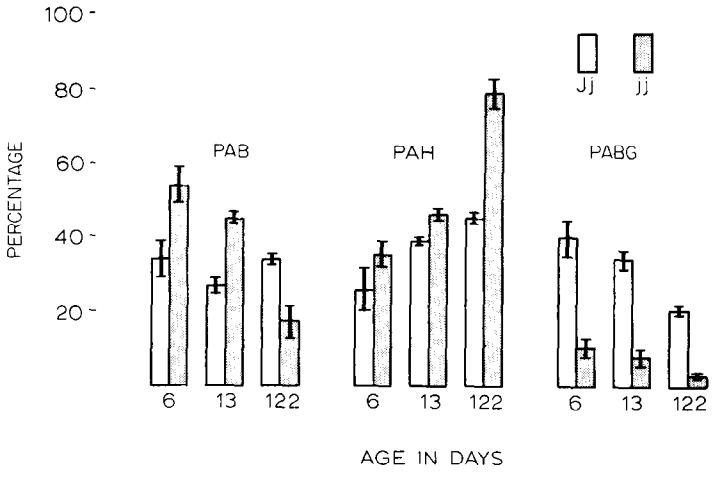

Fig. 2. Urinary excretion of PAB, PAH and PABG 0–3 hours after PAB was given to the Gunn rat. Male and female rats were injected i.p. with PAB at a dose of 400 mg/kg of body weight. The data are expressed as the percentage of the total excretion of PAB, PAH and PABG. Each column represents the mean ± S.E.M. from a minimum of seven rats.

percentage of PAB excreted as the glucuronide with a corresponding increase in the amount excreted as PAH. The glucuronide of PAB was significantly lower in the urine of the homozygous rat, and the majority of PAB was excreted either as PAB or as PAH. In the 6-day-old homozygous rat, most of the administered PAB was excreted as the parent compound, whereas in the adult the glycine conjugate is the major urinary metabolite. In the adult rats 0–24 hr post-treatment urine samples were collected and the percentages of diazotized metabolites were PAB, 22 per cent; PAH, 58 per cent; and PABG, 20 per cent for the heterozygous rat; and PAB, 17 per cent; PAH, 80 per cent; and PABG, 3 per cent for the homozygous rat. The values for PAH and PABG metabolites were significantly different between genotypes.

Plasma half-life values for injected PAH were similar for both genotypes. The 2-day-old rats had $T_{\frac{1}{2}}$ values of 28 and 25 min, while the adult rats had values of 11 and 14 min, respectively, for the heterozygous and homozygous rats.

Glycine conjugation *in vivo* was determined by measuring plasma levels of PAH after i.p. injection of PAB. The data in Table 1 indicated that the 2-, 7- and 32-day-old homozygous rats had significantly lower plasma levels of PAH than did the heterozy-

Table 1. Glycine conjugation *in vivo*: plasma levels of *p*-aminohippuric acid in the Gunn rat after PAB and glycine administration*

			PAH (μmoles/100 ml plasma)						
			Hr after PAB injection						
Age	Genotype	N†	0.5	1.0	1.5	2.0	3.0	5.0	7.0
2 Day	Jj	3	13 ± 2		32 ± 2		38 ± 5	35 ± 2	20 ± 5
	jj	3	6 ± 1‡		6 ± 1‡		6 ± 2‡	5 ± 1‡	4 ± 4‡
7 Day	Jj	5	8 ± 2	14 ± 2	14 ± 2	18 ± 2	26 ± 2	22 ± 1	12 ± 2
	jj	5	2 ± 1‡	2 ± 1‡	3 ± 1‡		6 ± 1‡	4 ± 0‡	4 ± 2‡
7 Day-gly	Jj	3	9 ± 2		20 ± 1		22 ± 1		
	jj	3	3 ± 1‡		3 ± 1‡		5 ± 1‡		
32 Day	Jj	5	21 ± 2	20 ± 2	13 ± 2	10 ± 2	0 ± 0		
	jj	4	12 ± 0‡	9 ± 1‡	12 ± 1	9 ± 1	0 ± 0		
100 Day	Jj	5	16 ± 2	20 ± 3	15 ± 2	11 ± 2	1 ± 1		
	jj	5	12 ± 2	14 ± 2	16 ± 2	14 ± 2			
100 Day-gly	Jj	3	35 ± 5§	38 ± 4§	23 ± 6	12 ± 2	0 ± 0		
	jj	3	48 ± 2§	42 ± 2§	19 ± 11	6 ± 3	0 ± 0		

* PAB was injected i.p. at a dose of 200 mg/kg of body weight. A group of 7- and 100-day-old rats was injected with glycine i.p. at a dose of 500 mg/kg of body weight 30 min prior to the injection of PAB. Values are expressed as mean ± S.E.M.

† N = the number of determination, at each time period, representing two to three rats/determination for the 2- and 7-day-old groups; the number of rats sacrificed/time period for the 32-day-old group; and the number of rats from which repeated blood samples were taken for the 100-day-old group.

‡ Values were significantly different ($P < 0.05$) from heterozygous values of the same age.

§ Values were significantly different ($P < 0.05$) from the non-glycine-treated values of the same age and genotype.

Table 2. Plasma half-life of PAB plus total diazotizable compounds in the Gunn rat*

Age (days)	N†	Plasma $T_{\frac{1}{2}}$ (min) Jj	jj
2	3	244	351‡
7	5	297	326
31	4	64	70
100	5	74	82

* PAB was injected i.p. at a dose of 200 mg/kg of body weight.

† N = the number of determinations, per genotype at each sampling period (two to three rats/determination) for the 2- and 7-day-old groups; the number of rats sacrificed/genotype at each sampling period for the 31-day-old group; and the number of rats/genotype from which repeated blood samples were taken for the 100-day-old group.

‡ The value was significantly different ($P < 0.05$) from the heterozygous value for the same age group.

gous rats. There were no apparent differences in genotypes for the adult groups studied. The administration of glycine to the 7-day-old rats did not alter plasma PAH levels or reduce the plasma half-life of total diazo compounds for either genotype. However, in the adult the plasma PAH levels were significantly increased in the glycine-treated rats for both genotypes.

The $T_{\frac{1}{2}}$ values for total plasma diazo reactants after the administration of PAB in the four groups of rats studied are given in Table 2. The 2-day-old homozygous rats had a significantly longer plasma half-life than the heterozygous rats of the same age.

The studies *in vitro* indicated that glycine conjugation in the liver was quite low in the newborn Gunn rat, while almost reaching adult levels at about 30 days of age (Table 3). Hippuric acid synthesis was significantly lower in the 2-day-old homozygous rats when compared to the heterozygous littermates; however, in the 32-day- and 107-day-old rats the homozygous male rats were significantly higher than the heterozygous males. Within genotype, the formation of hippuric acid was significantly higher in the adult heterozygous female and the adult homozygous male.

DISCUSSION

It was reported previously [8] that *p*-aminobenzoic acid was found to be significantly more toxic to the 2-day-old icteric Gunn rat than to its non-icteric littermate. Initially, it was hypothesized that the mechanism for toxicity in the newborn icteric rat was the drug-mediated displacement of bilirubin from plasma proteins resulting in increased mortality due to kernicterus. The administration of PAB or PAH has been shown [15] to reduce plasma bilirubin levels in the adult icteric Gunn rat; however, more recent data from our laboratory (D. R. Davis, unpublished data) suggested that the displacement of bilirubin in the icteric newborn was not as marked as in the adult. Studies *in vitro* [15] indicated that neither PAB nor PAH was a strong competitor for the bilirubin–albumin binding site. Although PAB was found to be more toxic to the icteric newborn rats, preliminary findings showed (R. A. Yeary, unpublished data) that PAH, the major metabolite of PAB, was not significantly more toxic to these rats.

In view of the well-known UDP glucuronyltransferase deficiency in the icteric newborn Gunn rat for bilirubin and other substrates, the possibility exists for alterations in the metabolism of PAB in these young rats.

The urinary data clearly demonstrated a deficiency in the ability of the homozygous Gunn rat to conjugate PAB with glucuronic acid. The data also indicated a possible compensatory increase in the excretion of PAH especially as the rat matures. These findings are in agreement with the report of Schmid *et*

Table 3. Glycine conjugation *in vitro* of *p*-aminobenzoic acid in the Gunn rat*

Age (days)	Genotype	Sex†	N‡	Protein (mg/g of wet liver)	PAH (nmoles/mg liver protein/ 30 min)
2	Jj	B	6	154.0 ± 4.0	2.76 ± 0.21
	jj	B	6	153.3 ± 5.5	1.97 ± 0.23§
7	Jj	B	7	148.4 ± 3.5	3.99 ± 0.38
	jj	B	6	154.7 ± 5.6	3.49 ± 0.06
32	Jj	M	5	193.2 ± 11.4	34.50 ± 2.17
	Jj	F	5	216.8 ± 5.0	38.84 ± 3.50
	jj	M	5	194.2 ± 13.0	41.77 ± 2.80§
	jj	F	5	200.4 ± 8.8	34.43 ± 3.50
107	Jj	M	7	198.4 ± 5.9	35.92 ± 3.49
	Jj	F	5	226.2 ± 1.9	50.49 ± 1.38
	jj	M	8	192.6 ± 4.1	57.38 ± 3.49§
	jj	F	5	222.4 ± 3.2	47.30 ± 1.85
101	Wistar	M	4	196.0 ± 4.7	60.42 ± 4.34

* Data are expressed as the mean ± S.E.M.

† F = female, M = male and B = both sexes.

‡ Number of rats/group with the exception that for the 2- and 7-day-old groups, N equals the number of determinations where two to four rat livers were pooled/determination.

§ Values were significantly different ($P < 0.05$) from heterozygous values of the same age group and sex.

al. [7]. They observed both a significant decrease in the amount of glucuronic acid excreted after the administration of *o*-aminobenzoic acid and an increased excretion of hippuric acid in the jaundiced Gunn rat. The decreased quantity of the total diazotizable compounds excreted in 24 hr and the large percentage of the free compound excreted in the icteric newborn suggest a decreased metabolism of PAB in these young rats.

Preliminary findings (D. R. Davis, unpublished data) in this laboratory suggested that after an i.p. injection of PAB at a dose of 400 mg/kg of body weight, 5–10 per cent of the recovered urinary metabolites was acetylated. The level of acetylation was found to be inversely related to dose; however, no significant genotype differences were observed. Although the acetylation pathway is quite pronounced when small doses of PAB are given [11], this pathway apparently is readily saturable, and at the large doses used in this study, this pathway of excretion was considered to be insignificant.

The data in Table 1 illustrated that the level of plasma PAH after the administration of PAB was significantly lower in the homozygous rat at 2, 7 and 32 days of age. To our knowledge these are the first reported data to suggest that the hippuric acid-synthesizing system may be deficient in the newborn icteric Gunn rat. The similarity of the plasma half-lives of PAH in the two genotypes precludes the possibility of differences in renal clearance which could explain the observed differences in plasma PAH levels. The result of administering exogenous glycine on hippuric acid synthesis *in vivo* indicated that glycine may be rate-limiting in the adult but not in the newborn rat.

The longer plasma half-lives for the elimination of total diazotizable compounds in the homozygous rat further support the hypothesis of decreased PAB metabolism in these rats. The total diazotizable compounds is a composite of PAB, PAH and PABG, since each has a diazotizable aromatic amine.

The age dependency of hippuric acid synthesis as seen in Table 3 is in agreement with other reports [4, 5]. The synthesis of PAH is quite low in the newborn; however, the level increases very rapidly to near adult levels at 30 days of age. The significantly lower synthesis in the 2-day-old icteric rats is in agreement with the data *in vivo*. In the male rats, there was evidence of a compensatory increase in the synthesis of PAH as the rats matured (similar to the urinary data). PAH synthesis was significantly higher in the homozygous male rats at 32 and 107 days of age when compared to heterozygous male rats of the same age. This finding is in similar agreement with the recent report of Marniemi *et al.* [16], which found glycine-*N*-acyltransferase activity to be significantly higher in the homozygous Gunn rat when compared to the Wistar rat. Our data from the female rats showed no apparent genotype differences; however, with respect to both sexes there does appear to be some sex–genotype interaction which is not readily explained. Sex differences in drug metabolism in the rat, however, are not uncommon.

The apparent differences between the urinary data, which might be interpreted as evidence for increased synthesis of PAH in the homozygous rat and the data *in vivo* and *in vitro*, which suggest a deficiency of this pathway in the homozygous rat, may possibly be explained in view of the significantly lower excretion of total diazotizable compounds in the homozygous rat.

These data indicated that for the first few days of life the icteric newborn rat is deficient in the glycine conjugation of PAB; however, as the rat matures, hippuric acid synthesis appears to be normal. Levy and Ertel [17] have reported normal glycine conjugation in hyperbilirubinemic children, ages 3 and 9 years, with Crigler–Najjar syndrome. If the decreased glycine conjugation in the icteric newborn rat is associated with the relatively high levels of serum bilirubin, then it is possible that a similar decrease may occur in the jaundiced newborn infant. Although Vest and Salzberg [6] have reported hippuric acid synthesis in premature infants to be approximately one-fourth adult values, no serum bilirubin levels were reported.

It is suggested from these studies that an increased toxicity of PAB to the icteric newborn rat [8] may be the result of a decreased metabolism of this compound by the glucuronidation and glycine conjugation pathways.

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