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# Bilirubin Binding to Hepatic Y and Z Protein (LIGANDIN): Tissue Bilirubin Concentrations in Phenobarbital Treated Gunn Rats[1] (38466)

DAVID R. DAVIS AND ROGER A. YEARY

*Department of Veterinary Physiology and Pharmacology, The Ohio State University, Columbus, Ohio 43210*

In view of its inductive effect on bilirubin glucuronyltransferase to lower plasma bilirubin levels in the hyperbilirubinemic infant, phenobarbital therapy has recently been widely reported as an aid in the prevention and/or treatment of neonatal jaundice. DeLeon and coworkers (1) reported that in the hyperbilirubinemic Gunn rat that phenobarbital treatment for 2 or 12 wk had no effect on plasma bilirubin levels. However, repeated experiments in our laboratory with the icteric Gunn rat demonstrated a significant decrease in plasma bilirubin levels after only 4 days of phenobarbital treatment.

Bilirubin along with many other organic anions have been shown to be bound to cytoplasmic proteins Y and Z in the liver (2). These proteins are thought to facilitate both the uptake and the storage of organic anions by the liver. The induction of Y protein (3), the major organic anion binding protein in the liver, by phenobarbital suggests a possible mode of action of phenobarbital on tissue bilirubin distribution which is independent of its microsomal enzyme inductive effects. This study investigated the effects of phenobarbital treatment on the tissue distribution of bilirubin as affected by bilirubin binding to cytoplasmic liver proteins Y and Z in the icteric Gunn rat.

*Materials and Methods.* Gunn and Sprague–Dawley rats 70–90 days of age were used. Experimental animals received daily ip injections of sodium phenobarbital (NaP) (80 mg/kg) while the controls were given an equivalent volume of saline.

Plasma bilirubin levels were measured in male and female homozygous (jj) Gunn rats injected for 14 days with either NaP or saline. Blood samples were collected with heparinized capillary tubes from the infraorbital sinus under light ether anesthesia. Plasma bilirubin levels were determined by the method of Jendrassik and Grof (4) as modified by Nosslin (5) and Michaelson (6).

The effects of NaP on liver UDP-glucuronyltransferase activity were studied in male rats of the Sprague–Dawley heterozygous (Jj) and homozygous (jj) Gunn strains. Rats were terminated by decapitation approximately 24 hr following the last of four or seven daily injections. The livers were rapidly excised and placed on ice in chilled 0.154 *M* KCL. A 10% (w/v) homogenate was prepared in cold 0.154 *M* KCL using a motor-driven Potter–Elvhjem homogenizer with a Teflon pestle. Duplicate enzyme analyses for glucuronyltransferase activity were performed on each rat liver using *p*-nitrophenol and bilirubin as substrates. Glucuronidation of *p*-nitrophenol was determined by a modification of the method of Yeary and Fleming (7), using a final UDPGA concentration of 3.0 m*M*. Enzyme activity is expressed as μmoles of *p*-nitrophenol that disappeared per g of liver during the 15 min incubation. The assay system for bilirubin glucuronyltransferase activity was a modification of the method of DeLeon and coworkers (1). The incubation system contained 0.044 *M* sodium phosphate buffer pH 7.4, 8.8 m*M* $mgcl_2$, 8 mg of bovine serum albumin, 0.30 m*M* bilirubin, 2.65 m*M* UDPGA and 100 mg of liver in a final volume of 3.4 ml. The determination of bilirubin glucuronide was made by the method of Van Roy and Heirwegh (8). Enzyme activity is expressed as the increase in optical density at 530 nm corrected for an incubated reagent blank.

The crystallization of $^{14}$C-bilirubin from dog bile following the iv administration of δ-aminolevulinic acid-4 $^{14}$C was performed as described by Barrett (9). Three groups of male homozygous (jj) Gunn rats were used in this phase of the study. Twenty-four hours after the last of four or 14 daily injections of

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NaP or saline all rats were injected iv with $^{14}$C-bilirubin. Twenty-four hours were allowed for equilibration of the labelled bilirubin with the total miscible bilirubin pool before the rats were terminated. Rats in Groups I and II were both injected for 4 days with either NaP or saline, while Group III was injected for 14 days. Groups I and II differed in that Group I was exsanguinated by cardiac puncture followed by decapitation while Group II was exsanguinated by cardiac puncture followed by perfusion of the rats with cold saline. Following the correction (10) of the data from Groups I and II for residual tissue plasma, based on a blood vol of 60 ml/kg BW, the data were pooled. Two controls and three treated rats were utilized in each of the groups. All rats in Group III were perfused with cold saline and similarly corrected for residual tissue plasma. Tissues were excised, rinsed in saline, blotted, weighed, and frozen. Approximately 100 mg of tissue were solublized in 1.0 ml of Soluene (Packard Instrument Co.) and liquid scintillation counting was performed using Aquasol (New England Nuclear) as the scintillation cocktail.

Liver anion-binding proteins Y and Z were isolated by sephadex G-75 column chromatography (2). An aliquot of 110,000 *g* supernatant, equivalent to 4 g of liver was placed on the column. The supernatant was eluted from the column with 0.01 *M* phosphate buffer, pH 7.4, at 4°. The radioactivity and the absorbance at 460 and 280 nm were determined for each 3.5 ml fraction to substantiate that $^{14}$C-radioactivity represented bilirubin binding to Y and Z proteins.

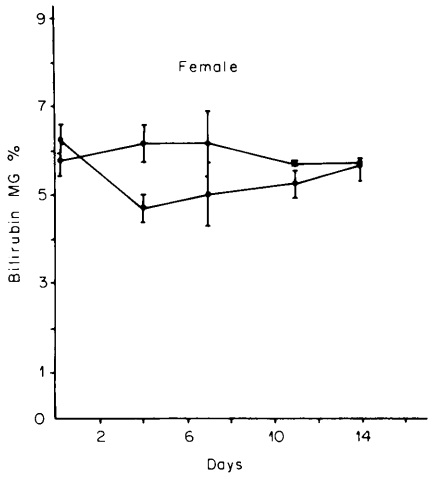


FIG. 1. The effects of phenobarbital on plasma bilirubin levels in the adult homozygous Gunn rat. The rats were given daily injections of sodium phenobarbital (80 mg/kg ip) or an equivalent volume of saline for 14 days. The data are expressed as the mean ± SEM of three control (○——○) and five treated (●——●) rats.

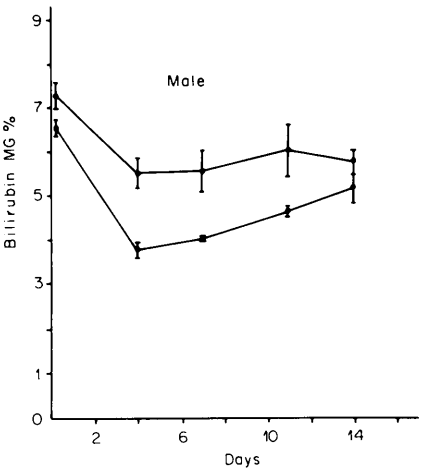


FIG. 2. The effects of phenobarbital on plasma bilirubin levels in the adult homozygous Gunn rat. The rats were given daily injections of sodium phenobarbital (80 mg/kg ip) or an equivalent volume of saline for 14 days. The data are expressed as the mean ± SEM of three control (○——○ upper curve) and five treated (●——●) rats.

Statistical comparisons of the data were made by Student's *t* test. The level of significant difference was set at $P < 0.05$.

*Results.* A comparison of the changes in plasma bilirubin concentration for the control and NaP treated rats from zero time concentration indicated significant effects of drug treatment. Daily NaP treatment of the (jj) Gunn rat resulted in a significant lowering of plasma bilirubin in both sexes after 4 days of NaP treatment (Figs. 1 and 2). There was a tendency for bilirubin levels to return to pretreatment values by the 14th day of continuous treatment.

Phenobarbital treatment resulted in similar increases in *p*-nitrophenol glucuronidation for both Gunn rat genotypes (Fig. 3). However, the increase above control values of

135 and 136% for the heterozygous and homozygous Gunn rats respectively was considerably less than the 207% increase exhibited by normal Sprague–Dawley rats. Bilirubin glucuronidation increased 260% above control values in the Sprague–Dawley, 126% in the heterozygous but did not increase in the homozygous Gunn rats following 7 days of phenobarbital treatment (Fig. 4). Measurable bilirubin glucuronide was not found in the incubation system for the homozygous (jj) Gunn rat.

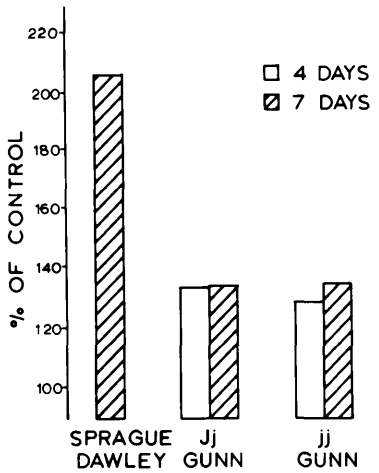


FIG. 3. The effects of phenobarbital on *in vitro* glucuronidation of *p*-nitrophenol in liver rat homogenates. A minimum of four adult male rats per treatment were given four or seven daily injections of sodium phenobarbital (80 mg/kg ip) or an equivalent volume of saline.

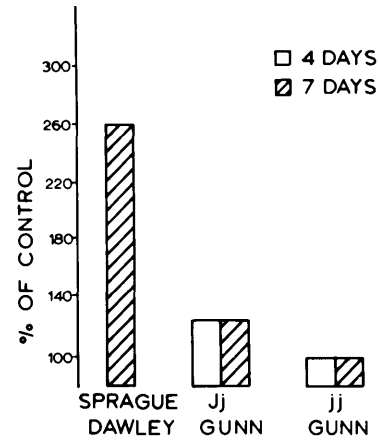


FIG. 4. The effects of phenobarbital on *in vitro* glucuronidation of bilirubin in rat liver homogenates. A minimum of four adult male rats per treatment were given four or seven daily injections of sodium phenobarbital (80 mg/kg ip) or an equivalent volume of saline.

The distribution of $^{14}$C-bilirubin in the untreated (jj) Gunn rat (Tables I and II) indicated that the major bilirubin binding tissues were liver, carcass, skin, gastrointestinal tract and blood. NaP treatment for 4 days (Table I) resulted in a significant increase in the percent of recovered $^{14}$C-bilirubin found in the liver and a significant decrease in the gastrointestinal tract. However, the decrease in the gastrointestinal tract can be accounted for by differences in $^{14}$C-bilirubin recovered in the feces. Although the decreased tissue bilirubin levels of blood and carcass were not significant, they account for most of the increased recoverable $^{14}$C-bilirubin observed in the liver tissue.

The treatment of (jj) Gunn rats for 14 days

TABLE I. DISTRIBUTION OF $^{14}$C-BILIRUBIN IN THE GUNN RAT FOLLOWING PHENOBARBITAL TREATMENT.[a]

| Tissue | Control[b] | Na phenobarbital[b] |
|---|---|---|
| Heart | 0.15 ± 0.01 | 0.13 ± 0.01 |
| Lung | 0.73 ± 0.12 | 0.57 ± 0.05 |
| Liver | 11.46 ± 0.56 | 14.01 ± 0.40[c] |
| Spleen | 0.20 ± 0.03 | 0.19 ± 0.04 |
| Kidney | 1.35 ± 0.10 | 1.30 ± 0.07 |
| Testes | 0.54 ± 0.02 | 0.55 ± 0.03 |
| Brain | 0.05 ± 0.01 | 0.07 ± 0.02 |
| Epididymal fat | 0.59 ± 0.02 | 0.47 ± 0.05 |
| Renal fat | 0.51 ± 0.02 | 0.44 ± 0.03 |
| Carcass | 9.95 ± 0.83 | 8.70 ± 0.73 |
| Skin | 11.51 ± 0.68 | 12.03 ± 1.05 |
| Pancreas-mesentery | 0.91 ± 0.09 | 0.71 ± 0.08 |
| GI tract + contents | 30.93 ± 3.68 | 22.89 ± 2.51[c] |
| Urine | 6.25 ± 0.49 | 6.44 ± 0.39 |
| Feces | 7.77 ± 3.73 | 16.53 ± 3.33 |
| Blood | 17.09 ± 1.45 | 14.99 ± 1.24 |

[a] Rats were given four daily injections of either sodium phenobarbital (80 mg/kg ip) or an equivalent volume of saline (Group I and II).

[b] Data are the mean and standard error of the mean expressed as the % of the recovered $^{14}$C-bilirubin from four control and six treated rats.

[c] Significantly different from the control rats ($P < 0.05$).

with phenobarbital resulted in a more pronounced alteration in tissue bilirubin levels (Table II). The percent of recovered $^{14}$C-bilirubin was significantly increased in the liver, while bilirubin levels significantly decreased in kidney, skin and blood tissues.

TABLE II. DISTRIBUTION OF $^{14}$C-BILIRUBIN IN THE GUNN RAT FOLLOWING PHENOBARBITAL TREATMENT.[a]

| Tissue | Control[b] | Na phenobarbital[b] |
|---|---|---|
| Heart | 0.25 ± 0.06 | 0.16 ± 0.00 |
| Lung | 1.21 ± 0.06 | 1.67 ± 0.27 |
| Liver | 13.41 ± 0.53 | 18.86 ± 1.08[c] |
| Spleen | 0.21 ± 0.01 | 0.27 ± 0.03 |
| Kidney | 1.60 ± 0.03 | 1.37 ± 0.10[c] |
| Testes | 0.64 ± 0.04 | 0.53 ± 0.08 |
| Brain | 0.06 ± 0.00 | 0.06 ± 0.00 |
| Epididymal fat | 0.87 ± 0.06 | 1.16 ± 0.28 |
| Renal fat | 0.67 ± 0.10 | 0.52 ± 0.10 |
| Carcass | 11.99 ± 1.35 | 9.98 ± 1.65 |
| Skin | 14.17 ± 0.90 | 11.39 ± 0.84[c] |
| Pancreas-mesentery | 1.59 ± 0.29 | 1.35 ± 0.20 |
| GI tract + contents | 17.91 ± 4.61 | 23.41 ± 2.45 |
| Urine | 3.27 ± 0.17 | 3.58 ± 0.08 |
| Feces | 14.53 ± 4.93 | 10.36 ± 1.65 |
| Blood | 17.63 ± 0.79 | 15.33 ± 0.73[c] |

[a] Rats were given 14 daily injections of either sodium phenobarbital (80 mg/kg ip) or an equivalent volume of saline (Group III).

[b] Data are the mean and standard error of the mean expressed as the % of the recovered $^{14}$C-bilirubin from four control and four treated rats.

[c] Significantly different from the control rats ($P < 0.05$).

The effects of phenobarbital treatment on bilirubin binding to liver proteins Y and Z are indicated in Table III. Bilirubin binding to both Y and Z proteins was significantly increased with phenobarbital treatment in both the 4- and 14-day treated groups. A more pronounced increase in bilirubin binding to Y and Z proteins was present after the longer phenobarbital treatment period.

*Discussion.* The data in this report demonstrated that NaP significantly lowered plasma bilirubin levels in the (jj) Gunn rat with only 4 days of treatment. These findings are in disagreement with the publication by DeLeon and coworkers (1) which reported no significant effects of NaP on serum bilirubin levels in the Gunn rat. However, these workers may have missed the initial effects of NaP on plasma bilirubin because of the sampling schedule employed. The glucuronidation data are in agreement with the reports of others (1, 11).

Phenobarbital treatment for 4 days resulted in a redistribution of bilirubin from the blood to the liver tissue. The data in Table I do not show a significant decrease in the percent of $^{14}$C-bilirubin recovered in blood with NaP treatment, however, the plasma bilirubin concentration was signifi-

TABLE III. THE EFFECTS OF PHENOBARBITAL TREATMENT ON THE BINDING OF $^{14}$C-BILIRUBIN TO LIVER ANION BINDING PROTEINS Y AND Z.

| Treatment | | μg bilirubin/4 g liver[a] | | μg bilirubin/100 g BW[a] | |
|---|---|---|---|---|---|
| | | Y | Z | Y | Z |
| 4 day[c] | | | | | |
| Control | (4) | 24.10 ± 3.05 | 4.99 ± 1.68 | 19.71 ± 2.76 | 4.23 ± 1.31 |
| Treated | (6) | 46.12 ± 6.73[e] | 7.87 ± 1.29 | 54.32 ± 7.33[e] | 8.55 ± 1.41[e] |
| 14 day[d] | | | | | |
| Control | (4) | 14.40 ± 5.07 | 4.67 ± 1.21 | 14.88 ± 5.27 | 4.82 ± 1.26 |
| Treated | (4) | 58.55 ± 4.80[e] | 12.32 ± 0.89[e] | 80.39 ± 7.68[e] | 15.56 ± 2.52[e] |

[a] Data are mean and standard error of the mean.

[b] (N) = number of rats per group.

[c] Rats were given four daily injections of sodium phenobarbital (80 mg/kg ip) or an equivalent volume of saline (Group I and II).

[d] Rats were given 14 daily injections of sodium phenobarbital (80 mg/kg ip) or an equivalent volume of saline (Group III).

[e] Significantly different from control rats ($P < 0.05$).

cantly lower. The loading effect of the injected $^{14}$C-bilirubin appears to equalize plasma bilirubin concentrations in the treated animals.

A number of possible mechanisms exist for explaining the decrease in plasma bilirubin following NaP treatment. However, such mechanisms as biotransformation (12), increased biliary excretion (12, 13), displacement (14) and changes in membrane permeability have not been demonstrated in NaP treated (jj) Gunn rats.

If the distribution of tissue bilirubin is dependent on the number and the apparent affinity of the binding sites for this substance, then the induction of liver organic anion binding protein Y by phenobarbital (3) is of considerable interest. Uptake of bilirubin and a variety of organic anions by the liver cell is facilitated by a process involving cytoplasmic proteins Y and Z (2, 3). Furthermore, these proteins appear also to be involved in intracellular transport and storage of organic anions.

It is suggested that NaP treatment resulted in the induction of Y protein synthesis in the liver and an associated increased capacity for uptake and storage of bilirubin by this organ. The accompanying decrease in bilirubin concentrations in blood and skin, though transient, are consistent with redistribution and the establishment of a new tissue bilirubin equilibrium.

*Summary.* NaP treatment significantly reduced plasma bilirubin levels in the (jj) Gunn rat. It is suggested that NaP significantly increased extravascular bilirubin binding in the liver by induction of the synthesis of Y anion binding protein. The changes in bilirubin concentration of blood and other tissues subsequent to NaP treatment were consistent with the establishment of a new tissue bilirubin equilibrium.

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