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Bilirubin neonate
hyperbilirubinemia sulfadimethoxine

Effect of Sulfadimethoxine on Tissue Distribution of [^{14}C]Bilirubin in the Newborn and Adult Hyperbilirubinemic Gunn Rat

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Extract

Sulfadimethoxine significantly reduced plasma bilirubin levels and altered the tissue bilirubin distribution in both the newborn and the adult Gunn rat. The majority of the unbound bilirubin appeared to distribute preferentially to the intestine and liver in the newborn, whereas in the adult the unbound bilirubin was taken up primarily by the liver. The bilirubin content of brain tissue from both age groups was significantly higher after sulfadimethoxine treatment.

Speculation

A decrease in the available extravascular bilirubin binding sites, indicated by an increased concentration of bilirubin in the carcass and an apparent decreased capacity of the liver of the newborn Gunn rat to bind bilirubin, would suggest that kernicterus in the Gunn rat neonate is associated with increased bilirubin concentrations in the brain. However, the similar uptake of bilirubin by the brain tissue of both the newborn and the adult Gun rat raises the question of increased sensitivity of the newborn brain to bilirubin toxicity.

Sulfonamide therapy in hyperbilirubinemic newborn infants has resulted in a significant increase in the incidence of kernicterus accompanied by a decrease in serum bilirubin levels (21). Johnson (9) reported a similar syndrome for the young hyperbilirubinemic Gunn rat. The significance of the decrease in plasma bilirubin levels during sulfonamide therapy was not recognized until Odell (16, 17) proposed that certain anions including sulfonamides were capable of displacing protein-bound bilirubin from albumin. This concept stimulated widespread interest in the competitive protein binding of anions and shifted the clinical interests from total plasma bilirubin levels to free or unbound plasma bilirubin (5, 10, 14).

Since it is the unbound bilirubin which freely diffuses into extravascular compartments such as neural tissue (2, 20), where its more serious toxic effects may be manifest, it is logical to examine the tissue distribution of unbound bilirubin in the presence of a drug that can displace bilirubin from serum albumin at therapeutic serum levels. Furthermore, since kernicterus is a comparatively rare phenomenon in hyperbilirubinemic adults and there is disagreement on age-related permeability of the blood-brain barrier to bilirubin, comparable studies in newborn and adult animals were undertaken. For this purpose the Gunn rat was chosen as the animal model. We have found (23) sulfadimethoxine to be significantly more toxic when injected into the 2-day-old icteric Gunn rat (LD_{50} = 63 mg/kg, 95% confidence

limits 49–82) than in the nonicteric heterozygote (LD_{50} = 770 mg/kg, 95% confidence limits 358–1655). However, the acute toxicity in the adult rats of either genotype was similar (LD_{50} = 1,110 mg/kg). The authors are aware of only limited studies of tissue distribution of bilirubin in the Gunn rat (3, 4, 19) and its redistribution in the presence of drugs that interfere with the binding of bilirubin to serum albumin (6). This is a report of studies designed to examine the tissue distribution of [^{14}C]bilirubin after its displacement from albumin by sulfadimethoxine in the hyperbilirubinemic newborn and adult Gunn rat.

METHODS

Icteric homozygous (jj) Gunn rats selected from our breeding colony were used for this study. Two groups of 2-day-old rats of both sexes and one group of adult female rats were employed.

Crystalline [^{14}C]bilirubin was prepared from dog bile after the intravenous administration of δ-[4-^{14}C]aminolevulinic acid (24) as described by Barrett (1). The injectable bilirubin was prepared by dissolving crystalline [^{14}C]bilirubin in 0.1 N sodium hydroxide and adjusting to pH 8.0–8.5 with 0.15 M phosphate buffer and 1% glacial acetic acid. This solution was made up to volume with heterozygous Gun rat serum. Plasma bilirubin levels were determined by the method of Jendrassik and Grof (8) as modified by Nosslin (15) and Michaelson (13). The specific activity of bilirubin was determined at the time of killing each animal by dividing the ^{14}C radioactivity per ml plasma by the micrograms of bilirubin per ml plasma, as determined by the Jendrassik method. The total bilirubin pool was determined by dividing the total ^{14}C radioactivity recovered by the specific activity of the plasma and correcting for the bilirubin injected and the bilirubin excreted during the equilibration period. It is recognized that the radioactivity measurement of [^{14}C]bilirubin does not permit identification of the form of bilirubin, however, because of the nature of the experiment it was not feasible to recrystallize bilirubin from individual tissues.

Experiment I consisted of five control and five treated 2-day-old rats. Each rat received 0.03 μCi (7.85 μg) of [^{14}C]bilirubin subcutaneously. The rats were then returned to their dams for 24 hr to allow the [^{14}C]bilirubin to equilibrate with the total miscible bilirubin pool The treated rats were then given sulfadimethoxine (25), 25 mg/kg intraperitoneally and the controls were given equivalent injections of the vehicle. Two hours after the sulfadimethoxine treatment, all rats were killed by decapitation.

Experiment II consisted of four control and five treated 2-day-old rats of both sexes. Each rat was injected subcutaneously with 0.029 μCi (7.5 μg) of [^{14}C]bilirubin. The rats were then placed in a 40° incubator for 22 hr without their dams. The 2-day-old rats were removed from their dams in order that the individual excretion of [^{14}C]bilirubin in the urine and feces could be determined. Each 2-day-old rat was placed on an absorbant pad in the 40° incubator for the duration of the equilibration period. [^{14}C]Bilirubin in the urine and feces was recovered by extracting each pad with a chloroform-methanol solution. An aliquot of the extracting solution was counted for radioactivity. At the end of the 22 hr equilibration period, the rats were injected intraperitioneally with either sulfadimethoxine, 50 mg/kg, or an equivalent volume of the vehicle. Two hours after the sulfadimethoxine injection all rats were killed by decapitation. A volume of 60 ml/kg body weight was used to calculate blood volume for the 2-day-old rats of both *experiments I* and *II*.

Experiment III consisted of three control and three treated adult female rats. Each rat was injected intravenously with 0.063 μCi (186 μg) [^{14}C]bilirubin/100 g body weight. All of the rats were placed in metabolic cages for the separate collection of urine and feces with the rats having access to water only, for the 24-hr period after the [^{14}C]bilirubin injection. At the end of the 24-hr equilibration period, the rats were injected intravenously with either sulfadimethoxine, 25 mg/kg, or an equivalent volume of the vehicle. Thirty minutes after sulfadimethoxine treatment the rats were anesthetized with ether and blood for chemical determination of bilirubin and for radioassay of [^{14}C]bilirubin activity was collected by cardiac puncture. The rats were then perfused with cold saline until the perfusate was clear. Blood volume was calculated by dividing total radioactivity recovered in the collected blood and perfusate by the radioactivity per ml whole blood.

The tissues from all rats were excised, rinsed in saline, blotted, weighed, and frozen for bilirubin determination by liquid scintilation counting. Approximately 100 mg tissue were solubilized in 1.0 ml Soluene-100 (26). Aquasol scintillator cocktail (24) was added and the radioactivity measured with a Packard Tri-Carb scintillation spectrophotometer (26).

Statistical comparisons were made by Student's *t*-test. The level of significance was set at $P < 0.05$.

The data are expressed either as the absolute amount of bilirubin per tissue or as the percentage of the recovered radioactivity found in a given tissue. The absolute bilirubin values given were calculated from the radioactivity of the tissue and the specific activity of the plasma. The percentage of recovered radioactivity values are reported on the basis of total tissue weight, calculated from the radioactivity of a representative sample and the total weight of the tissue. The average percentages of injected radioactivity recovered were 85% and 95% for the adult and newborn Gunn rats, respectively.

RESULTS

A dose of sulfadimethoxine (25 or 50 mg/kg) that produces therapeutic blood levels in rats significantly lowered plasma bilirubin levels in the 2-day-old and adult Gunn rat (Table 1). The total bilirubin pool for the 2-day-old and adult Gunn rats was calculated by dividing the total recovered radioactivity by the specific activity of the serum bilirubin at sacrifice and correcting for injected [^{14}C]bilirubin. The data in Table 2 demonstrate the relative size of the bilirubin pool in terms of micrograms of bilirubin. On a micrograms of bilirubin per 100 g body weight basis, the tissues of the 2-day-old control rats contained significantly more bilirubin than those of the adult.

Statistical comparisons of the newborn data in Table 3 show a significant decrease in tissue bilirubin from controls for the heart,

Table 1. *Effects of sulfadimethoxine on plasma bilirubin levels in icteric Gunn rats*

Experiment	Time, hr	SDM, mg/kg	Bilirubin (mg/100 ml)[1] (*n*)	Control	(*n*)	Treated
I Newborn	2	25	(5)	7.75 ± 0.58	(5)	4.31 ± 0.51[2]
II Newborn	2	50	(4)	14.33 ± 1.20	(5)	2.95 ± 0.27[2]
III Adult	0	25	(3)	13.50 ± 0.80	(3)	14.22 ± 1.44
	0.5		(3)	13.65 ± 1.08	(3)	7.25 ± 0.54[3]

[1] Values expressed as the mean ± 1 SEM.

[2] Significantly different from control animals ($P < 0.05$).

[3] Significantly different from pretreatment values ($P < 0.05$).

Table 2. *Total bilirubin pool in 2-day-old and adult Gunn rats*

Experiment	SDM, mg/kg	Bilirubin, μg[1]		Bilirubin, μg/100 g body wt[1]	
		Control	Sulfadimethoxine[2]	Control	Sulfadimethoxine[2]
II. Newborn	50	377 ± 17	248 ± 24	5,998 ± 383[3]	4,004 ± 357
III. Adult	25	6,536 ± 186	7,457 ± 390	4,282 ± 214	4,762 ± 267

[1] Values expressed as the mean ± 1 SEM, corrected for injected bilirubin.
[2] Values from rats killed 2 hr after sulfadimethoxine injection for the newborn and 30 min after injection for the adult.
[3] Significantly different from the adult animals ($P < 0.05$).

Table 3. *Distribution of [^{14}C]bilirubin in 2-day-old Gunn rats after sulfadimethoxine treatment*

Tissue	Control[1]	Sulfadimethoxine, 25 mg/kg[1]
Heart	0.41 ± 0.01	0.35 ± 0.02[2]
Lung	1.12 ± 0.13	0.91 ± 0.08
Liver	4.39 ± 0.31	5.05 ± 0.35
Spleen	0.20 ± 0.02	0.27 ± 0.02[2]
Kidney	1.77 ± 0.05	2.07 ± 0.13[2]
Brain	0.38 ± 0.04	0.58 ± 0.06[2]
Gastrointestinal tract + contents	28.88 ± 4.68	46.54 ± 4.18[2]
Carcass	51.99 ± 3.75	36.66 ± 3.36[2]
Blood	10.87 ± 0.54	7.56 ± 0.57[2]

[1] Data are the mean ± 1 SEM from five rats per group expressed as the percentage of recovered [^{14}C]bilirubin in *experiment I* in which newborn rats remained with their dams.
[2] Significantly different from control animals ($P < 0.05$).

Table 4. *Distribution of [^{14}C]bilirubin in 2-day-old Gunn rats after sulfadimethoxine treatment*

Tissue	Control[1]	Sulfadimethoxine, 50 mg/kg[1]
Heart	0.62 ± 0.05	0.62 ± 0.05
Lung	1.55 ± 0.09	1.98 ± 0.28
Liver	3.63 ± 0.09	9.51 ± 2.72[2]
Spleen	0.08 ± 0.00	0.17 ± 0.01[2]
Kidney	1.15 ± 0.09	1.16 ± 0.07
Brain	0.87 ± 0.07	1.05 ± 0.09
Gastrointestinal tract + contents	9.83 ± 3.08	25.91 ± 4.95[2]
Carcass	51.15 ± 1.98	36.12 ± 3.33[2]
Urine and feces	19.55 ± 5.52	15.32 ± 5.05
Blood	11.57 ± 0.83	8.15 ± 0.57[2]

[1] Data are the mean ± 1 SEM from four control and five treated rats expressed as percentage of recovered [^{14}C]-bilirubin in *experiment II* in which newborn rats were separated from their dams.
[2] Significantly different from control animals ($P < 0.05$).

Table 5. *Distribution of [^{14}C]bilirubin in adult Gunn rats after sulfadimethoxine treatment*

Tissue	Control[1]	Sulfadimethoxine, 25 mg/kg[1]
Heart	0.22 ± 0.04	0.24 ± 0.04
Lung	0.54 ± 0.01	0.57 ± 0.15
Liver	16.22 ± 1.92	22.86 ± 1.68[2]
Spleen	0.31 ± 0.02	0.28 ± 0.03
Kidney	1.44 ± 0.23	1.49 ± 0.13
Reproductive tract	0.32 ± 0.08	0.36 ± 0.07
Brain	0.09 ± 0.00	0.20 ± 0.02[2]
Renal fat	0.93 ± 0.14	1.14 ± 0.22
Pelvic fat	0.58 ± 0.12	0.99 ± 0.31
Pancreas-mesentery	1.14 ± 0.20	1.64 ± 0.79
Skin	11.95 ± 0.88	10.53 ± 0.41
Gastrointestinal tract + contents	28.95 ± 0.73	25.55 ± 1.40[2]
Carcass	15.12 ± 1.89	16.09 ± 1.95
Urine	2.85 ± 0.69	1.69 ± 0.20
Feces	1.99 ± 0.85	5.39 ± 3.68
Blood	17.35 ± 0.48	10.99 ± 1.40[2]

[1] Data are the mean ± 1 SEM from three control and three treated rats expressed as percentage of recovered [^{14}C]bilirubin in *experiment III*.
[2] Significantly different from control animals ($P < 0.05$).

carcass, and blood with a corresponding significant increase in tissue bilirubin of the spleen, kidney, brain, and gastrointestinal tract after sulfadimethoxine treatment. The treatment of the 2-day-old rat with 50 mg/kg sulfadimethoxine resulted in a similar redistribution of bilirubin from blood to extravascular tissues. The data of Table 4 demonstrate that tissue bilirubin decreased significantly from controls for the carcass and blood and increased significantly in the liver, spleen, and gastrointestinal tract.

Tissue bilirubin concentrations of the sulfadimethoxine-treated adult Gunn rats were decreased significantly from the controls for both the blood and the gastrointestinal tract and increased significantly in the liver and brain (Table 5). The major redistribution of tissue bilirubin in the adult rat after sulfadimethoxine (25 mg/kg) treatment was the movement of bilirubin from the blood to the liver. This is in contrast to the major redistribution of bilirubin in the 2-day-old rat where the shift of bilirubin was from the carcass to the gastrointestinal tract.

Table 6 compares the percentage of the recovered [^{14}C]bilirubin located in the tissues of the untreated 2-day-old and adult Gunn rats. The majority of the bilirubin pool in the 2-day-old rat was located in the carcass and the gastrointestinal tract plus urine and feces. However, on a micrograms of bilirubin per g tissue basis, the concentration of bilirubin was highest in the gastrointestinal tract and blood (Table 7). In the adult, the percentage of recovered bilirubin was more evenly distributed among the liver, skin, blood, gastrointestinal tract, and carcass tissues with the highest concentration per g tissue located in liver, gastrointestinal tract, feces, and blood.

DISCUSSION

Sulfadimethoxine decreased plasma bilirubin levels significantly in both the 2-day and the adult Gunn rat, presumably by its displacement from serum albumin. The majority of the unbound bilirubin appeared to distribute preferentially to the intestine and liver in the newborn, whereas in the adult the unbound bilirubin was taken up by the liver.

When the total bilirubin pool for the 2-day-old and adult control rats was calculated on a body weight basis, a comparison of the

Table 6. *Comparison of percentage of recovered [^{14}C]bilirubin located in tisue of untreated newborn and adult Gunn rats*

	Experiment		
	I Newborn	*II* Newborn	*III* Adult
Heart	0.42 ± 0.01[1]	0.62 ± 0.05[1]	0.22 ± 0.04[1]
Lung	1.12 ± 0.13	1.55 ± 0.09	0.54 ± 0.01
Liver	4.38 ± 0.31	3.63 ± 0.09	16.22 ± 1.92
Spleen	0.20 ± 0.02	0.08 ± 0.00	0.31 ± 0.02
Kidney	1.76 ± 0.05	1.16 ± 0.09	1.44 ± 0.23
Reproductive tract			0.32 ± 0.08
Brain	0.38 ± 0.04	0.87 ± 0.07	0.09 ± 0.00
Renal fat			0.93 ± 0.14
Pelvic fat			0.58 ± 0.12
Pancreas-mesentery			1.14 ± 0.20
Skin			11.95 ± 0.88
Gastrointestinal tract + contents	28.87 ± 4.68	9.83 ± 3.07	28.95 ± 0.73
Feces	[2]	19.55 ± 5.51[3]	1.99 ± 0.85
Carcass	51.98 ± 3.76[4]	51.15 ± 1.98[4]	15.12 ± 1.89
Urine	[2]		2.85 ± 0.69
Blood	10.87 ± 0.54	11.57 ± 0.83	17.35 ± 0.48

[1] Data are the mean ± 1 SEM.
[2] [^{14}C]Bilirubin excreted in the feces and urine was not recoverable.
[3] Urine and feces were collected together.
[4] Skin included in the carcass data.

Table 7. *Comparison of tissue concentration of bilirubin in untreated newborn and adult Gunn rats*

	Bilirubin, μg/g wet tissue[1]		
Tissue	Exp. I Newborn	Exp. II Newborn	Exp. III Adult
Heart	17.6 ± 1.4	43.7 ± 4.1	24.2 ± 4.5
Lung	21.0 ± 1.4	53.9 ± 2.4	28.0 ± 3.1
Liver	38.4 ± 1.6	61.2 ± 3.4	141.8 ± 11.7
Spleen	14.4 ± 1.0	30.2 ± 3.0	47.4 ± 0.9
Kidney	49.1 ± 4.5	52.6 ± 3.1	74.4 ± 3.1
Reproductive tract			34.8 ± 2.2
Brain	2.9 ± 0.2	8.9 ± 0.5	3.4 ± 0.2
Renal fat			46.8 ± 3.6
Pelvic fat			44.7 ± 4.9
Pancreas mesentery			35.0 ± 8.7
Skin			32.1 ± 1.9
Gastrointestinal tract + contents	113.5 ± 16.1	110.2 ± 33.3	121.9 ± 14.9
Carcass	25.7 ± 1.9[2]	44.1 ± 1.9[2]	12.8 ± 2.2
Urine			8.1 ± 1.2
Feces			142.5 ± 58.9
Blood	62.6 ± 4.8	117.7 ± 9.9	140.5 ± 13.5

[1] Data are mean ± 1 SEM.
[2] Skin included in carcass data.

values indicated that the tissues of the 2-day-old rat contained a significantly higher concentration of bilirubin. This finding could be toxicologically important in the management of hyperbilirubinemia in the newborn. In both *experiments I* and *II* (Tables 3 and 4), the 2-day-old rats had approximately 50% of the recoverable bilirubin located in the carcass and skin, whereas liver of these newborn rats contained only 27–43% as much bilirubin per g tissue as the adult (Table 7). The smaller bilirubin pool for the 2-day-old sulfadimethoxine-treated rats compared with the controls may be because the action of sulfadimethoxine on the unbound bilirubin gave a diazo-negative derivative of bilirubin in the presence of smaller concentrations of serum albumin in the newborn. This action of sulfadimethoxine on the chemical stability of unbound bilirubin has been reported (22).

With the higher sulfadimethoxine dose there was significant uptake of bilirubin by the liver in the 2-day-old rat. This observation, coupled with the low percentage of recovered bilirubin found in the liver, suggests that the apparent binding capacity of the 2-day-old liver for bilirubin was reduced. The limited hepatic uptake of bilirubin by the newborn is in agreement with those reports (11, 18) which suggest that hepatic uptake of bilirubin is facilitated by cytoplasmic proteins Y and Z. These proteins have been shown to be in low concentration in the newborn (7, 12), and thus would have a comparatively limited capacity for uptake of displaced bilirubin.

The concentration of bilirubin in brain tissue was significantly higher in the sulfadimethoxine-treated rats for both the newborn and the adult (Tables 3 and 5). The concentration of bilirubin per g brain tissue was 2.92 ± 0.15 μg/g and 5.03 ± 0.56 μg/g (mean ± SEM) in the newborn and 3.37 ± 0.22 μg/g and 8.09 ± 0.67 μg/g in the adult for the control and sulfadimethoxine-treated rats, respectively. These data suggest that displaced bilirubin moves into brain tissues of both the newborn and the adult Gunn rat. The higher concentration of bilirubin in the adult Gunn rat brain coupled with the 18-fold difference in LD_{50} values for sulfadimethoxine in newborn and adult icteric Gunn rats suggests that displaced bilirubin is more toxic to the newborn. However, since sulfadimethoxine was also more toxic in the nonicteric newborn, not all of the increased toxicity can be attributed to bilirubin. It remains to be established whether there is a greater sensitivity of the brain of the neonate to bilirubin toxicity.

It is suggested that in the icteric Gunn rat that plasma bilirubin exists in a delicate equilibrium with the various extravascular bilirubin binding tissues and that these change as the rat matures. This maturation of extravascular bilirubin binding compartments undoubtedly is a determinant of the plasma bilirubin concentration for glucuronidation is undetectable at any age. The treatment of icteric Gunn rats with sulfadimethoxine, a strongly albumin-bound sulfonamide, resulted in marked changes in the tissue distribution of bilirubin in both newborn and adult Gunn rats, but because of the nature of available extravascular binding sites and possible increased sensitivity of the newborn brain to bilirubin, the newborn was apparently at a greater risk for sulfadimethoxine toxicity.

SUMMARY

These studies indicate that a single therapeutic dose of sulfadimethoxine decreased serum bilirubin levels significantly, resulting in significant changes in tissue bilirubin levels for the 2-day-old and adult icteric Gunn rat. A significant redistribution of bilirubin from the plasma and carcass to the gastrointestinal tract was observed in the 2-day-old rat. In contrast, in the adult a significant decrease in plasma bilirubin was accompanied by a significant increase in the liver concentration of bilirubin. The major bilirubin binding tissues were similar for both age groups: namely, blood, liver, skin, carcass, and gastrointestinal tract. The data suggest differences in tissue distribution patterns between the newborn and adult icteric rats which may reflect the maturation of extravascular bilirubin binding compartments.

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Letters to the Editor

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I would like to comment on the paper of Fosmire *et al.* (1) on "Some Effects of Postnatal Zinc Deficiency on Developing Rat Brain." It is well recognized that zinc deficiency results in a loss of appetite as acknowledged in the paper and as previously noted in children (2). It would thus seem likely that the pups of the zinc-deficient mothers had reduced appetite and ate even less than the pups of the pair-fed, adequate zinc-intake mothers. As generalized undernutrition can cause all of the findings the authors attribute to zinc deficiency, I believe it dangerous to attribute their findings to a lack of zinc.

The authors' main argument, that it is the zinc deficiency and not generalized undernutrition, is based on the difference in brain weights at 6 days in 12 pups from the zinc-deficient dams and 12 pups from the pair-fed dams, whereas body weights of 8 different pups from each maternal group did not differ. One wonders (*1*) why the authors did not combine body weights from the numerous groups studied rather than use just one group of 8 animals, and if this was to be done, why was this group not comprised of the same animals whose brains were studied at that age; (*2*) how the 12 animals were chosen for brain weights at 6 days; (*3*) whether the vertical lines in Figure 2 are SEM's as in Figure 3, and if so, are these really different at $P < 0.01$? No statistics are given for DNA values (Fig. 3), and although quantities are stated to be different in the three groups, one also wonders if these were "statistically" different at all ages.

Would not the proper experiment be to pair-feed pups from birth on diets identical in content other than in zinc, using artificial feeding methods such as described by Miller and the group from MIT? Until this is done it would seem dangerous to implicate zinc deficiency as a cause of poor brain growth, especially when Hambidge *et al.* (2) did not find this to be a problem in the children they studied.

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