Relationship between Protein Binding of Bilirubin, Salicylic Acid, and Sulfisoxazole in Serum of Unmedicated and Phenobarbital-Treated Rats

AVRAHAM YACOBI, SVEIN ØIE, and GERHARD LEVY ˣ

Abstract □ The relationship between the protein binding of bilirubin, salicylic acid, and sulfisoxazole in the serum of phenobarbital-treated and untreated rats was studied. The free fraction of bilirubin in serum varied about threefold between animals but was not related to the albumin concentration, the free fraction of salicylate, or the free fraction of sulfisoxazole. However, there was a strong positive correlation between the free fraction values of salicylate and sulfisoxazole. The free fraction value for each of these drugs showed a significant negative correlation with the serum albumin concentration. Treatment of the animals with phenobarbital had no apparent effect on the serum protein binding of bilirubin, salicylic acid, and sulfisoxazole.

Keyphrases □ Bilirubin—protein binding, relationship to binding of salicylic acid and sulfisoxazole, effect of phenobarbital, rat serum □ Salicylic acid—protein binding, relationship to binding of bilirubin and sulfisoxazole, effect of phenobarbital, rat serum □ Sulfisoxazole—protein binding, relationship to binding of salicylic acid and bilirubin, effect of phenobarbital, rat serum □ Protein binding—bilirubin, salicylic acid, and sulfisoxazole, effect of phenobarbital, rat serum □ Binding, protein—bilirubin, salicylic acid, and sulfisoxazole, effect of phenobarbital, rat serum □ Phenobarbital—effect on protein binding of bilirubin, salicylic acid and sulfisoxazole, rat serum

Drugs that displace bilirubin from plasma protein binding sites can cause brain damage and death in premature infants with neonatal jaundice (1–3). Classical bilirubin displacing agents, in terms of clinical experience and experimental interest, are sulfisoxazole and salicylic acid. Administration of these drugs to humans and animals during hyperbilirubinemia causes a redistribution of bilirubin from plasma to extravascular tissues, including the brain (4–9).

The rat has been used in this laboratory as a model for investigating the pharmacokinetics of the interaction of salicylic acid and sulfisoxazole with bilirubin. As an extension of this research, the relationship between the protein binding of bilirubin, salicylic acid, and sulfisoxazole in the serum of individual rats has been determined. A previous study revealed that there are pronounced interindividual differences in the serum protein binding of bilirubin in rats (10).

Neonatal unconjugated hyperbilirubinemia is treated sometimes with the enzyme inducer phenobarbital (11, 12). *In vitro* addition of phenobarbital to hyperbilirubinemic serum does not change the total binding capacity of albumin (13), but it remains to be determined if enzyme induction *per se* affects the serum protein binding of bilirubin, perhaps by changing the formation and/or elimination kinetics of endogenous inhibitors of bilirubin binding. Accordingly, protein binding studies were carried out with serum from unmedicated and phenobarbital-pretreated rats.

EXPERIMENTAL

Twenty adult male Sprague–Dawley rats, ~350 g, were divided into two groups. One group received daily injections of phenobarbital, 75 mg/kg ip, for 4 days; the other group received daily injections of normal saline solution. On the 5th day, all blood was removed from the aorta under light ether anesthesia and the liver was excised and weighed. Serum was separated and assayed for total protein (14), using crystalline rat albumin as the standard. The fraction of albumin was determined by electrophoresis[1].

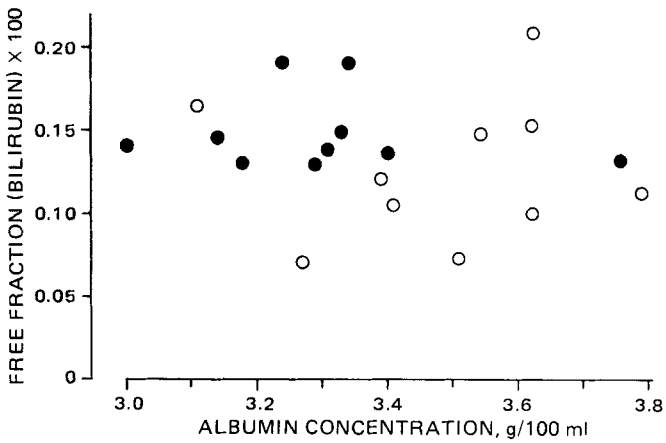

Figure 1—*Relationship between free fraction of bilirubin and albumin concentration in serum of phenobarbital-treated (●) and control (○) rats. Correlation coefficient = 0.36 (not significant) for combined data.*

The protein binding of salicylic acid and sulfisoxazole was determined by equilibrium dialysis at 37° as previously described (15). The buffer solution contained an initial concentration of 30 mg of salicylic acid or 10 mg of sulfisoxazole/100 ml. The protein binding of bilirubin was determined by measuring the rate of the peroxidase-catalyzed degradation of the pigment by peroxide (16). Since only unbound bilirubin reacts with peroxide, the reaction rate is proportional to the free bilirubin concentration.

Bilirubin solution[2], 10 μl, was added to 0.5 ml of serum from a rat to

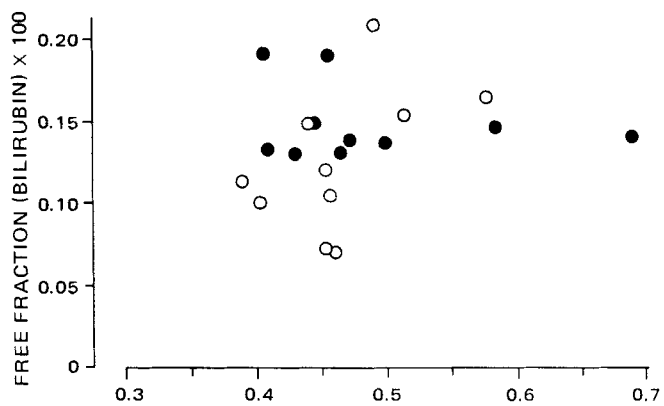

Figure 2—*Relationship between free fraction values of bilirubin and salicylic acid in serum of phenobarbital-treated (●) and control (○) rats. Correlation coefficient is 0.185 (not significant) for combined data and 0.518 (not significant) for control data.*

[1] Gelman serum protein electrophoresis system.

[2] Crystalline bilirubin (Sigma) dissolved in water with the aid of a small quantity of 2 *M* sodium hydroxide.

Table I—Effect of Treatment with Phenobarbital on Total Protein and Albumin Concentrations[a] in Serum of Rats

	Before Treatment		After Treatment	
	Control	Phenobarbital	Control	Phenobarbital
Total protein, g/100 ml	8.10 ± 0.24	8.01 ± 0.35	7.94 ± 0.26	8.13 ± 0.20
Albumin, g/100 ml	3.96 ± 0.22	3.77 ± 0.21	3.49 ± 0.20	3.30 ± 0.20
	N.S.		N.S.	
	$p < 0.001$			
		$p < 0.001$		

[a] Mean ± *SD*, $n = 10$.

obtain a serum bilirubin concentration of 8.7–9.5 mg/100 ml. A 0.1-ml aliquot of 0.1 *M* phosphate buffer, pH 7.4, containing 0.01 *M* edetate disodium was evaporated to dryness in a 10-ml centrifuge tube. Plasma, 0.1 ml, was added, and the dried buffer was dissolved in it by vortexing. Peroxidase solution, 10 μl (8 purpurogallin units of horseradish peroxidase[3]/ml of 0.1 *M* phosphate buffer, pH 7.4), was then added.

The reaction was started by adding 5 μl of peroxide solution (0.2% ethyl hydrogen peroxide in 0.1 *M* phosphate buffer, pH 7.4) and stopped after 1–2 min by addition of 1 ml of 6% ascorbic acid in 0.2 *M* phosphate buffer, pH 8.2. The entire reaction was carried out in a water bath at 37°. The concentration of total unconjugated bilirubin before and after the reaction was determined spectrophotometrically after chloroform extraction (17), using a commercial bilirubin control[4] as the standard.

All protein binding data are expressed as free fraction values, *i.e.*, the concentration of free (unbound) divided by the concentration of total (free and protein bound) drug or bilirubin in serum.

RESULTS

Treatment with phenobarbital caused a significant increase in liver weight (3.70 ± 0.35 *versus* 3.09 ± 0.16 g/100 g of body weight, $p < 0.001$) but had no apparent effect on the concentrations of total protein and albumin in serum (Table I). There was a small but statistically significant decrease in the serum albumin concentration in both the control and phenobarbital-treated groups during the treatment period. Treatment with phenobarbital had no statistically significant effect on the serum protein binding of bilirubin, sulfisoxazole, and salicylic acid (Table II).

There were appreciable intersubject differences in the binding of bilirubin and the concentration of albumin in serum but no apparent correlation between the free fraction values of bilirubin and the albumin concentration in serum (Fig. 1). There was also no apparent correlation between the free fraction values of bilirubin and those of salicylic acid (Fig. 2) or sulfisoxazole (Fig. 3). On the other hand, a strong and statistically highly significant correlation existed between the free fraction values of salicylic acid and sulfisoxazole in individual animals (Fig. 4). There was also a statistically significant negative correlation between

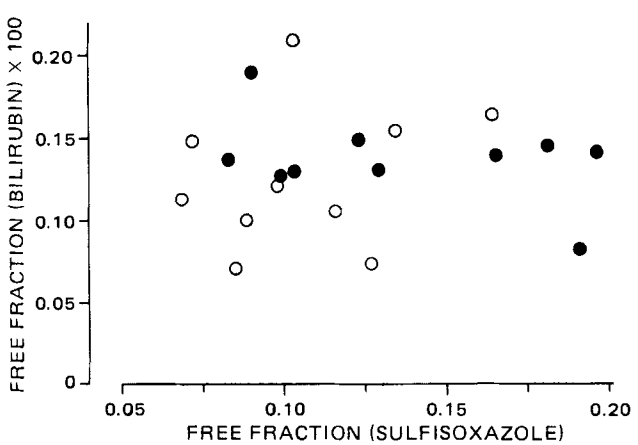

Figure 3—*Relationship between free fraction values of bilirubin and sulfisoxazole in serum of phenobarbital-treated (●) and control (○) rats. Correlation coefficient is 0.074 (not significant) for combined data.*

[3] Sigma type I.
[4] Dade Division, American Hospital Supply Corp., Miami, Fla.

Table II—Effect of Treatment with Phenobarbital on Protein Binding of Bilirubin, Salicylic Acid, and Sulfisoxazole in Serum of Rats

	Free Fraction[a]	
	Control	Phenobarbital
Bilirubin	0.00126 ± 0.00043 (0.00071–0.00209)	0.00149 ± 0.00023[b] (0.00131–0.00191)
Sulfisoxazole	0.106 ± 0.030 (0.0717–0.164)	0.125 ± 0.042[b] (0.0816–0.196)
Salicylic acid	0.463 ± 0.053 (0.390–0.573)	0.484 ± 0.088[b] (0.405–0.690)

[a] Mean ± *SD* (range), $n = 10$. [b] Not significantly different from control value (Student *t* test).

the serum albumin concentration and the free fraction of salicylic acid ($r = -0.635$, $p < 0.005$) and sulfisoxazole ($r = -0.600$, $p < 0.005$).

DISCUSSION

Binding of bilirubin to albumin in serum, which is technically difficult to determine because it is so extensive (>99%), cannot be predicted by the extent of serum protein binding of two other weak acids, salicylic acid and sulfisoxazole, at least in the rat. There are significant intersubject differences in the protein binding of bilirubin, salicylic acid, sulfisoxazole, and other drugs in the rat and also in humans (10, 15, 18, 19, and unpublished data). This fact must be considered in the design and interpretation of studies on species differences in serum protein binding of drugs.

No apparent relationship between the concentration of albumin and the free fraction of bilirubin in serum of rats was found. A similar lack of correlation was observed previously with two other extensively albumin-bound compounds, warfarin (18) and dicumarol (20). However, the

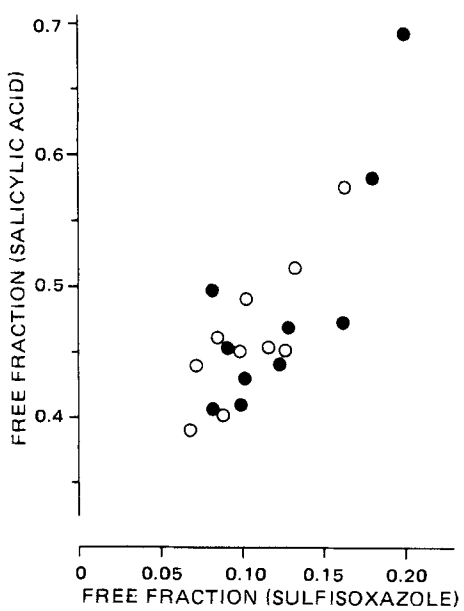

Figure 4—*Relationship between free fraction values of salicylic acid and sulfisoxazole in serum of phenobarbital-treated (●) and control (○) rats. Correlation coefficient is 0.818, p < 0.001, for combined data.*

free fraction values of salicylic acid and sulfisoxazole, which are much less extensively protein bound, exhibited a statistically significant negative correlation with the concentration of albumin in rat serum.

Phenobarbital treatment, which is an effective means of lowering the serum bilirubin concentration in certain types of unconjugated hyperbilirubinemia (11, 12), has no apparent effect on the serum protein binding of bilirubin in rats. This information will facilitate the interpretation of results of phenobarbital–bilirubin interaction studies in normal rats. It remains to be determined if phenobarbital treatment affects bilirubin binding in the Gunn rat, the most important animal model of physiological unconjugated hyperbilirubinemia.

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ACKNOWLEDGMENTS AND ADDRESSES

Received August 13, 1976, from the *Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214.*

Accepted for publication September 17, 1976.

Supported in part by Grant GM 19568 from the National Institute of General Medical Sciences, National Institutes of Health.

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Rapid GLC Determination of Therapeutic Plasma Glycerin Levels

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Abstract □ A new rapid and inexpensive method for the determination of therapeutic plasma glycerin concentrations is described. In this method, acetic anhydride and pyridine are added to 15 μl of plasma. After brief incubation and centrifugation, an aliquot of the supernate is injected directly onto the 3% OV-1 column. A linear calibration curve was found in the 0.05–3-mg/ml range, with the precision of the assay estimated to be ±5.5% (*RSD*). The method was used in determining preliminary pharmacokinetic data in the rabbit.

Keyphrases □ Glycerin—GLC analysis, plasma □ GLC—analysis, glycerin in plasma □ Hyperosmolar dehydrating agents—glycerin, GLC analysis in plasma

Cerebral edema, or swelling of brain tissue, increases intracranial pressure, which, if not medically corrected, leads to herniation of brain tissue with irreversible brain damage and subsequent death. Deterioration of brain function and progressive deepening of coma in acute neurologic emergencies are often results of increased intracranial pressure due to cerebral edema. Medical management is a triad composed of hypothermia, hypocapnea, and hyperosmolar dehydration of cerebral tissues (1). Glycerin has been particularly useful for the treatment of cerebral edema by hyperosmolar dehydration because of its physical properties, but a lack of clinical knowledge has hindered its maximum use (2, 3).

At present, empirical relationships govern the use of glycerin in life-threatening situations. The efficacy of treatment with glycerin probably could be improved with quantitative assessment of glycerin therapy, including monitoring of plasma levels. It is now possible, and preferable, to individualize the dosage regimen for each patient based on a desired therapeutic plasma concentration range known to produce a desired response. In life-threatening situations, such as increased intracranial pressure, it is imperative to have rapid assessment of plasma glycerin levels for effective therapy.

Currently, the most commonly used methods for the determination of plasma glycerin levels are enzymatic (4–11). The first such method (4) was developed for determining endogenous glycerin levels, normally in the 5–17-μg/ml range. For assaying therapeutic plasma glycerin levels (milligram per milliliter range) with enzymatic methods, it is necessary to find the proper dilution factor for each sample, which is both time consuming and expensive. Thus, another method was needed that would be