Binding of Bilirubin to Erythrocytes[1] (37185)

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(Introduced by J. E. Smith)

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Since clearing the circulation of toxic materials is characteristic for the liver, the level of bilirubin in serum and the clearance rate of intravenously administered pigments or dyes are used as criteria by which overall functioning of the liver is evaluated. In such liver function tests, binding of a circulatory compound to erythrocytes (1, 2) would affect the level of that compound in serum and its clearance rate.

In the present work, erythrocytes were incubated with different concentrations of bilirubin and albumin. Bilirubin binding to erythrocytes was related to the level of nonalbumin-bound pigment, determined by ultracentrifugation. Salicylate, which competitively decreases the extent of albumin–bilirubin complexing (3, 4) and thus elevates the level of unbound bilirubin, increased bilirubin binding to erythrocytes accordingly. Use of ^{14}C-bilirubin allowed measurement of low concentrations of nonalbumin-bound bilirubin established in mixtures containing albumin and bilirubin in physiologically encountered molar ratios.

Materials and Methods.. Bilirubin, dissolved in 0.1 *N* NaOH–0.9% NaCl and stored on ice, salicylic acid, and bovine serum albumin, fraction V were used as provided by Nutritional Biochemical Corp., Cleveland, OH.

Purified ^{14}C-bilirubin was isolated from bile of dogs injected with δ-aminolevulinic acid-4-^{14}C (5, 6). When stored in the dark in an evacuated desiccator at −15°, its specific activity (about 4 μCi/mg) remained constant for periods in excess of 4 mo.

Red blood cells were obtained from heparinized bovine blood by centrifuging for 10 min at 1000*g* and removing plasma and buffy coat. The erythrocytes were washed twice with 0.9% NaCl and once with Krebs–Ringer bicarbonate buffer (pH 7.4).

Erythrocytes (about 13%, v/v) were incubated for 10 min at 37° in Krebs–Ringer bicarbonate buffer (pH 7.4) that contained albumin (0.75, 1.5, or 3.0%, w/v), varying amounts of ^{14}C-bilirubin (16 to 148 μg/ml), and, in some cases, salicylate (20 mg/100 ml). Media, sampled before the addition of erythrocytes and after incubation, were prepared for counting by solubilizing 1 part sample and 2 parts Biosolv (BB-3, Beckman, Fullerton, CA) in 10 ml of scintillation cocktail of 0.4% 2,5-diphenyloxazole (PPO) and 0.01% 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) in toluene. Using a Packard Tri-Carb liquid scintillation spectrometer, ^{14}C was determined within a 1% probable error including quench correction by internal standardization.

Levels of nonalbumin-bound bilirubin were established by ultracentrifugation of media, devoid of erythrocytes, in a Type 50 Ti, fixed-angle rotor at average force of 226,400*g* for 14 hr at 10°. Levels of ^{14}C-bilirubin were measured in three samples taken from the homogenous mixture before centrifugation and in five samples taken from the clear layer above the sedimented albumin after centrifugation. The completeness of albumin sedimentation was ascertained by measuring in supernatant: (i) Absence of TCA-precipitable protein; (ii) presence of only a trace of Folin–Ciocalteu-positive material; and

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(iii) homogenous distribution of nonalbumin-bound bilirubin at various depths in the centrifuge tube. The sedimented albumin could be solubilized by gentle shaking. In the absence of albumin, no bilirubin sedimented out.

Data were analyzed by analysis of variance, with $p < 0.05$ considered significant.

Results and Discussion. The 10-min incubation period was indicated by preliminary experiments showing that no additional bilirubin was bound to erythrocytes during the last 5 min of incubation.

At a given albumin concentration, erythrocytes and albumin bound constant proportions of bilirubin, independent of bilirubin concentrations used (Table I). Reducing the albumin concentration lowered the extent of bilirubin binding to the protein and increased the proportions of bilirubin that were bound to erythrocytes (Table I). Similarly, salicylate increased the proportion of nonalbumin-bound bilirubin and enhanced adsorption of the pigment to erythrocytes (Table I). No influence was observed from small variations in packed erythrocyte volumes (11.3–14.2%) on extent of bilirubin adsorption.

Various workers (4, 7, 8) have noted an apparent discrepancy, that only slight decreases in the extent of bilirubin–albumin binding (after salicylate administration) result in large-scale displacement of serum bilirubin. The data presented in Table I could account, in part, for that observation: increasing the proportion of nonalbumin-bound bilirubin by only 3% resulted in a 5-fold increased bilirubin binding to erythrocytes. A similar enhancement was observed in hepatic clearance rate of administered bilirubin when the level of nonalbumin-bound bilirubin in the circulation was raised only slightly—either by lowering albumin levels or by treating with salicylate (9).

Binding of bilirubin to erythrocytes must be considered among factors that lower the serum bilirubin level when liver function is to be evaluated from observed rates of bilirubin clearance from the circulation. As shown in Fig. 1, disappearance of bilirubin from medium (binding to erythrocytes) was linearly related to the extent of bilirubin–albumin complexing. Hence, variations in the extent of serum bilirubin lowering caused by binding to erythrocytes under various conditions (hypoalbuminemia; salicylate treatment) can be evaluated arithmetically (Fig. 1) when the proportion of nonalbumin-bound bilirubin is measured.

Salicylate did not influence the binding of bilirubin to erythrocytes other than was predictable from increased unbinding of albumin–bilirubin complex (Fig. 1).

Summary. Bovine erythrocytes were incubated in buffered media (pH 7.4) containing ^{14}C-bilirubin and bovine serum albumin in molar ratios from 0.04 to 1.5. After equilibration, erythrocytes and media were separated by centrifugation and the level of ^{14}C-bilirubin in media was determined. Bilirubin binding to erythrocytes was then correlated with the proportion of nonalbumin-bound

TABLE I. Bilirubin Binding to Erythrocytes and Albumin.[a]

Incubation medium		No. of blood samples	Fraction of bilirubin			
			Bound to erythrocytes		Not bound to albumin	
Albumin (g/100 ml)	Salicylate (mg/100 ml)		%	SE	%	SE
3.0	0	11	2.9	0.55	1.68	0.10
1.5	0	8	7.3	0.63	2.32	0.10
0.75	0	11	8.5	0.55	3.10	0.13
3.0	20	8	12.2	0.77	3.25	0.02
0.75	20	8	15.4	0.77	4.60	0.06

[a] Packed erythrocyte volume was about 13%. Ranging bilirubin levels between 16 and 148 μg/ml, at each of the albumin concentrations used, did not affect the fractions of added bilirubin binding either to erythrocytes or to albumin.

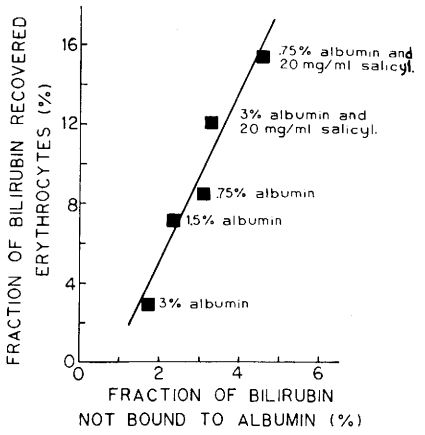

FIG. 1. Dependency of bilirubin adsorption to erythrocytes on the level of nonalbumin-bound bilirubin. Average values of 8–11 measurements were expressed as percentages of bilirubin levels with which erythrocytes and albumin were equilibrated.

bilirubin in media, determined ultracentrifugally.

At a given albumin concentration, erythrocytes and albumin bound constant proportions of bilirubin, despite varying bilirubin concentrations. Upon changing albumin concentrations, a linear relationship was found between extents of bilirubin binding to erythrocytes and to albumin, whereby an increase of only 3% in nonalbumin-bound bilirubin resulted in 5-fold increased bilirubin binding to erythrocytes.

Adding salicylate did not influence bilirubin binding to erythrocytes other than was predictable from increased unbinding of albumin–bilirubin complex.

In evaluating functioning of the liver from observed rates at which an administered compound is cleared from serum, the extent of binding to erythrocytes must be considered. It would appear that such binding can be estimated arithmetically when the proportion of nonalbumin-bound compound has been measured.

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