DO HEME AND BILIRUBIN HAVE IDENTICAL BINDING SITES ON THE ALBUMIN MOLECULE?[1]

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SUMMARY

A competition of heme with bilirubin for binding on the albumin molecule has often been assumed. High plasma concentrations of heme are, therefore, thought to promote deposition of bilirubin in the brain and to cause kernicterus. Studies of competitive binding of heme and bilirubin in vitro have, however, not been reported and are the subject of the present paper. The molar binding ratios of bilirubin:albumin and heme:albumin are 1.0, when less than 0.5 percent of albumin is filtered on Sephadex G-25, 0.05M sodium phosphate buffer at pH 8.3. This molar binding ratio holds regardless of whether or not albumin has previously been complexed with either of the pigments. This finding strongly suggests two distinctly different binding sites on the albumin molecule for bilirubin and for heme.

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Bilirubin, added to serum, is bound exclusively by albumin (1). Deposited in the brain of guinea pig neonates or adult Gunn rats, this pigment may return to the circulation, upon infusion with albumin (2). The toxic effects of bilirubin can thus be partially prevented (3). Heme, like bilirubin, depresses the oxygen uptake of minced rat brain in vitro (4). In addition, high plasma levels of heme, which is also bound by albumin (methemalbumin), are considered to predispose the newborn to kernicterus (5), because of competition with bilirubin for binding on the albumin molecule. While salicylates, sulfonamides and free fatty acids have been shown to decrease the affinity of albumin for bilirubin (3, 6), experimental evidence has not been adduced for displacement of bilirubin by heme. This communication reports on the molar binding ratio of bilirubin:protein after incubation of albumin containing an equimolar amount of ^{59}Fe-heme.

MATERIALS AND METHODS

Human serum albumin was purified from the plasma of a healthy adult male by preparative zone electrophoresis on Pevikon (7). When analyzed for purity, albumin showed a single protein band on polyacrylamide gel electrophoresis, and a single arc on immunoelectrophoresis precipitation, when tested with polyvalent antiserum. Albumin concentrations were determined* by the micro-Kjeldahl technique (assuming a 16 percent N content for the protein).

Bilirubin crystals (Nutritional Biochemicals Corp.) were dissolved in a minimal amount of 0.1 N NaOH; and the solution brought to a final pH of 8.3 with 0.15M sodium phosphate buffer. Bilirubin concentrations were determined by the alkaline

* Courtesy of Dr. W.O. Weigle, Department of Experimental Pathology, Scripps Clinic and Research Foundation.

diazo method of Fog (8), using as a standard an elevated bilirubin control serum (Hyland).

^{59}Fe-heme was prepared as previously described (9), crystallized according to Labbe and Nishida (10), and the concentrations were measured spectrophotometrically as the pyridine hemochromogen (11). Radioactivity was assessed in a well-type scintillation counter; the specific activity was 1.2×10^7 dpm/μmole.

Sephadex G-25 superfine (Pharmacia) was used for chromatography. The column, 25 x 1 cm, was wrapped in aluminum foil and kept in dim light to avoid bilirubin degradation. Mixtures of pigments and albumin in molar ratios ranging from 0.2 to 2.0 were incubated for 30 minutes at 37° C immediately prior to application.

RESULTS AND DISCUSSION

The results obtained by gel filtration (Fig. 1) show clearly that only one molecule of either bilirubin or heme is bound by each molecule of albumin. Applying pigment:albumin in molar ratios exceeding 1.0 lead to retention of excess pigment in the gel.

Previous studies on the molar binding ratio of bilirubin: albumin employing gel filtration indicate that this ratio is strictly dependent upon the experimental conditions. Schmid et al. (12) observed a ratio of 2.0 when applying 4 percent albumin solutions and eluting with 0.0625M phosphate buffer at pH 8.3. Kučerova et al. (13) realized the dependency of the molar binding ratio on the protein concentration. At an albumin concentration of 4 percent, the ratio was 2.0, and at 0.5 percent only 1.0. Bratlid and Fog (14) found one bilirubin molecule tightly

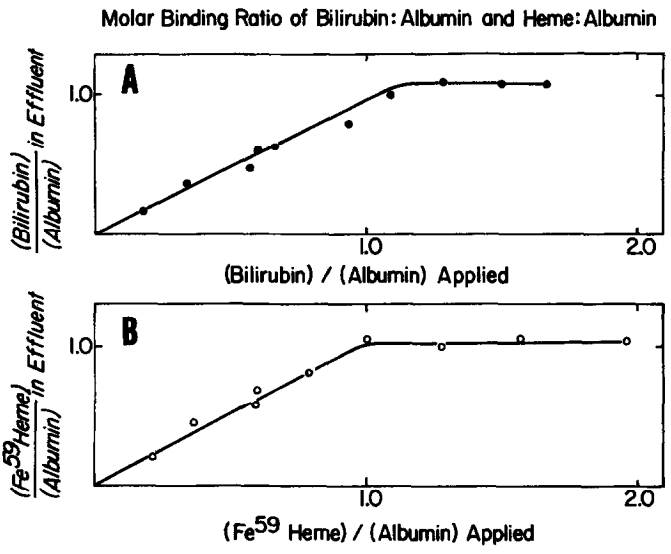

Fig. 1: Molar binding ratios of bilirubin:albumin and heme:albumin in the effluent of gel filtrations of a human albumin solution incubated with varying amounts of either bilirubin or ^{59}Fe-heme.

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bound and another loosely bound per mole of albumin. In their experiments, albumin (0.05 to 0.2 percent) was incubated with a constant amount of bilirubin and eluted with Tris buffer, $\Gamma/2$ = 0.1, of pH values varying from 7.4 to 8.3. The binding ratios reported on experiments obtained by equilibrium dialysis (Martin et al., 15), and by spectrophotometry (Watson, 16)vary considerably, ranging from 1.9 to 3.3.

A detailed study on the molar binding ratio of heme:albumin was published by Rosenfeld and Surgenor (17). Employing spectrophotometry at 38°, a molar ratio of 1.0 was found upon addition of albumin to heme, and ratios of 2.0 upon addition of heme to albumin. These binding ratios were not affected by moderate changes in ionic strength, nor by variations in pH between 7.0 and 10.0. These authors state that equilibrium dialysis is not applicable to a study of heme:protein ratios, because of

the marked tendency of heme to aggregate -- a finding subsequently confirmed by others (18-21).

Table 1 records the studies of the competition of bilirubin and heme for binding on the albumin molecule. Regardless of which pigment had previously been complexed with albumin prior to incubation with the other pigment, the molar binding ratio was 1.0 for heme:albumin and 1.0 for bilirubin:albumin. It is, therefore, highly unlikely that the two pigments compete for the

TABLE 1

GEL FILTRATION OF ALBUMIN INCUBATED WITH HEME AND BILIRUBIN

COMPOSITION OF SAMPLES			
BEFORE FILTRATION		AFTER FILTRATION[1]	
Fe^{59}Heme-Albumin[2] + Bilirubin		Fe^{59}Heme : Albumin : Bilirubin	
μM	μM		
42.1	0	0.96 : 1	--
42.1	62.2	0.96 : 1 :	0.71
42.1	74.6	1.0 : 1 :	0.71
Bilirubin-Albumin[2] + Fe^{59}Heme		Bilirubin : Albumin : Fe^{59}Heme	
μM	μM		
36.7	0	0.91 : 1	--
36.7	41.7	0.89 : 1 :	0.91
36.7	55.6	0.89 : 1 :	0.90
36.7	69.5	0.87 : 1 :	0.92

1 Ratios in pooled effluent peak.

2 Pigment:albumin ratio is 1.0.

same binding site. Our data do not, however, exclude competition of bilirubin with heme for a second binding site of lesser affinity than that of the first site. According to Little and Neilands (22), this may involve histidine residues of the albumin molecule interacting with heme.

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