[CONTRIBUTION FROM THE DEPARTMENT OF PHYSICAL CHEMISTRY, HARVARD MEDICAL SCHOOL]

Preparation and Properties of Serum and Plasma Proteins. XXI. Interactions with Bilirubin[1,2]

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Many of the small molecules circulating in the blood are "bound" to one or another of the protein components of the plasma. Among these is bilirubin, one of the intermediates of porphyrin metabolism. Bennhold,[4] using the electrophoretic techniques available to him in 1932, demonstrated that, in icteric serum, bilirubin migrated with the albumin fraction. Pedersen and Waldenström[5] made similar observations in the course of ultracentrifugal studies. Since then it has been assumed that the "bound" bilirubin in the blood serum was attached to the circulating albumins.[6]

In recent years a number of components of human plasma have been isolated in highly purified form. These include crystallized human serum albumins, a crystallized β_1 metal-combining globulin and electrophoretically pure γ-globulins. In addition, a number of α-globulins and of other β-globulins have been concentrated in various fractions.[7] This investigation was undertaken to study the interactions of bilirubin with these proteins.

Materials[8]

Human Serum Albumin.—Three different preparations were used. The first had been recrystallized five times from ethanol–water mixtures containing chloroform.[9] Another preparation had been recrystallized as a mercury compound.[10] The third preparation was made from crystallized serum albumin by guanidination of the free amino groups by reaction with methyl isourea, according to a modification of the method of Greenstein.[11] The modified, or guanidinated, preparation had approximately fifty-four of its amino groups replaced with guanidine groups.[12]

Bovine serum albumin was Armour's crystallized preparation.

α_1-Globulin.—This globulin was concentrated from Fraction V-1 of human plasma. It was the component responsible for the indirect van den Bergh reaction[13] and, as isolated, already contained stably-bound bilirubin.[14]

α_2-Globulins.—The first preparation was isolated from Fraction IV-6[15] and was 97% α_2-globulin by electrophoresis. It contained approximately 6% carbohydrate. A second preparation was isolated from Fraction IV-7 and was 74% α_2-globulin by electrophoresis. It was estimated to contain 3% carbohydrate.[16]

β_1 Metal-Binding Globulin.—This protein was purified from fraction IV-7 and crystallized from ethanol–water mixtures.[16]

γ-Globulin was 98% pure by electrophoresis.[17]

Bilirubin (Armour Laboratories) was isolated from ox bile. In chloroform, ethylene dichloride and acetone it had a well-defined absorption maximum at 450 mμ with a molecular extinction coefficient of 55,300. This is in good agreement with the value of 54,900 given by Heilmeyer.[18]

Methods

Dissolved in 0.01 N sodium hydroxide at room temperature, the bilirubin had an ill-defined absorption maximum lying between 425 and 440 mμ whose height tended to decrease rapidly on standing. Preservation at 0° in the absence of light delayed but did not inhibit degradation. Advantage was taken of the ability of ascorbic acid to stabilize solutions of bilirubin.[19] Solutions were prepared by dissolving weighed amounts of bilirubin in 0.01 N sodium hydroxide at 0°, and immediately adding phosphate buffer containing sufficient ascorbate so that the final ratio of ascorbate to bilirubin was twenty to one. The extinction of solutions prepared in this manner decreased about 10% in forty-eight hours while the peak remained reasonably well defined at 430 to 435 mμ (Fig. 1). That ascorbic acid did not interfere with the interactions being studied was indicated by the fact that, within the experimental error, no difference was observed in the amount of bilirubin interacting with albumin in the presence or absence of ascorbate. Furthermore, in weight concentrations of ascorbate of from 5 to 30-fold the bilirubin concentration appeared to have no apparent effect on the extent of the interaction.

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(2) This work was originally supported by grants from the Rockefeller Foundation and from funds of Harvard University. It was aided early in 1941 by grants from the Committee on Medicine of the National Research Council which included a grant from the American College of Physicians. From August, 1941, to July, 1946, it was carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University. Since then it has been aided by a grant recommended by the Panel on Hematology of the National Institute of Health.

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(4) H. Bennhold, *Ergeb. inn. Med. Kinderheilk.*, **42**, 273 (1932).

(5) K. O. Pedersen and J. Waldenström, *Z. physiol. Chem.*, **245**, 152 (1937).

(6) I. Snapper and W. M. Bendien, *Acta. Med. Scand.*, **98**, 77 (1938).

(7) E. J. Cohn, L. E. Strong, W. L. Hughes, Jr., D. J. Mulford, J. N. Ashworth, M. Melin and H. L. Taylor, THIS JOURNAL, **68**, 459 (1946).

(8) The proteins used in these studies were, with the exception of the bovine serum albumin, prepared in the Department of Physical Chemistry during the course of investigations leading to the isolation and purification of the protein components of plasma. Acknowledgment is made to the various members of the department for making these components available. The bovine serum albumin was obtained through the courtesy of Dr. J. D. Porsche of the Armour Laboratories.

(9) E. J. Cohn, W. L. Hughes, Jr., and J. H. Weare, THIS JOURNAL, **69**, 1753 (1947).

(10) W. L. Hughes, Jr., *ibid.*, **69**, 1836 (1947).

(11) J. P. Greenstein, *J. Biol. Chem.*, **109**, 529 (1925).

(12) H. A. Saroff, W. L. Hughes, Jr., and A. L. Carney, in preparation. Earlier studies on this reaction were carried out by Hughes in 1940–1941. See *Chem. Revs.*, **28**, 395 (1941), footnote 10, p. 413.

(13) E. J. Cohn, *Blood*, **3**, 471 (1948).

(14) The purification of the albumin in Fraction V is described in reference 7. Fraction V-1 is that part of Fraction V which was insoluble at 10% ethanol, $\Gamma/2$ 0.01 and pH 4.7 at −5°. We are indebted to Dr. G. Derouaux for the preparation of Fraction V-1.

(15) D. M. Surgenor, L. E. Strong, H. L. Taylor R. S. Gordon, Jr., and D. M. Gibson, THIS JOURNAL, **71**, 1223 (1949).

(16) B. A. Koechlin, in preparation.

(17) J. L. Oncley, M. Melin, D. A. Richert, J. W. Cameron and P. M. Gross, Jr., THIS JOURNAL, **71**, 451 (1949).

(18) L. Heilmeyer, *Biochem. Z.*, **232**, 229 (1931).

(19) A. Lambrecht and G. Barac, *Bull. soc. chim. biol.*, **21**, 1171 (1939).

Electrophoretic measurements were carried out in the apparatus of Tiselius,[20] using the 11-cm. analytical cell. The electrophoretic migration of the protein solutions containing bilirubin was studied and in those fractions which were not homogeneous it was possible to observe with which component or components the bilirubin traveled, thus obtaining confirmatory evidence of the interaction observed by other techniques.

Dialysis experiments were carried out in two chambered systems across cellophane membranes. The solutions were kept at 0° in the absence of light and oxygen. Equilibration was aided by repeated inversion of the tubes. The outer chamber contained bilirubin dissolved in aqueous buffer, stabilized with ascorbate, as described, and in contact with solid bilirubin. The inner chamber contained albumin dissolved in the same buffer. A control experiment in which the inner chamber contained the buffer without protein, was run simultaneously. It was thus possible to compensate for losses of bilirubin due to its instability, as well as for losses in handling. Equilibrium was considered to be attained when the concentrations of bilirubin inside and outside the membranes were the same in the control experiment. Because of the instability of the bilirubin the experiments were not run longer than forty-eight hours. Attempts to avoid dialysis were unsuccessful due to difficulties in determining free and bound bilirubin directly.

pH measurements were made with a glass electrode.

Absorption spectra were measured with a Beckman spectrophotometer using quartz cells.

Results

1. Studies on the Interaction of Bilirubin with Serum Albumin

a. Spectrophotometry.—Within the range of concentration used in this work, bilirubin, either alone or in the presence of protein, was found to obey Beer's law. In aqueous phosphate buffer the molecular extinction coefficient at 435 to 440 mμ was 46,700, taking 584 as the molecular weight of bilirubin, and was independent of *p*H between *p*H 7.0 and *p*H 8.5. In the presence of crystallized human or bovine serum albumin the absorption maximum was displaced to 460 mμ (Fig. 1), while the extinction coefficient remained the same. The wave length of the maximum, and the extinction coefficient of bilirubin in the presence of albumin, were also independent of *p*H between *p*H 7.0 and 8.5. The characteristic shift in the absorption maximum of bilirubin, resulting from the addition of albumin, was taken as evidence of interaction. No such shift occurred when the albumin was replaced by γ-globulin.

b. Electrophoresis.—Further evidence of interaction was obtained when a solution of albumin to which bilirubin had been added was analyzed in the electrophoresis apparatus of Tiselius. The pigment was found to migrate in sharp coincidence with and at the same mobility as the albumin peak. In the presence of γ-globulin, no such clearly defined pigment boundary was observed. Attempts to follow the migration of the bilirubin in the absence of protein were unsuccessful.

c. Dialysis Experiments.—The results of dialysis studies with crystallized human serum albumin are summarized in Table I. Under

(20) A. Tiselius, *Trans. Faraday Soc.*, **33**, 524 (1937).

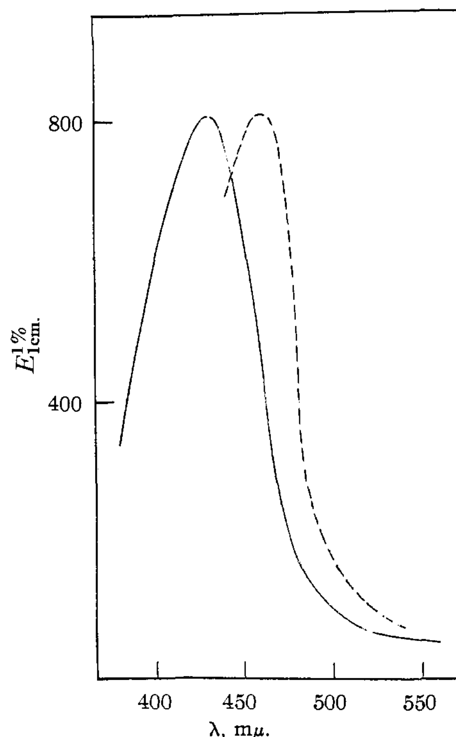

Fig. 1.—Absorption spectra of bilirubin alone and in the presence of excess albumin. *Solid line:* bilirubin, 1 mg./100 ml.; ascorbic acid 20 mg./100 ml. in *p*H 7.8, Γ/2 0.1 phosphate. *Dashed line:* Same in presence of 250 mg./100 ml. human serum albumin.

these experimental conditions, and within the limits of *p*H and concentrations studied, it was found that between 2 and 3 moles of bilirubin interacted with each mole of albumin. Human serum albumin crystallized as the mercury compound behaved in the same manner. However, the guanidinated albumin was found to bind only approximately half a mole of bilirubin per mole.

TABLE I

INTERACTION OF ALBUMINS AND BILIRUBIN IN Γ/2 0.10 PHOSPHATE BUFFER AT 0°

	Protein system at *p*H 7.4		Protein system at *p*H 7.6		Protein system at *p*H 7.9	
	inside bag	outside bag	inside bag	outside bag	inside bag	outside bag
Volume, ml.	5	45	5	45	5	45
Concn. of albumin: mg./100 ml.	330	0	600	0	600	0
Concn. of bilirubin: mg./100 ml.	5.3	0.1	13.4	1.0	19.6	2.7
Absorption maximum mμ	460	435	460	435	460	435
Concn. of bound bilirubin: mg./100 ml.	5.2	...	12.4	...	16.9	...
Moles bilirubin bound per mole albumin	1.9	...	2.5	...	3.3	...

d. Range of Stability of the Complex.—A bilirubin–albumin complex prepared at *p*H 7.6

TABLE II

INTERACTIONS OF BILIRUBIN WITH PURIFIED HUMAN SERUM PROTEINS AT pH 7.6, $\Gamma/2$ = 0.10 PHOSPHATE BUFFER

	Composition by electrophoresis in mg./100 ml.						Bound bilirubin mg./g. protein	Bilirubin assumed to be bound to albumin mg./g.	Bilirubin apparently bound to globulin mg./g.
		Globulins							
	Albumin	α_1	α_2	β_1	β_2	γ			
Albumin, crystallized	500	...	...	...	...	...	21.1	21.1	0
α_1-Globulin from Fraction V-1	100	250	100	50	...	...	16.4	4.2	12.2
α_2-Globulin from Fraction IV-6	5	...	485	10	...	...	2.8	0.2	2.6
α_2-Globulin from Fraction IV-7	40	30	370	60	...	...	11.4	1.8	9.6
β_1 Metal-combining globulins, crystallized from Fraction IV-7	...	...	...	500	...	...	0.1	0	0
γ-Globulin from Fraction II	Trace	...	...	...	...	500	Trace	Trace	Trace

and containing 2 moles of bilirubin per mole of albumin was examined over a pH range of 8 to 3.5. The absorption maximum remained constant at 460 mμ between pH 8 and 5. Dissociation, as evidenced by a gradual return of the maximum to 435 mμ and precipitation of bilirubin, occurred near pH 4.5.[21] This is approximately the same range as was found by Klotz for the interactions of bovine serum albumin with methyl orange and azosulfathiazole.[22,23] Further, Stenhagen and Teorell have shown that albumin and "yellow pigment" migrated together in electrophoresis between pH 4.18 and 8.0.[24]

2. Studies on the Interaction of Bilirubin with the Serum Globulins

When the interaction of bilirubin with some of the globulin-containing fractions of human plasma was studied, it was observed that in certain cases more bilirubin was bound by the protein than could readily be accounted for by the amounts of albumin present in the fractions. This was confirmed, in the case of Fraction IV-7, by observing the electrophoretic behavior of the fraction in the presence of bilirubin. This experiment suggested that the bilirubin was bound to the α_2-globulin, as well as to the albumin present in the fraction. The main component, β_1 metal-combining globulin, did not bind bilirubin under these conditions.

Accordingly, dialysis studies were made with a number of fractions rich in α-, β_1- and γ-globulins. The results are given in Table II. Since the molecular weights of some of these components are not known, the data have been calculated on a gram basis. In the fourth column, the amount of bilirubin that would normally be bound to the albumin in the fraction has been calculated. The difference between this and the total bound bilirubin (column 3) has been taken as a measure of the binding capacity of the globulin components present. This involves the assumption that the albumin present in these fractions behaved normally with respect to its interaction with bilirubin. It does not preclude the possibility of other phenomena which might give rise to interactions with bilirubin.

The results indicate that neither γ-globulin nor the β_1 metal-combining globulin bind, if any, not more than traces of bilirubin. The α_1-globulin from Fraction V-1 bound nearly four times as much bilirubin as would be anticipated from the amount of albumin present as impurity. Indeed, it is possible that the pure α_1-globulin from this fraction may be capable of binding more bilirubin per gram than albumin. Of the α_2-globulins studied, that isolated from Fraction IV-7 bound more bilirubin than the α_2-globulin from Fraction IV-6.[25]

Summary

From the present study it would appear that crystallized serum albumin forms a complex with bilirubin which is stable over a pH range including but extending well beyond physiological limits. The amount of this complex that formed was diminished when the free amino groups had been guanidinated by methyl isourea. Quantitative data suggest that, within the limits of bilirubin and protein concentration and in the range of pH used, the composition of the complex is of the order of 3 moles of bilirubin to 1 mole of albumin.

The studies on the interaction of bilirubin with fractions containing high proportions of α-globulins suggest that complex formation can take place between α-globulins and bilirubin. This is most marked with the α_1-globulin isolated from Fraction V-1 but is evident with the α_2-globulins isolated from Fractions IV-6 and IV-7 as well. The crystallized β_1 metal-combining globulin and the γ-globulin examined did not appear to interact with bilirubin to any significant extent.

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(21) It is possible that the dissociation of the complex was favored, at pH 4.5, by the extremely low solubility of bilirubin.

(22) I. M. Klotz, F. M. Walker and R. B. Pivan, THIS JOURNAL, **68**, 1486 (1946).

(23) I. M. Klotz, *ibid.*, **68**, 2299 (1946).

(24) E. Stenhagen and T. Teorell, *Nature*, **141**, 415 (1938).

(25) Since this paper was completed we have learned of studies on the state of bilirubin in serum now being conducted by Dr. Ulrich Westphal at the University of Tübingen in Germany (French Zone). Dr. Westphal states: "Electrophoresis diagrams using the Philpot method (by courtesy of Dr. E. Wiedemann, Basel) demonstrate that bilirubin when added to human serum, is bound partly to the albumins, and partly to the α_2 globulin fraction."

(26) Original manuscript received March 12, 1947.