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Solubility of Bilirubin in Aqueous Solutions.* (27201)

RICHARD C. BURNSTINE† AND RUDI SCHMID

Department of Pediatrics, Boston University School of Medicine, and Thorndike Memorial Laboratory, Boston City Hospital, Harvard Medical School

Recent studies concerning the bilirubin-binding properties of serum proteins(1) have made it necessary to reexamine the solubility of unconjugated bilirubin in aqueous protein-free solutions of varying salt concentrations. In dialysis experiments, Martin(2) noted that only 0.1 mg % bilirubin was soluble in phosphate buffer of ionic strength 0.1. By potentiometric titration, Overbeek *et al.*(3) found that less than 0.5 mg % bilirubin was soluble at pH 7.4 in solutions with an ionic strength ranging from 0.003 to 0.006; but Lucassen(4) encountered difficulties in repeating these determinations unless acetone was added to stabilize the reaction mixture. On the other hand, Odell's studies(1) appeared to indicate that at pH 7.4, larger amounts of bilirubin may be soluble in protein-free ultrafiltrates possessing a higher ionic strength. Consequently, the solubility of bilirubin was determined in buffers of varying ionic strengths and pH.

Materials and methods. Pyrex™ glassware was used which was prepared as follows: (a) soaked in 0.4N NaOH for 15 minutes; (b) rinsed with distilled water × 5; (c) soaked in 1:100 solution of 7×™ for at least 18 hours; (d) rinsed with distilled water × 20; (e) soaked in 6N HCl for at least 24 hours; (f) rinsed with distilled water × 20; (g) soaked in 3% bovine albumin for 15 min.; (h) rinsed with distilled water × 25. For centrifugation in the Spinco Model L ultracentrifuge, the Lusteroid™ tubes were prepared as in steps c, d, g, h, and discarded after each experiment. The screw caps were first treated with Siliclad™ and then prepared in the same manner as the Lusteroid tubes .

Cold phosphate buffer solutions of 0.01, 0.05, 0.10 and 0.20 molarity, corresponding at pH 7.4 to an ionic strength of 0.024, 0.120, 0.240 and 0.479 respectively(5) were prepared at various pH values ranging from 7.1 to 7.9. In all subsequent steps the solutions were protected from direct light. Bilirubin (Pfanstiehl) was dissolved in cold sodium hydroxide of appropriate normality to give an approximate pigment concentration of 100 mg %. One ml of this freshly prepared sodium bilirubinate solution was added immediately to 10 ml of the corersponding buffer, mixed by inversion, placed in a Lusteroid™ tube and centrifuged for 60 minutes in the dark at room temperature at 30,000 rpm. (G_{avg} 78,410). The pH of the clear supernatant was determined in a Beckman Model G pH Meter, care being taken to standardize the instrument before each determination. An aliquot of the supernatant was analyzed for bilirubin concentration by the Malloy and Evelyn method(6), modified as follows: the aliquot of the supernatant was diluted with appropriate amounts of 60% aqueous methanol (v/v); the diazo reagent was made up in 0.6N HCl; optical density was determined at 555 mμ in a Beckman DU Spectrophotometer. The results obtained were compared with a standard curve, prepared by the same

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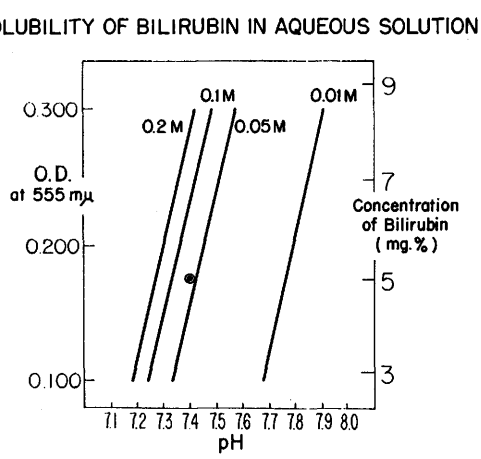

FIG. 1. Relationship of bilirubin solubility to pH in 0.2, 0.1, 0.05 and 0.01 M phosphate buffer. At pH 7.4, corresponding ionic strengths are 0.479, 0.240, 0.120 and 0.024. Approximate ionic strength of plasma ultrafiltrate at pH 7.4 is 0.150 (●).

methods using weighed amounts of bilirubin.

Preliminary investigations showed that during the time interval required for completion of these experiments significant deterioration of bilirubin in the light-protected phosphate buffer did not occur.

Results. Over the pH range from 7.2 to 7.9 decreasing hydrogen ion concentration of the buffers resulted in an abrupt increase in bilirubin solubility. The pH at which this change in solubility occurred was dependent on the molarity of the buffer. Fig. 1 gives the pH range over which large changes in bilirubin solubility occurred in buffers of 4 different molarities. For bilirubin concentrations, giving an optical density between 0.1 and 0.3, the average slope of the 4 regression lines (Fig. 1) was 0.834374 with a mean residual sum of squares S(y/x), of 0.001706 (7).

At 0.240 ionic strength replacement of up to one-half of the phosphate in the buffer by equivalent amounts of sodium chloride or sodium salicylate failed to give significantly different results in bilirubin solubility. The absorption spectrum of bilirubin in 0.1M phosphate buffer at 7.4 revealed a peak at 442 mμ, which remained unchanged on addition of more alkali, confirming an earlier observation of With(8). Furthermore, addition of sodium salicylate to this buffer in a final concentration of 400 mg % produced no change in the absorption spectrum of the dissolved bilirubin.

Discussion. These findings indicate that in aqueous solutions approximating physiological molarity and pH, bilirubin is far more soluble than has hitherto been assumed(9). Martin's values(2) were obtained with solutions of ionic strength below that of plasma; furthermore, his system contained albumin on one side of the semipermeable membrane, which has strong bilirubin-binding properties (1,2). Overbeek *et al.*(3), working at much lower ionic strength, obtained solubility values which, as would be predicted from the present findings, fall to the right of regression lines in Fig. 1. The dependency of bilirubin solubility on the ionic strength of the buffer is a manifestation of the "salting-in effect" (10).

It is evident that at the ionic strength of extracellular fluid (approximately 0.15), relatively minor changes in pH significantly affect bilirubin solubility. From Fig. 1 it is apparent that at pH 7.4, solubility of bilirubin in protein-free ultrafiltrates of plasma is of the order of magnitude of 5 mg %, but rapidly decreases on slight reduction of pH.

It is attractive to speculate that the bilirubin crystals present in the interstitial tissue of the renal papilla of Gunn rats(11,12) and the bilirubin "infarcts" of the renal medulla in hyperbilirubinemic infants(13) may result from local reduction in pH, since the salt concentration in the papillary region is markedly elevated(14). The present findings invalidate previous criticism(9,15) of Odell's studies(1) insofar as the solubility of bilirubin in protein-free aqueous solutions is concerned.

Summary. Solubility of unconjugated bilirubin was studied in protein-free aqueous solutions of various molarity and pH. At salt concentrations approximating that of extracellular fluid, bilirubin was found to be more soluble than has hitherto been assumed. Solubility of bilirubin in aqueous solution depends critically on pH and ionic strength.

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Immunocytochemical Reactions of Serum from Ulcerative Colitis Patients.* (27202)

DAVID KOFFLER, JOHN GARLOCK, AND WALTER ROTHMAN
(Introduced by Hans Popper)
Departments of Pathology and Surgery, The Mount Sinai Hospital, New York City

The association of chronic ulcerative colitis with arthritis, urticaria, erythema nodosum, conjunctivitis, hepatic and renal damage suggests the presence of an altered immunologic apparatus(1). The colon participates in nonspecific allergic reactions of the Arthus(2), and Auer types(3). The serum contains precipitating(4), agglutinating(5) and complement fixing antibodies(6) which react primarily with extracts of colonic mucosa, but also of liver and kidney. However, this has been disputed(10). Antigenic specificity resides in the microsomal fraction of colonic mucosa(7,8) although lesions have been produced with colotoxic serum only rarely(9), and an attempt at eliciting autoimmune disease of the gastro-intestinal tract was not successful(11). Therefore sera of patients with ulcerative colitis and regional enteritis were studied for gamma globulin factors bound to human colonic mucosa and hepatic ductules by the indirect fluorescent antibody technic of Coons.

Material and methods. A few drops of the sera from 25 patients with ulcerative colitis, 8 with regional ileitis, 27 with other diseases, and 26 of normal controls (Table I) were applied to cryostat sections of normal human adult colon at room temperature for 30 minutes(12). Following 3 washes with buffered saline, they were treated for 30 minutes with fluorescinated rabbit anti-human gamma globulin absorbed with liver powder. Control tissues were first blocked by unlabeled rabbit anti-human gamma globulin before use of fluorescinated rabbit anti-human gamma globulin. All sections were then washed, mounted in buffered glycerin and examined under a fluorescence microscope.

Patient's sera which showed positive binding with adult colon were then applied to the following sections using the indirect technics: sterile fetal colon, patient's own resected colon, Brunner's glands from normal duodenum and liver with malignant hepatitis showing proliferation of bile ductules (Table II). Additional sections of adult colon were incubated 30 to 45 minutes with 0.5% periodic acid to inactivate polysaccharide substance and then treated by the indirect method. Isopentane and dry ice fixed surgical specimens of acute ulcerative colitis, regional

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