Interaction of Bilirubin with Reconstituted Collagen Fibrils

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1. Interaction of bilirubin with collagen fibrils was explored in a two-phase system where collagen was present as an opaque rigid gel composed of striated fibrils, and bilirubin as an aqueous solution. 2. The K_a value of the binding of bilirubin to collagen fibrils is $5.4 \times 10^3 \text{ M}^{-1}$. The interaction of bilirubin with collagen fibrils depends on temperature. Below 5°C , the binding is greatly diminished and denaturation of collagen fibril aggregates at $52\text{--}53^\circ\text{C}$ into a dissolution state abolishes binding of bilirubin. 3. Salicylate and sulphanilamide do not affect the binding of bilirubin to reconstituted collagen fibrils. 4. Serum albumin (40–80 mM), known to reverse the binding of bilirubin to lipids, dissociates only 50% of the bilirubin bound to collagen fibrils. This suggests that sites located on collagen participate in some tight binding of bilirubin and the corresponding binding sites on albumin do not compete with them. 5. Urea (4M) abolishes more than 70% of the binding of bilirubin to collagen. Urea and thermal denaturation studies indicate the importance of conformation and organization of collagen fibrillar aggregates for the binding of bilirubin.

Skin epithelium or skin strips of rat, mouse, guinea pig and human appear to be involved in the homeostasis of circulating bilirubin (Kapoor *et al.*, 1973a,b, 1974). Preliminary heat-inactivation studies indicated that the binding of bilirubin to skin strips required participation by collagen (Kapoor *et al.*, 1973b; Kapoor & Krishna Murti, 1973). It was therefore decided to study the interaction of bilirubin with collagen fibrils. A preliminary report of this work has appeared elsewhere (Kapoor, 1974).

Materials and Methods

Chemicals and reagents

Crystalline bilirubin was a product of E. Merck, Darmstadt, Germany. The purity of the sample was checked by the procedure of McDonagh & Assisi (1972). It was stored in the dark at 2°C in a desiccator. Tris was obtained from Mann Research Laboratories, New York, N.Y., U.S.A. All other chemicals used were of AnalaR quality. Triple-glass-distilled water was used throughout.

Human serum albumin and bovine serum albumin

Recrystallized (four times) human serum albumin and bovine serum albumin were prepared as described by Cohn *et al.* (1947) and fatty acids were removed by charcoal treatment by the method of Chen (1967). After charcoal treatment the suspension was twice

centrifuged in a Spinco model L ultracentrifuge at 30000 rev./min (59 364g; r_{av} , 5.9 cm) for 30 min at 2°C , to obtain a clear supernatant. The preparation was dialysed at 2°C for 8 h against several changes of 1 litre of glass-distilled water. The absorbance of human serum albumin and bovine serum albumin at pH 7.0 did not change significantly during storage at $0\text{--}5^\circ\text{C}$ for 1 week. Values of $E_{1\%}^{1\text{cm}}$ at 278–279 nm for human serum albumin and bovine serum albumin at room temperature (28°C) were 5.3 and 6.4, very near to the values reported by Blauer *et al.* (1972) and Blauer & King (1970).

Animals

Adult male albino rats were taken from the stock colony of the Central Drug Research Institute, Lucknow, India.

Solubilization and purification of collagen

Freshly removed tail tendon of adult rats was dissolved in 1% acetic acid at 2°C and the collagen extracted was processed by the procedure of Glimcher & Krane (1964) and purified by dialysis against $0.02 \text{ M-Na}_2\text{HPO}_4$ (Jackson & Cleary, 1967).

Determination of hydroxyproline content of collagen

The purified preparation of collagen was hydrolysed in 6M-HCl in sealed tubes for 3 h at $138 \pm 2^\circ\text{C}$ (Jackson & Cleary, 1967), and the hydroxyproline content of the hydrolysate was determined by the chloramine-T oxidation procedure of Stegemann (1958). The hydroxyproline content was utilized to assess the

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purity of collagen (mammalian collagen contains 13.5% hydroxyproline; Woessner, 1961).

Preparation of reconstituted collagen fibrils

The property of aggregation of tropocollagen to form collagen fibrils in solution, as described by Gross & Krick (1958), was used in the preparation of the fibrils. An opaque rigid gel composed of striated fibrils was formed from a 0.1% cold neutral collagen solution by dialysis at 2°C against several changes (200ml each time) of 200mM-Tris-HCl buffer, pH 8.6. Dialysed collagen solution (equivalent to 0.5mg) was kept at 37°C for 1h in a test tube (10mm×100mm).

Procedure for the binding of bilirubin to reconstituted collagen fibrils

Portions of collagen fibrils were incubated for 1h in 5ml of 100mM-Tris-HCl buffer, pH 8.6, containing different amounts of bilirubin or different molar ratios of bilirubin and albumin, in a metabolic shaker (60 oscillations/min, amplitude 2cm), at 37°C in the dark. The collagen fibrils were then recovered by centrifugation and washed four times with 5ml of 100mM-Tris-HCl buffer at the temperature used for incubation and finally suspended in acetone-ethanol-water-acetic acid (7:7:4:2, by vol.), and the bilirubin liberated was determined without further delay by the procedure of Van Roy *et al.* (1971).

Results

Binding of bilirubin to reconstituted collagen fibrils

Results of a study of the interaction of bilirubin and collagen fibrils as a function of time of incubation are given in Table 1. The binding of bilirubin to collagen fibril aggregates was a linear function of time up to 60min and of concentration up to 340μM

Table 1. *Binding of bilirubin by reconstituted collagen fibrils as a function of time*

Collagen fibrils (0.5mg) were incubated in 5ml of 100mM-Tris-HCl buffer (pH 8.6) containing 425μM-bilirubin at 37°C in a metabolic shaker. At selected intervals bilirubin-binding to collagen fibrils was established as described in the Materials and Methods section. Each result is a mean value from two separate experiments.

Time (min)	Bilirubin (nmol/mg of collagen fibril)
15	94
30	232
45	332
60	400
120	412
180	434
240	464

Table 2. *Effect of temperature of incubation on binding of bilirubin to collagen fibrils*

Collagen fibrils (0.5mg) were incubated at different temperatures in 5ml of 100mM-Tris-HCl buffer (pH 8.6) containing 425μM-bilirubin for 1h in a metabolic shaker. Bilirubin bound to collagen fibrils was determined as described in the Materials and Methods section. Each result is the mean of the values from two separate experiments.

Temperature of incubation medium (°C)	Bilirubin (nmol/mg of reconstituted collagen fibrils)
5±1	10
15±1	102
25±1	234
37±1	380

approaching saturation at about 500μM. Extrapolation of Scatchard plots gave an apparent K_a value of $5.4 \times 10^3 \text{ M}^{-1}$ (Fig. 1). The bilirubin-collagen fibril complex did not readily dissociate in Tris-HCl buffer. Table 2 summarizes the results of a study of the effect of temperature of incubation on the binding of bilirubin to collagen fibrils. The binding increased progressively from 4° to 37°C. The amount of bilirubin bound with unheated collagen fibrils at 37°C was 380nmol/mg of collagen fibrils; however, after heat denaturation of collagen fibrils at 52–53°C for 1h into dissolution state (Gross, 1964) and then incubation with bilirubin at 37°C, there was negligible binding affinity for bilirubin (<10nmol of bilirubin/mg of collagen fibrils).

Effect of organic anions on the binding of bilirubin to reconstituted collagen fibrils

A number of drugs, including salicylate and sulphanilamide, are known to cause displacement *in vitro* (Odell, 1970; Bratlid, 1972) of bilirubin from albumin. This can lead to an increase in free bilirubin concentration and a consequent increase in the incidence of bilirubin toxicity (Silverman *et al.*, 1956). Therefore the effect of different concentrations of salicylate and sulphanilamide was studied on the interaction of bilirubin with reconstituted collagen fibrils. Salicylate and sulphanilamide (1–20mM) did not significantly affect the binding (Table 3). The effect of salicylate on the relative distribution of bilirubin between albumin and reconstituted collagen fibrils was studied at different molar ratios of bilirubin and albumin. Salicylate at more than 1:1 molar ratio of bilirubin to albumin revealed significant displacement of bilirubin from albumin to collagen fibrils.

Effect of human serum albumin on the dissociation of bilirubin from collagen fibril aggregates

The results presented in Fig. 2 outline the binding affinity of collagen fibrils for bilirubin. The dis-

Fig. 1. Binding of bilirubin to reconstituted collagen fibrils

(a) Collagen fibrils (0.5mg) were incubated at 37°C for 1 h in 5 ml of 100mM-Tris-HCl buffer, pH8.6, containing increasing concentrations of bilirubin. The vertical bars represent s.d. of the mean value for three separate experiments in duplicate. (b) Scatchard plot for the binding of bilirubin to collagen fibrils (Scatchard, 1949). B, Bound bilirubin; F, free bilirubin.

Table 3. Additive effect of salicylate and sulphanilamide on the binding of bilirubin to reconstituted collagen fibrils

Collagen fibrils (0.5mg) were incubated in 5 ml of 100mM-Tris-HCl buffer (pH8.6) containing 425 μM-bilirubin at 37°C for 1 h in a metabolic shaker. Salicylate and sulphanilamide were added to the final concentration and bilirubin bound to collagen fibrils was measured as described in the Materials and Methods section. Each result is the mean of the values from two separate experiments.

Drug added (μmol)	Bilirubin (nmol/mg of collagen fibril)	
	Salicylates	Sulphanilamide
None	390	390
5	393	392
25	410	394
100	400	404

sociation of bilirubin from collagen fibrils was followed in the presence of 40–80mM-human serum albumin (0.2–0.4 μM). Up to 1 h the total dissociation was 70%. The dissociation of bilirubin from collagen fibrils was not affected significantly by increasing the concentration of human serum albumin in the incubation medium. This would suggest that there are sites located on collagen fibril aggregates providing loci for a relatively strong binding for bilirubin.

Effect of urea on the binding of bilirubin to collagen fibrils

As is evident from the above results collagen fibril aggregates apparently bind bilirubin strongly.

Fig. 2. Effect of human serum albumin on the dissociation of bilirubin from bilirubin-collagen fibril complex

Bilirubin-collagen fibril complexes were prepared by incubating collagen fibrils (0.5mg) in 5 ml of 100mM-Tris-HCl buffer, pH8.6, containing 425 μM-bilirubin at 37°C for 1 h and washed free from medium as described in the Materials and Methods section. The fibrils were then incubated in 5 ml of 100mM-Tris-HCl buffer (pH8.6) in the presence of (●) 40mM- and (▲) 80mM-human serum albumin. The retention of bilirubin in collagen fibrils was followed for 4 h. ■, Initial concentration of bilirubin in bilirubin-collagen fibril complex. Each result is the mean value from three separate experiments.

Urea (8.5M) is known to alter the cross-linking characteristic of collagen fibrils (Gross & Krick, 1958). The effect of urea on the binding of bilirubin to collagen fibrils was therefore studied. Data illustrated in Fig. 3 show that urea (4M) in the incubation medium abolished more than 70% of the binding of bilirubin. The results suggest that the helical structure of collagen is perhaps the active conformation required for the binding of bilirubin.

Discussion

Collagen has hitherto been considered to have a purely structural function, although it is known from circumstantial evidence to play a dynamic role in the metabolism of neonatal animals (Grobstein & Cohen, 1965; Hauschka & Konigsberg, 1966; Stuart & Moscona, 1967). The extensive yellow discoloration of skin occurring in neonatal hyperbilirubinaemia suggests such a role for skin in the homeostasis of circulatory bilirubin. Sites are located on skin itself which account for the large binding of bilirubin, and the binding is sensitive to temperature (Kapoor *et al.*, 1973b). Collagen, the major structural protein of skin, is known to undergo thermal denaturation. The evidence obtained in the present study would suggest that collagen is a major component of skin responsible for binding of bilirubin. At the pH used in the present study collagen may be assumed to exist in a poly-

meric form (Veis *et al.*, 1967; Hayashi & Nagai, 1973) and hence is ideally suited to provide the conformation probably necessary for binding of bilirubin, which depends on the temperature of incubation. Collagen fibrils show decreased cross-linking characteristics at low temperature (Gross, 1964). However, on thermal denaturation of collagen fibrils (52–53°C) into a dissolution state (Gross, 1964) there is a negligible binding of bilirubin. The nature of binding sites on collagen also seems to be different from those on albumin. Albumin binds strongly to bilirubin and prevents bilirubin toxicity as long as the molar ratio is less than 1:1 (Jacobsen, 1969; Mustafa *et al.*, 1969; Odell, 1970). Even at the 1:1 molar ratio of bilirubin and albumin, when one molecule of bilirubin is bound to one molecule of albumin, toxicity and kernicterus have been reported in some neonatal animals (Harris *et al.*, 1958; Gartner *et al.*, 1970). Salicylate and sulphanilamide have been reported to cause displacement of bilirubin from albumin *in vitro* (Odell, 1970; Bratlid, 1972), as well as an increase in the incidence of kernicterus (Silverman *et al.*, 1956). The data presented here indicate that the effect of salicylate on the distribution of bilirubin between albumin and collagen fibrils depends on the molar ratio of bilirubin to albumin.

The molar ratios used in the present study were selected as representative of concentration of bilirubin encountered in the hyperbilirubinaemic newborn. Previous reports (Kapoor *et al.*, 1973a,b, 1974) suggest a dynamic role for skin in the influx and efflux of bilirubin by virtue of its large surface area. In situations where administration of drugs such as salicylate or sulphanilamide can lead to displacement of bilirubin from circulatory albumin and the bilirubin thus released can potentiate toxic reaction, fibrillar aggregates of collagen can still sequester the bilirubin and provide an effective detoxication system. Collagen seems to be involved in this process as the most important constituent of skin. Urea and thermal denaturation studies reveal the indispensability of specific conformation and organization of the native collagen polymers for establishing optimum binding of bilirubin. Gelatin (denatured collagen) does not bind bilirubin.

Fig. 3. Effect of urea on the interaction of bilirubin with collagen fibrils

Collagen fibrils (0.5mg) were incubated at 37°C for 1 h in 5 ml of 100mM-Tris-HCl buffer, pH 8.6, containing increasing concentrations of urea (1–4M), and binding of bilirubin to collagen fibrils was measured as described in the Materials and Methods section. Each result is the mean value of three separate experiments.

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