Bilirubin is the end product of heme catabolism in man and most animals and is ultimately excreted into the bile.1 The compound has a low solubility in aqueous media. Almost all of the bilirubin transported in the blood is tightly bound to serum albumin.2 It is assumed that charged residues of histidine, arginine, and tyrosine are involved in the binding,3 while one lysine group is close to the binding site.4 The nature of the binding of bilirubin to serum albumin is still unknown, although recent data suggest that salt linkages and hydrogen bonds rather than hydrophobic interactions are the main factors in the binding.5

Recently, we prepared6 two polymers (Scheme I) with an imidazole ring in each repeating unit, viz., poly(carbonylhistidine), 1a, and poly(carbonylhistamine), 1b. Their official names are poly[3-(4-imidazolyl)-2-isocyanopropanoic acid] and poly[2-(4-imidazolyl)-1-isocyanoethane], respectively. Trivial names are used for the sake of simplicity. These polymers might serve as models for the interaction of bilirubin with charged and uncharged imidazole sites in serum albumin. The two polymers have appreciably different pK\textsubscript{a}(Im) values: pK\textsubscript{a}(1a) = 9.4 and pK\textsubscript{a}(1b) = 5.2.6 Therefore, at pH of blood plasma, the imidazole groups in poly(carbonylhistidine) are nearly completely protonated, whereas in poly(carbonylhistamine) these groups are nearly completely unprotonated. We wish to report here that at pH 7.3, bilirubin interacts only with the polymer having unprotonated imidazole groups, suggesting that salt linkages are probably not of major importance in the binding of bilirubin to histidyl residues of serum albumin.

Degassed solutions of bilirubin (Merck p.a.) in 0.01 mol/l. aqueous NaOH were added at 0°C to solutions of polymer 1a or 1b in aqueous phosphate buffer of pH 7.3. In the solution containing polymer 1b, a precipitate was formed immediately. After vigorous shaking, a fine suspension was obtained, which had a \( \lambda_{\text{max}} \) at 445 nm in the visible spectrum. A reference solution containing bilirubin and buffer only showed a \( \lambda_{\text{max}} \) at 438 nm (\( \epsilon = 41,000 \)). In the solution containing polymer 1a, neither a precipitate nor a \( \lambda_{\text{max}} \) shift was observed. The immediate formation of a precipitate with 1b indicates an interaction between bilirubin and this polymer. The shift in the absorption maximum from 438 to 445 nm supports such an interaction. There is a remarkable increase in the stability of the bilirubin when attached to the polymer: the precipitate did not change on prolonged standing at room temperature, whereas free bilirubin does. However, on treatment of the precipitate with acid, the bilirubin was set free quantitatively.

The composition of the polymer–bilirubin complex was determined as follows. Samples of bilirubin in 10% ethanol/0.005 mol/l. NaOH were taken from a cooled (0°C) and degassed stock solution (concentration of bilirubin, 6.0 \( \times 10^{-4} \) mol/l) and added to 25 ml of a cooled (0°C) and degassed aqueous phosphate buffer of pH 7.3 and ionic strength of 0.14 mol/l. containing polymer 1b. The volume was adjusted to 35 ml with 10% ethanol/0.005 mol/l. NaOH. The final concentration of 1b was 2.0 \( \times 10^{-3} \) mol/l. The concentration range of bilirubin was (0.8–17) \( \times 10^{-5} \) mol/l. Precipitates were formed at once. After shaking the reaction mixtures for 2 min, the precipitates were collected by centrifugation, dried over P\textsubscript{2}O\textsubscript{5}, and weighed. The results are given in Table I. The uv-visible spectrum of the supernatant showed that the remaining solution contained polymer and a negligible amount of bilirubin. The total weight of precipitate is plotted against the initial amount of bilirubin added in Fig.

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TABLE I
Composition of Poly(Carbylhistamine)-Bilirubin Complex

<table>
<thead>
<tr>
<th>Initial Bilirubin (mg)</th>
<th>Total Precipitate (mg)</th>
<th>Bilirubin in Precipitate(^a) (mmol ( \times 10^{-4} ))</th>
<th>Polymer(^b) in Precipitate(^a) (mmol ( \times 10^{-5} ))</th>
<th>Molar Ratio of Bilirubin to Polymer</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.18</td>
<td>1.4</td>
<td>1.99</td>
<td>1.03</td>
<td>0.019</td>
</tr>
<tr>
<td>0.35</td>
<td>2.9</td>
<td>4.13</td>
<td>1.43</td>
<td>0.029</td>
</tr>
<tr>
<td>0.70</td>
<td>3.6</td>
<td>9.58</td>
<td>2.09</td>
<td>0.046</td>
</tr>
<tr>
<td>1.06</td>
<td>4.7</td>
<td>15.8</td>
<td>2.61</td>
<td>0.061</td>
</tr>
<tr>
<td>1.41</td>
<td>5.9</td>
<td>24.0</td>
<td>2.96</td>
<td>0.081</td>
</tr>
<tr>
<td>1.76</td>
<td>5.5</td>
<td>28.1</td>
<td>3.16</td>
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</tr>
<tr>
<td>2.11</td>
<td>6.8</td>
<td>31.7</td>
<td>3.31</td>
<td>0.096</td>
</tr>
<tr>
<td>2.81</td>
<td>8.2</td>
<td>46.5</td>
<td>3.97</td>
<td>0.12</td>
</tr>
<tr>
<td>3.52</td>
<td>9.6</td>
<td>59.3</td>
<td>4.55</td>
<td>0.13</td>
</tr>
</tbody>
</table>

\(^a\) As measured by uv spectroscopy. See text for experimental details.
\(^b\) Expressed per polymer unit.

1. Apart from the initial stage, probably related to a precipitate of different composition, the plot is linear. The linearity suggests that the complex formed has a fixed ratio of bilirubin to polymer. This ratio can be calculated from the slope \( s \) of the plot in Fig. 1. This slope is related to the number of bilirubin molecules bound per polymer repeating unit \( \nu \) as follows:

\[
(s - 1)\nu = \frac{M_\text{r polymer repeating unit}}{M_\text{r bilirubin}}
\]

Taking 584 and 121 as the molecular weights of bilirubin and polymer repeating unit, respectively, \( \nu \) was calculated to be 0.20 ± 0.02. It is worth noting here that this value of \( \nu \) strongly deviates from the bilirubin:polymer ratio, which can be expected when bilirubin is distributed statistically over the polymer. This ratio ranges from 0.004 for the lowest to 0.085 for the highest bilirubin addition.

![Fig. 1. Total weight of precipitate, P (in mg), as a function of initial amount of bilirubin, \( b_0 \), added to the polymer solution.](image)
The composition of the complex was also determined by uv spectroscopy. The precipitates were dissolved in 10 ml 0.1 mol/l aqueous HCl, and these solutions were extracted immediately with chloroform. The amounts of bilirubin and polymer were calculated from the absorbances of the chloroform and water solutions, respectively. We determined the molecular extinction coefficients of bilirubin in chloroform at 450 nm and of 1b in 0.1 mol/l HCl at 300 nm to be 51,000 and 1440, respectively. The molar ratios of bilirubin to polymer are given in Table 1. A plot of the molar amount of bilirubin versus the molar amount of polymer, expressed per polymer unit, in the precipitates gave a curve which was very similar to the one in Fig. 1. The number of bilirubin molecules bound per polymer repeating unit was calculated from the slope of its linear part to be \( \nu = 0.21 \pm 0.01 \). This is in agreement with the value of \( \nu = 0.20 \) obtained from Fig. 1.

Polymers like 1b have a rigid-rod helical structure with approximately four repeating units, \( R—N=C, \) per helical turn.\(^7\) In polymer 1b, \( R = \text{ImCH}_2\text{CH}_2—. \) Thus the binding of one molecule of bilirubin to five polymer units is equivalent to slightly less than one molecule of bilirubin per helical turn. Probably each of both ends of a bilirubin molecule is attached to an imidazole group. These ends could be coordinated to imidazole rings of either the same polymer molecule or different polymer molecules. In the former case, four bilirubin molecules will be bound to the polymer along its longitudinal axis over four to five helical turns in a very close packing. In the latter case the bilirubin acts as a crosslinking agent. We prefer the latter case over the former one because of the nonstatistical distribution of bilirubin over the polymer. Such a distribution can be visualized more easily for an orderly network than for a single chain complex.

At pH 7.3 bilirubin is a dianion.\(^5\) From the fact that this dianion binds to the neutral compound 1b and not to protonated 1a, one can conclude that the binding of bilirubin to polymer-bound imidazole does not involve salt linkages. (Catalytic experiments with polymer 1a show that the repulsive effect by the polymer COO" groups on charged substrates is small.\(^8\) Instead we prefer hydrogen bonds because such bonds are quite possible. It is well known that a neutral imidazole can act both as a hydrogen acceptor and a hydrogen donor. Bilirubin itself is a hydrogen bond donor through the weakly acidic protons in its lactam rings and an acceptor through the slightly basic pyrrolic nitrogens, as well as its COO" functions (Fig. 2).\(^9\)

Our findings with the polymeric model systems 1a and 1b have interesting implications for the type of binding of bilirubin to serum albumin. At the pH of blood plasma bilirubin might interact with the albumin mainly through the uncharged imidazole group of histidine in the protein. The function of imidazole in the binding process will be that of a proton donor and proton acceptor. If present, salt linkages will only occur to arginine and lysine residues.

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**References**


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