A comparative study on the extraction of membrane-bound bilirubin from erythrocyte membranes using various methods

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Abstract

In this study, we used three different methods for the extraction of membrane-bound bilirubin (EMB) from erythrocyte membranes. Use of 2.5% albumin, pH 7.4, for elution of EMB resulted in only 34% of the total EMB which was estimated after the solubilization of bilirubin-loaded erythrocyte membranes (BLEMs) with 1% SDS. On the other hand, incubation of BLEMs with 38 mM sodium carbonate solution containing 5 mM EDTA, pH 11.0, yielded 77% of the total EMB. Application of Fog’s reaction for the estimation of EMB directly on the BLEMs resulted in the estimation of 75% of the total EMB. These results suggest that either of the above methods, i.e. use of albumin or high pH, or direct Fog’s reaction cannot estimate the total EMB correctly. Increase in ionic strength from 0.15 to 0.45 did not release any EMB from erythrocyte membranes. Therefore, the best method for the estimation of total EMB is the solubilization of membrane with 1% SDS followed by Fog’s reaction method. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Bilirubin; Erythrocyte membrane; Sodium dodecyl sulfate; Human serum albumin

1. Introduction

Studies on the binding of bilirubin to the erythrocyte membranes have been made in order to understand the mechanism of bilirubin entry into cells which may open the way
for developing various preventive measures against bilirubin encephalopathy [1,2]. In most of these studies, radiolabelled bilirubin has been used [1–3]. Alternatively, unlabelled bilirubin has also been used after its incubation with the membranes and eluting the bound bilirubin with either chloroform or albumin [1,2,4]. However, both of these methods (chloroform/albumin extraction) suffer from a serious drawback of being incapable of removing the total membrane-bound bilirubin under different conditions (unpublished observation). Further, modified Jendrassik and Groff method (Fog’s method) [5] has not been used directly to determine the membrane-bound bilirubin. In view of the bilirubin dissociating potential of sodium benzoate and caffeine (present in Fog’s reagent) from the albumin–bilirubin complex [6,7], it seems probable that these compounds may dissociate bilirubin from the membranes, thus making it available to diazotized sulfanilic acid. Other factors such as change in pH and ionic strength may also dissociate bilirubin from the membranes. In this paper, we have compared the eluting potential of various reagents for the extraction of bound bilirubin from erythrocyte membranes.

2. Materials and methods

2.1. Materials

Bilirubin, caffeine anhydrous, sulfanilic acid, sodium benzoate and sodium nitrite were purchased from SD Fine Chemicals (India). Sodium potassium tartrate and sodium hydroxide were obtained from Qualigens Fine Chemicals (India). Bovine serum albumin, fraction V was procured from Sigma (USA). Human serum albumin was isolated by the method of Tayyab and Qasim [8]. Other reagents used were of analytical grade. Human blood (in 1.32% sodium citrate and 1.47% dextrose) was obtained from the Blood Bank of J.N. Medical College, Aligarh Muslim University, Aligarh.

2.2. Methods

Protein concentration was determined by the method of Lowry et al. [9] using bovine serum albumin as the standard.

2.2.1. Preparation of erythrocyte membrane suspension

Human erythrocytes were collected by centrifugation of blood at 1000 × g for 20 min, followed by triple washings with 50 mM Tris–HCl buffer, pH 7.4, containing 100 mM NaCl. Erythrocytes were diluted with equal volume of 50 mM Tris–HCl buffer, pH 7.4, containing 100 mM NaCl to obtain 50% hematocrit value. The membranes were isolated at 4°C from these erythrocytes following the method of Dodge et al. [10]. Finally, the membranes were washed with 50 mM Tris–HCl buffer, pH 7.4, and mixed with the same volume of the buffer equivalent to the volume of blood used.
2.2.2. Bilirubin binding experiments

Bilirubin solution was prepared by dissolving few crystals of bilirubin in 38 mM sodium carbonate solution containing 5 mM EDTA, pH 11.0. The concentration of bilirubin solution was determined by Fog’s method [5] using a calibration curve. No difference was noticed in the calibration curve for determining bilirubin concentration under all conditions used for the extraction of membrane-bound bilirubin (EMB). The bilirubin solution was protected from light and used within 1 h. All the experiments were carried out under yellow light.

The binding of bilirubin to erythrocyte membranes was studied by incubating 200 μl of stock bilirubin solution of desired concentration with 1.0 ml of erythrocyte membrane suspension and the final volume was adjusted to 1.5 ml with 50 mM Tris±HCl buffer, pH 7.4. After 30 min incubation at 37°C with intermittent shaking, the mixture was centrifuged in a Remi-10 microfuge, at 10,000 × g for 5 min at room temperature and the supernatant discarded. Membranes were washed several times with 50 mM Tris–HCl buffer, pH 7.4, until the last supernatant was devoid of yellow color. After final washing, the membrane-bound bilirubin was extracted and determined by each of the following methods.

2.2.2.1. Direct Fog’s reaction

Two milliliters of Fog’s reagent I (containing 30 g sodium benzoate, 20 g caffeine anhydrous and 50 g sodium acetate in 400 ml of water) and 0.5 ml of reagent II (prepared fresh by mixing three drops of 0.5% (w/v) sodium nitrite (reagent IIb) in 5.0 ml of reagent IIa (prepared by dissolving 0.5 g of sulfanilic acid in 1.5 ml of concentrated HCl, then diluted with 100 ml of water)) were directly added to 1.0 ml of bilirubin-loaded erythrocyte membrane (BLEM) suspension. The mixture was shaken well before incubation. If required, the reaction was carried out with 1.0 ml of diluted BLEM suspension. After 10 min incubation at room temperature, the mixture was centrifuged at 8000 × g for 20 min. To the pink-colored supernatant obtained above, 1.5 ml of Fog’s reagent III (containing 30 g sodium hydroxide and 105 g sodium potassium tartarate in 300 ml of water) was added and the green-colored alkaline azobilirubin was determined spectrophotometrically at 600 nm.

2.2.2.2. Sodium dodecyl sulfate (SDS) treatment

In another experiment, BLEMs were dissolved in 1% (w/v) SDS (to a final volume of 1.5 ml) by incubating the contents at 60°C for 1 h and Fog’s reaction was carried out with 1.0 ml of the solubilized BLEMs to determine bilirubin concentration. The turbidity caused by SDS was removed by centrifugation at 8000 × g for 20 min. The clear green-colored supernatant was collected and the absorbance was measured at 600 nm. The precipitate obtained after centrifugation was white and contained no bilirubin.

2.2.2.3. Elution with various media

Membrane-bound bilirubin was also eluted by incubating BLEMs with 1.0 ml each of either 3.8% albumin solution (final concentration, 2.5%) or 38 mM sodium carbonate solution containing 5 mM EDTA, pH 11.0, or with NaCl solution of different ionic strengths for 30 min at 37°C. The final volume of the incubation mixture was made up to
1.5 ml with the buffer. Then, the mixture was centrifuged at 10 000 × g for 5 min and 1.0 ml of the supernatant containing eluted bilirubin was subjected to Fog’s reaction for the determination of bilirubin concentration in the eluent. Necessary volume corrections were made in determining the bilirubin concentration. The pellet left was washed again to remove the eluted bilirubin and then dissolved in 1% (w/v) SDS (to a final volume of 1.5 ml) and the amount of bilirubin present in the final pellet was determined by Fog’s method [5].

3. Results and discussion

Table 1 shows the comparison of various eluting media for their ability to release bilirubin from BLEMs with the amount of bilirubin released after solubilizing the BLEMs in 1% (w/v) SDS and measuring the bilirubin directly by Fog’s method. It should be noted that the amount of bilirubin released after the solubilization of BLEMs with 1% (w/v) SDS represented the total amount of EMB. As can be seen from Table 1 at a given bilirubin load in the incubate (72 μM), the amount of bilirubin released after SDS solubilization of BLEMs was much higher than that eluted with the other media tested. Use of 2.5% albumin, pH 7.4, solution eluted only 34% of total EMB. These results suggest that 2.5% albumin solution which is reported to elute about 95% of bound bilirubin from intact erythrocytes [11], cannot be used as a bilirubin eluting medium from erythrocyte membranes. In view of the higher amount of bilirubin bound with lysed erythrocyte membranes compared to the sealed membranes [3] and dual nature of the binding of bilirubin to erythrocyte membranes (i.e. bilirubin dianion to polar heads of phospholipids and bilirubin acid binding to lipophilic regions of bilayer) [12], it seems that albumin removes that fraction of EMB which is bound to polar heads. This view is also supported by the observation that albumin could not remove aggregated bilirubin acid from membrane [13].

Use of 38 mM sodium carbonate solution containing 5 mM EDTA, pH 11.0, a

<table>
<thead>
<tr>
<th>Eluting medium</th>
<th>Estimated bilirubin* (μM)</th>
<th>Total estimated bilirubin (μM)</th>
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<tbody>
<tr>
<td></td>
<td>In eluate</td>
<td>EMB left after elution</td>
</tr>
<tr>
<td>1% SDS</td>
<td>41.9±0.9</td>
<td>41.9±0.9</td>
</tr>
<tr>
<td>2.5% Albumin</td>
<td>13.6±0.8</td>
<td>26.9±0.9</td>
</tr>
<tr>
<td>0.15-0.45 M NaCl</td>
<td>0</td>
<td>41.9±0.5</td>
</tr>
<tr>
<td>38 mM Na₂CO₃ + 5 mM EDTA, pH 11.0</td>
<td>32.0±0.8</td>
<td>10.1±0.9</td>
</tr>
<tr>
<td>Fog’s reagents I and II</td>
<td>30.1±0.5</td>
<td>12.4±0.4</td>
</tr>
</tbody>
</table>

The total bilirubin concentration in the incubate was 72 μM. Each value represents a mean of three observations from three independent experiments.

*Bilirubin was estimated by Fog’s method [5].
commonly used bilirubin solvent, resulted in the elution of about 76.4% of total EMB which was much higher than the albumin eluted bilirubin from BLEMs. These results were in agreement with the previous reports on the pH-dependent binding of bilirubin to erythrocyte membranes, i.e. decreased binding of bilirubin to erythrocyte membranes with the increase in pH of the incubation medium [1] and reversibility of bilirubin aggregate at high pH [13]. Since 24% of total EMB still remained bound with the membranes as measured by SDS treatment it can be said that increase in pH cannot dislodge all the bound bilirubin from erythrocyte membranes. Therefore, use of 38 mM sodium carbonate solution containing 5 mM EDTA, pH 11.0, cannot be a successful treatment for the estimation of total EMB.

Incubation of BLEM with solutions of different ionic strengths, i.e. 0.15–0.45 M NaCl, did not release any amount of bilirubin from the membranes as no bilirubin was detected in the eluent. These results suggest that the binding forces between bilirubin and membranes cannot be weakened by the increase in ionic strength. This was in agreement with an earlier observation that binding between bilirubin and membranes is hydrophobic [14]. Incubation of BLEM with Fog’s reagents I and II (containing sulfanilic acid, caffeine and sodium benzoate) also resulted in the removal of bound bilirubin in the form of azobilirubin. However, the amount of bilirubin estimated from the direct reaction of Fog’s reagents with BLEM was found to be 72% of the total EMB. The variation in either the incubation time of Fog’s reagents I and II with BLEM from 10 to 120 min (Table 2) or addition of Fog’s reagent III directly into the reaction mixture did not result in any significant change in the amount of bilirubin estimated. In all the above treatments, the fraction of EMB left unattacked by eluting medium was released by SDS treatment and the total sum of the amount of bilirubin released by various eluting media and SDS treatment was found to be within the range of bilirubin directly estimated after SDS solubilization of BLEM (see Table 1). These results strongly suggest that the estimation of total EMB by Fog’s method is most reliable when the estimation is carried out directly after solubilization of BLEM with 1% (w/v) SDS.

In yet another experiment, erythrocyte membranes were incubated with increasing concentrations of bilirubin (16.7–200 μM) and the amount of EMB was estimated either by direct reaction of Fog’s reagents with BLEM or with Fog’s reaction after SDS solubilization of the BLEM. As can be seen from Fig. 1, in both cases, the amount of

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Estimated EMB (μM)</th>
</tr>
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<tbody>
<tr>
<td>10</td>
<td>31.2±0.8</td>
</tr>
<tr>
<td>30</td>
<td>32.0±0.0</td>
</tr>
<tr>
<td>60</td>
<td>32.4±0.4</td>
</tr>
<tr>
<td>90</td>
<td>33.6±0.0</td>
</tr>
<tr>
<td>120</td>
<td>34.0±0.4</td>
</tr>
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</table>

*Time of incubation of BLEM with Fog’s reagents I and II.

Each value represents a mean of two observations from two different experiments.
EMB estimated increased with the increase in the concentration of bilirubin in the incubate. However, within the range of bilirubin concentration used in this study, the amount of EMB estimated by direct Fog’s reaction with intact BLEMs was always less than that of bilirubin estimated by the same method after the solubilization of BLEMs with 1% (w/v) SDS. From these results, there seems to be a competition between EMB and sulfate-binding proteins and lipids of membranes for diazotized sulfanilic acid, as diazotized sulfanilic acid is also known to bind membrane sulfate-binding proteins and lipids [15]. Further, sodium benzoate and caffeine (accelerators in Fog’s reaction) with a known potential of displacing bilirubin from the bilirubin–albumin complex [6,7], were found to be ineffective in dissociating bilirubin from the bilirubin–membrane complex. This may account for the difference in the estimated bilirubin by two different methods. In addition to the above, ineffectiveness of diazotized sulfanilic acid to enter into the bilayer membranes [15] might prevent the reaction of diazotized sulfanilic acid with some of the bound bilirubin which was reported to be hydrophobically inserted within the membrane bilayer [12]. On the other hand, the solubilization of BLEMs with SDS favored the estimation of total EMB as all the EMB was available for diazotized sulfanilic acid to react. From these results, it appears that SDS solubilization of membranes and estimation of EMB by Fog’s method can be applied successfully in the study of bilirubin binding to erythrocyte membranes.

Fig. 1. Comparison of the amount of EMB estimated by direct Fog’s reaction with BLEMs (●) and after 1% SDS solubilization of BLEMs (○).
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