Biochimica et Biophysica Acta, 690 (1982) 20–30
Elsevier Biomedical Press

BBA 71306

THE INTERACTION OF HEMIN AND BILIRUBIN WITH THE HUMAN RED CELL MEMBRANE

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(Received March 12th, 1982)

Key words: Hemolysis; Hemin; Bilirubin; (Erythrocyte membrane)

The incubation of 0.5% suspension of fresh normal erythrocytes with hemin or bilirubin resulted in substantial hemolysis. The amount of hemolysis achieved depended on the concentration of the lytic agents. In each concentration maximum hemolysis was reached within half an hour. The hemolytic effect was somewhat dependent on temperature. Comparison with the hemolytic effect of hemin on mice (Chau, A.C. and Fitch, C.D. (1980) J. Clin. Invest. 66, 856–858) showed that although both cells undergo hemolysis by hemin, the behaviour of each red cell type is different. Centrifugation and fluorescence quenching of membrane embedded probe revealed that both hemin and bilirubin bind to the red cell membrane, hemin having higher affinity. The reaction was found to be hydrophobic and therefore independent of ionic strength. The high affinity of the membrane for hemin was shown by its ability to compete successfully with globin for hemin. Electron microscopy of the red cells which underwent hemolysis indicated cell damage and some membrane destruction. Red cell ghosts were totally disrupted when saturated with hemin. These results suggest an explanation for hemolytic events occurring in cases such as elevation of serum bilirubin or abnormalities leading to hemin release by hemoglobin.

Introduction

Chau and Fitch [1,2] were able to demonstrate that mice red blood cells undergo lysis in the presence of hemin. They suggested that intracellular hemin which functions as a hemolytic agent may be liberated from abnormal hemoglobins or oxidized hemoglobin. In a recent study we were able to show that hemin can indeed be released from hemoglobin into phospholipid vesicles when globin-hemin interaction is weaker than in the native hemoglobin [3]. The possibility of hemin rather than whole hemoglobin as a precursor of lesions in the phospholipid bilayer was raised.

In the normal situation the concentration of free hemin in circulating red cells is extremely low due to the high affinity of globin and heme [4]. Upon oxidation of heme to hemin the free concentration of the latter will rise. Hemoglobin variants such as Hb-Köln are also known to liberate heme, moreover, in the last case hemolysis is known to occur [5]. The presence of free hemoglobin chains as occurring in thalassemia is known to be followed by hemolysis. Chains bind heme less strongly and also tend to be oxidized to a state similar to hemochromes which liberate easily their hemin [6]. In all these occasions one would expect the dissociated hemin to 'land' in the membrane core. While hemin is liberated intracellularly another hydrophobic molecule, in fact the catabolic proceder of hemin, bilirubin, is usually transported by the serum as an albumin conjugate. In cases of hyperbilirubinemia, the presence of unbound bilirubin was noticed to be dangerous. The toxic effect of bilirubin was demonstrated to result from its association with phospholipids [7]. It may

Abbreviations: Hb, hemoglobin; MetHb, methemoglobin

be therefore possible for bilirubin to associate with the red cell membrane from the outside. Tipping et al. [8] reported that hemin and bilirubin can bind to phospholipids such as phosphatidylcholine, phosphatidylserine and sphingomyelin, all known to be components of the red cell membrane [8]. Products of the bilirubin-phospholipid interaction were later identified by Eriksen et al. [9]. This accumulating information suggests that hemin and bilirubin undergo associations with various phospholipid components of the red cell membrane.

If binding to lipids is associated with the hemolytic effect of hemin, then bilirubin is also expected to serve as a lytic agent. Beaven and Gratzer [10] reported that hemin, but not bilirubin, binds to spectrin, the membrane cytoskeleton protein. It is possible that the interaction with such a protein and not with the phospholipids is responsible for the lytic effect of hemin. In order to discriminate between the two possibilities, the interaction of bilirubin with human red cell membrane and its hemolytic effects were studied together with those of hemin. The results are compared with data available on the lytic effect of hemin on mice red cells and discussed in light of the variations found between the two systems.

Materials and Methods

Materials. All reagents were of analytical grade. Hemin was purchased from Calbiochem, bilirubin from Fluka and anthroylstearic acid from Molecular Probes Inc.

Hemin preparation and determination. Hemin was dissolved in half the required volume of 10 mM NaOH, stirred well in the dark for at least 10 min, diluted with an equal volume of water to achieve the desired hemin concentration (2 mM in 5 mM NaOH), then centrifuged for 10 min at $40000 \times g$ and the pellet discarded. Hemin concentrations were estimated from the absorbance at 385 nm in a 5 mM NaOH solution and $\varepsilon_{mM} = 58.4$ [11]. In order to achieve greater accuracy and also quantitate this compound in red blood cell membranes, hemin was converted into porphyrin and the fluorescence intensity of the latter measured [12].

Bilirubin preparation and determination. Bilirubin was prepared essentially as described for hemin. Concentration was determined spectrophotometrically at 450 nm with $\varepsilon_{mM} = 59$ [13].

Red blood cell membrane concentration. Concentration of erythrocyte membranes was calculated on the basis of weight per volume using the following method. Freshly prepared erythrocyte membranes were suspended in buffer, dialyzed exhaustingly against water, lyophilized to dryness and weighed. The light absorption of the Fe (SCN)-phospholipid complex prepared according to Charles and Stewart [14] was determined at 488 nm. Concentrations of unknown membrane preparations were then quantitatively determined by the same method using the known dried membranes as standard.

Preparation of globin. Native globin was prepared by the method of Rossi-Fanelli et al. [15] and used within one week. Before use the globin solution was dialysed against the required solution and centrifuged to separate any unsoluble material. Globin concentration was determined from the absorbance at 280 nm using $\varepsilon_{mM} = 34.4$ [16].

Preparation of hemoglobin-free red blood cell ghosts. Hemoglobin-free ghosts were prepared by hypotonic lysis as previously described [17]. Fluorescence measurements were carried out using ghosts labelled with anthroylstearic acid as described elsewhere [17], at an excitation wave length of 340 nm and the emission read at 460 nm.

Preparation of Hb. Hb was prepared as described previously [17]. Concentration of Hb was determined using ε_{mM} at 415 nm = 125. MetHb concentration was calculated using ε_{mM} at 405 nm = 179.

Incubation of red blood cell membranes with hemin. Red blood cell ghosts pellet was diluted into an isotonic pH 7.3 phosphate buffer solution and shaken in the dark at 37°C. When temperature was reached, different amounts of hemin were added to the suspensions (zero time). At zero time and after 2 h, samples were withdrawn and centrifuged for 15 min at $74000 \times g$. The pellet was washed twice with incubation medium and hemin in pellet and supernatant determined.

Hemolysis of red blood cells induced by hemin or bilirubin. Freshly drawn erythrocytes were sedimented and washed twice with isotonic pH 7.3 solution. 0.5% cell suspensions were prepared. The experiments were carried out essentially as de-

scribed above for ghosts. Hemin or bilirubin were added to the desired concentrations and at timed intervals the cells were sedimented by centrifugation at $1000 \times g$ for 3 min and hemoglobin in the supernatant determined by measuring the absorbance at 415 nm. Hundred percent hemolysis values were taken by measuring the Hb released after hypotonic dilution (1:30) and centrifugation of the lysed cells for 30 min at $40000 \times g$.

Spectrum of MetHb. The spectrum of MetHb which developed after the addition of globin to hemin was drawn as a difference spectrum of the following: The cuvette in the sample compartment contained a mixture of hemin and globin in buffer or in buffered red blood cell membrane suspension, while the blank compartment contained either buffer alone or buffered membrane suspension. Free hemin concentration was estimated from the substraction of bound hemin from the total. Bound hemin was estimated in a preliminary experiment from the additional absorbance at 405 nm after adding globin.

Electron microscopy. Samples of cells or membranes from desired experiments were fixed in 1% isotonic glutaraldehyde and prepared for scanning electron microscopy according to the method of Sanders et al. [18]. Scanning electron microscopy was carried out using a JEOL-35 scanning electron microscope. Spectrophotometry was carried out with a Cary 219 spectrophotometer and fluorescence measured with a Perkin-Elmer MPF-44B spectrofluoremeter.

Results

Hemolysis of red blood cells induced by hemin and bilirubin

This set of experiments was carried out with fresh blood cells immediately after being drawn, as described in Materials and Methods. In this manner only up to 1% hemolysis occurred in controls where no hemin or bilirubin were added during the incubation time of 2.5 h at 37°C.

Time-course of hemolysis in the presence of various hemin or bilirubin concentrations is shown in Figs. 1 and 2. It can be observed that in the range of concentrations studied the amount of hemolysis reached depended on the concentration of hemin or bilirubin added. Comparison between percent hemolysis induced by hemin or bilirubin indicates that hemin is somewhat more effective

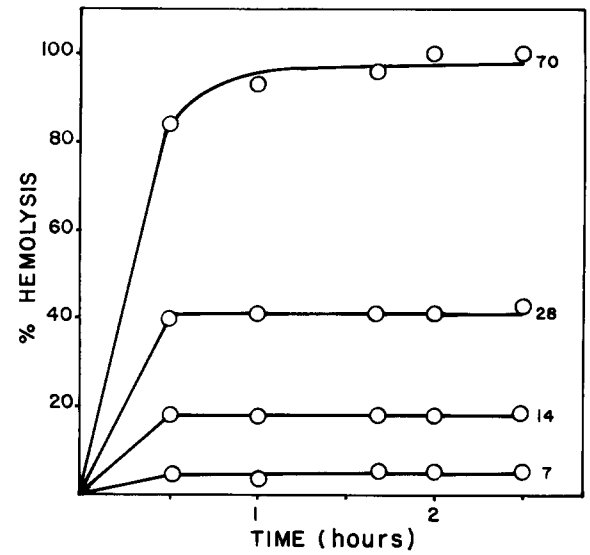

Fig. 1. Time-course of hemin-induced hemolysis of human red blood cells. 0.5% suspensions of erythrocytes in an isotonic pH 7.3 medium at 37°C in the dark. The numbers indicate the concentrations of hemin in μM.

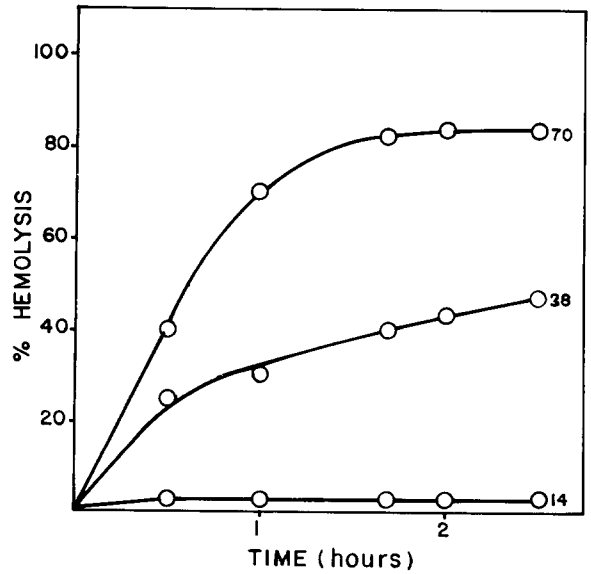

Fig. 2. Time-course of bilirubin induced hemolysis of human red blood cells. 0.5% suspensions of erythrocytes in an isotonic pH 7.3 medium at 37°C in the dark. The numbers indicate the concentrations of bilirubin in μM.

than bilirubin. 70 μM hemin brought about 100% hemolysis, while the same concentration of bilirubin caused 85% hemolysis. Moreover, in the case of hemin maximum hemolysis achieved at each concentration was reached within 0.5 h, while bilirubin appeared to exert its effect more slowly. In an additional experiment not reported here, it was observed that hemin brought about its effect within 15 min.

Fig. 3 shows the effect of temperature on the hemolysis. The data demonstrate that lowering the temperature down to 21°C did not prevent hemolysis by hemin. At lower concentrations of hemin (7 and 20 μM) hemolysis was reduced almost to half while at 70 μM hemin the final value was almost the same at 37°C and 21°C, moreover, the rate at 21°C may indicate that 100% hemolysis would have been reached after a longer time in the presence of 70 μM hemin. In other words, lowering the temperature reduced the rate of hemolysis rather than its maximal effect.

In Fig. 4 scanning electron micrographs of red blood cells incubated at 37°C in the presence of 14 μM hemin where 18% hemolysis was achieved and 38 μM bilirubin where 50% hemolysis occurred are

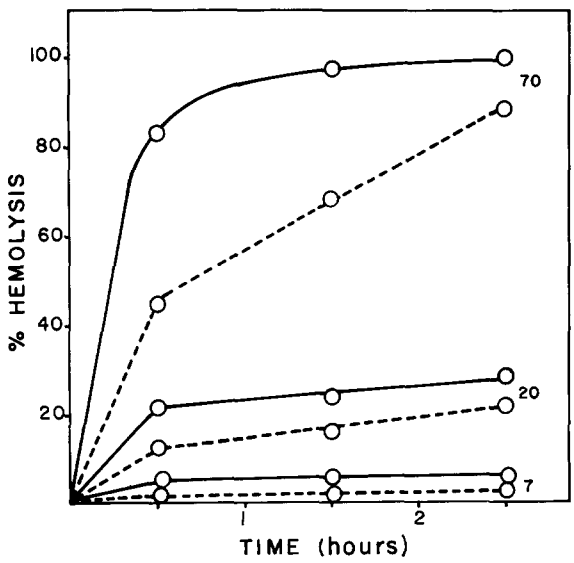

Fig. 3. Temperature dependency of human red blood cell hemolysis. Percent hemolysis achieved after incubation with various concentrations of hemin at 37°C (————) or at 21°C (— — —). The numbers indicate the various hemin concentrations in μM.

demonstrated. When morphology of the red blood cells treated with the hemolytic agents are compared to that of the control cells incubated in buffer alone (Fig. 4a), it is obvious that interaction with hemin or bilirubin brought about impressive morphological changes.

Saturation of red cell membranes with hemin

In order to see if a correlation exists between red cell hemolysis and binding of the hemolytic agents to the membranes, the following experiments were carried out. Membranes were prepared from freshly drawn blood and incubated under the same experimental condition as those of the hemolysis experiments. Various amounts of hemin were added to the different membrane suspensions and samples were drawn. The samples were immediately centrifuged and supernatant separated from pellet. These samples designated 'zero time' reflect an interval of about 0.5 h between mixing and separation of the supernatant and the precipitate. The amount of membranes in the precipitate was estimated in each case by quantitative phospholipid determination (as described in Methods). Hemin concentration in each phase was also determined quantitatively. The 'concentration' of hemin in the membrane is defined as μmol hemin per mg of membrane in the pellet. In Fig. 7 the 'concentration' of hemin in the pellet is demonstrated versus total concentration of hemin in the membrane suspension. It can be seen that the 'concentration' of hemin in the membrane phase increased, reaching a saturation value of 0.5 μmol of hemin per mg membranes.

After 2 h of incubation another set of samples were withdrawn and centrifuged under the same conditions as those of 'zero time'. The size of the pellet at higher (above 30 μM) hemin concentrations was smaller than that of the control namely membranes with no hemin added. Phospholipid determination revealed that the amount of membranes in the pellet was reduced. At the highest concentration of hemin added (300 μM), only 30% of the phospholipids pelleted under the centrifugation conditions applied. The morphology of the membranes precipitated is demonstrated by scanning electron microscopy in Fig. 8. When control membranes are compared to those where hemin was present, it is obvious that under the influence

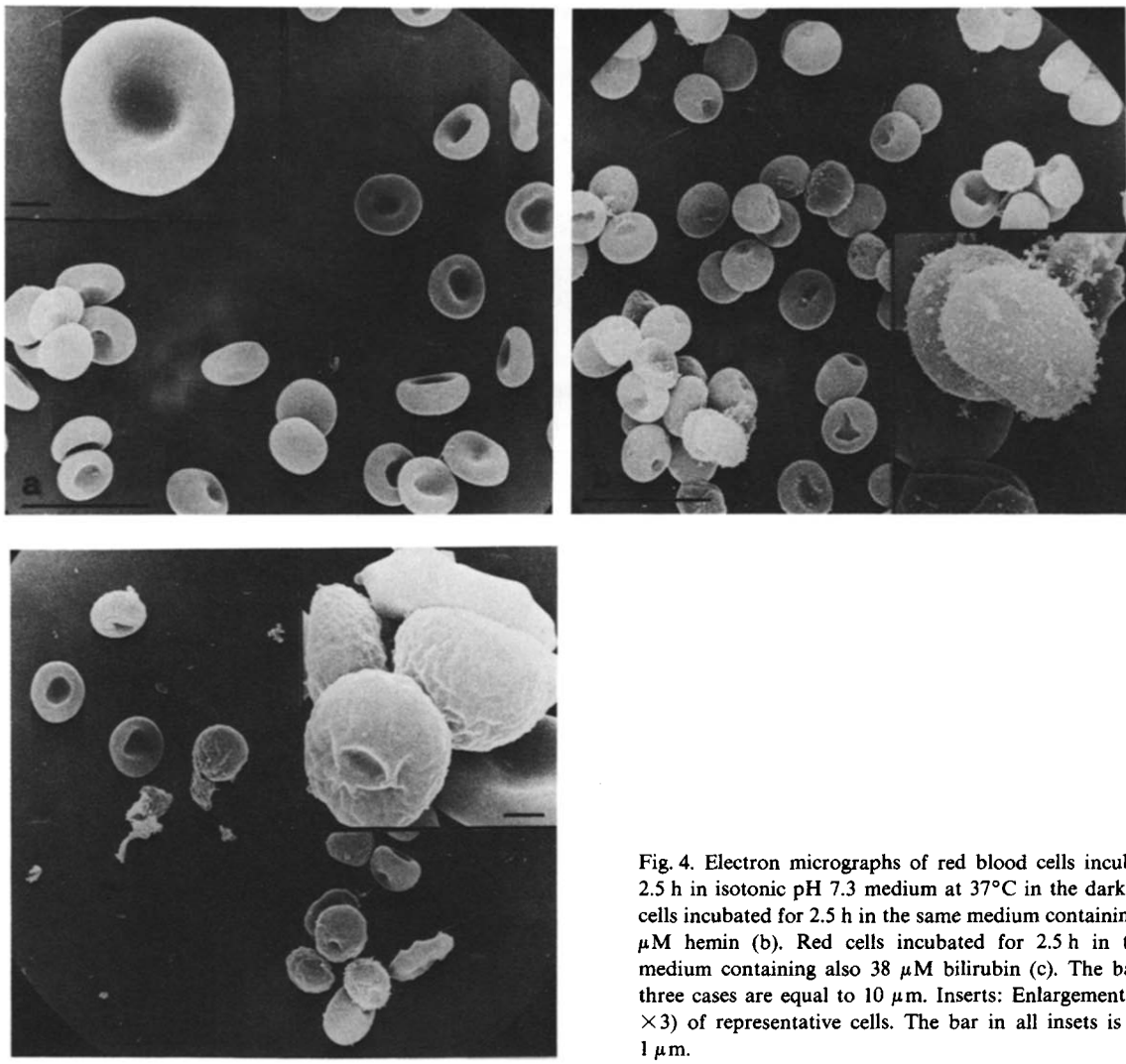

Fig. 4. Electron micrographs of red blood cells incubated for 2.5 h in isotonic pH 7.3 medium at 37°C in the dark (a). Red cells incubated for 2.5 h in the same medium containing also 14 μM hemin (b). Red cells incubated for 2.5 h in the same medium containing also 38 μM bilirubin (c). The bars in all three cases are equal to 10 μm. Inserts: Enlargement (approx. $\times 3$) of representative cells. The bar in all insets is equal to 1 μm.

of hemin the membranes could not maintain their regular morphology and total rupturing of the membrane organization resulted (Fig. 8b).

Binding of hemin and bilirubin to red cell membranes demonstrated by fluorescence measurements

The centrifugation studies indicate that hemin is able to bind to the red cell membrane. The mechanism of binding is more accurately observed in another set of experiments whereby the light absorption properties of both bilirubin and hemin is used to follow their binding to the red cell membrane. The red cell membranes were labelled with the fluorescent probe anthroylstearic acid. The emission band of the membrane probe anthroylstearic acid being at 440 nm [17], overlaps both hemin and bilirubin absorption spectra [11,13]. It should be noted that hemins Soret band is more ultraviolet shifted than that of bilirubin [11,13], and as a result overlaps the fluorescence emission band to a lesser extent. It is therefore expected to find a stronger quenching per molecule bound bilirubin than per molecule bound hemin. It is important to note that in all cases of Hb, hemin and bilirubin, the amount of fluorescence quenched reached its value within the mixing time of 1 min.

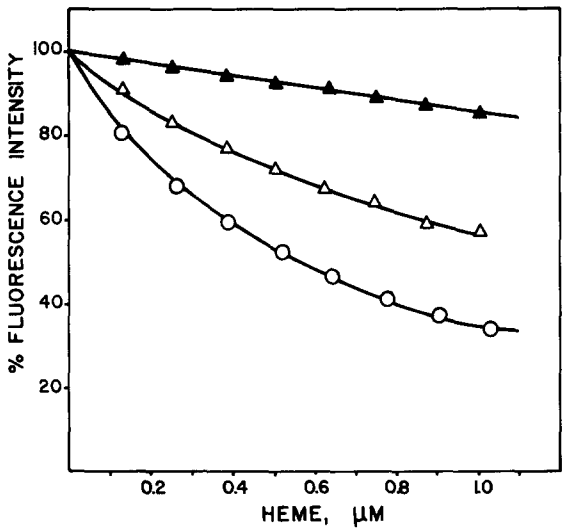

Fig. 5. Comparative binding of hemin and hemoglobin. The quenching of the fluorescence intensity of red blood cell membranes labeled with anthroylstearic acid (see text), temperature 21°C. Concentration on hemin basis is, given for both. △——△, Ghosts suspended in the presence of Hb in 5 mM phosphate buffer, pH 6.2, or ▲——▲, the same in addition to 8 mM NaCl. ○——○, Ghosts suspended in the presence of hemin in 5 mM phosphate buffer, pH 6.2, with or without 8 mM NaCl.

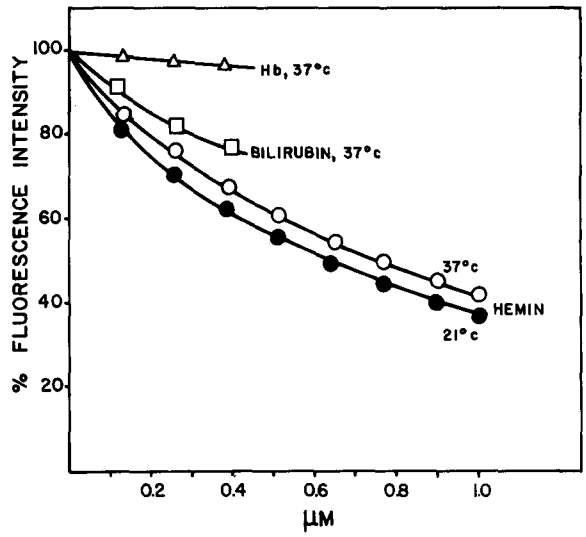

Fig. 6. Binding of hemin and bilirubin demonstrated by quenching of the fluorescence of anthroylstearic acid-labeled ghosts (see text). The ghosts were suspended in 5 mM phosphate and 145 mM NaCl, pH 7.3 at 37°C. △——△, Hb at 37°C; □——□, bilirubin at 37°C; ○——○, hemin at 37°C; ●——●, hemin at 21°C. Hypotonic conditions as in Fig. 5. Bilirubin at 21°C hypotonic pH 6.2 conditions and also above 0.4 μM could not be carried out since at low pH and higher concentrations quenching was immediately followed by recovery of the fluorescence.

Under hypotonic conditions and pH below 7.0 the ghosts lesions enable molecules even as large as proteins, which are added to the ghosts suspension, to interact with the inner membrane surface [19,20]. If ionic strength is raised to isotonicity, the ghosts reseal and therefore only the outer surface of their membrane is exposed to molecules added to their suspension. In addition, hypotonic low pH conditions encourage electrostatic interactions to occur while at isotonic pH 7 conditions electrostatically motivated associations with the membrane are appreciably reduced [21,22].

In Fig. 5 the interaction of hemin with the red cell membrane is compared to that of hemoglobin. The interaction is demonstrated by the quenching of the fluorescence intensity of anthroylstearic acid-labelled ghosts. The membrane bound hemoglobin was previously shown to exhibit Förster-type energy transfer from excited anthroylstearic acid to its heme [23]. The fact that the molar extinction coefficient of the free hemin is smaller than that of globin-heme and that the Soret band of the former is shifted to lower wavelength [8] implies that a smaller reduction in fluorescence intensity is expected per bound hemin than for a bound hemoglobin molecule. In fact, Fig. 5 shows that under hypotonic conditions hemin quenches the fluorescence intensity to a greater extent than Hb. This observation reflects higher affinity of

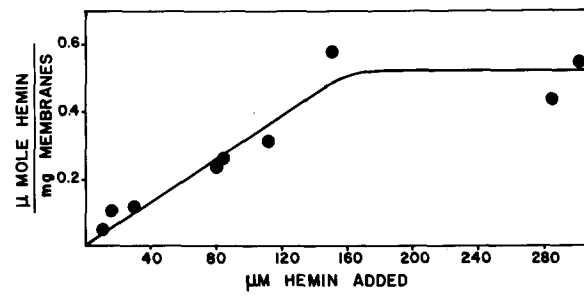

Fig. 7. Saturation of red blood cell membranes with hemin. Red cell ghosts pellet diluted into isotonic, pH 7.3 phosphate buffer solution. The ghosts were shaken in the dark at 37°C in the presence of various hemin concentrations. Samples were drawn and centrifuged as indicated in Methods.

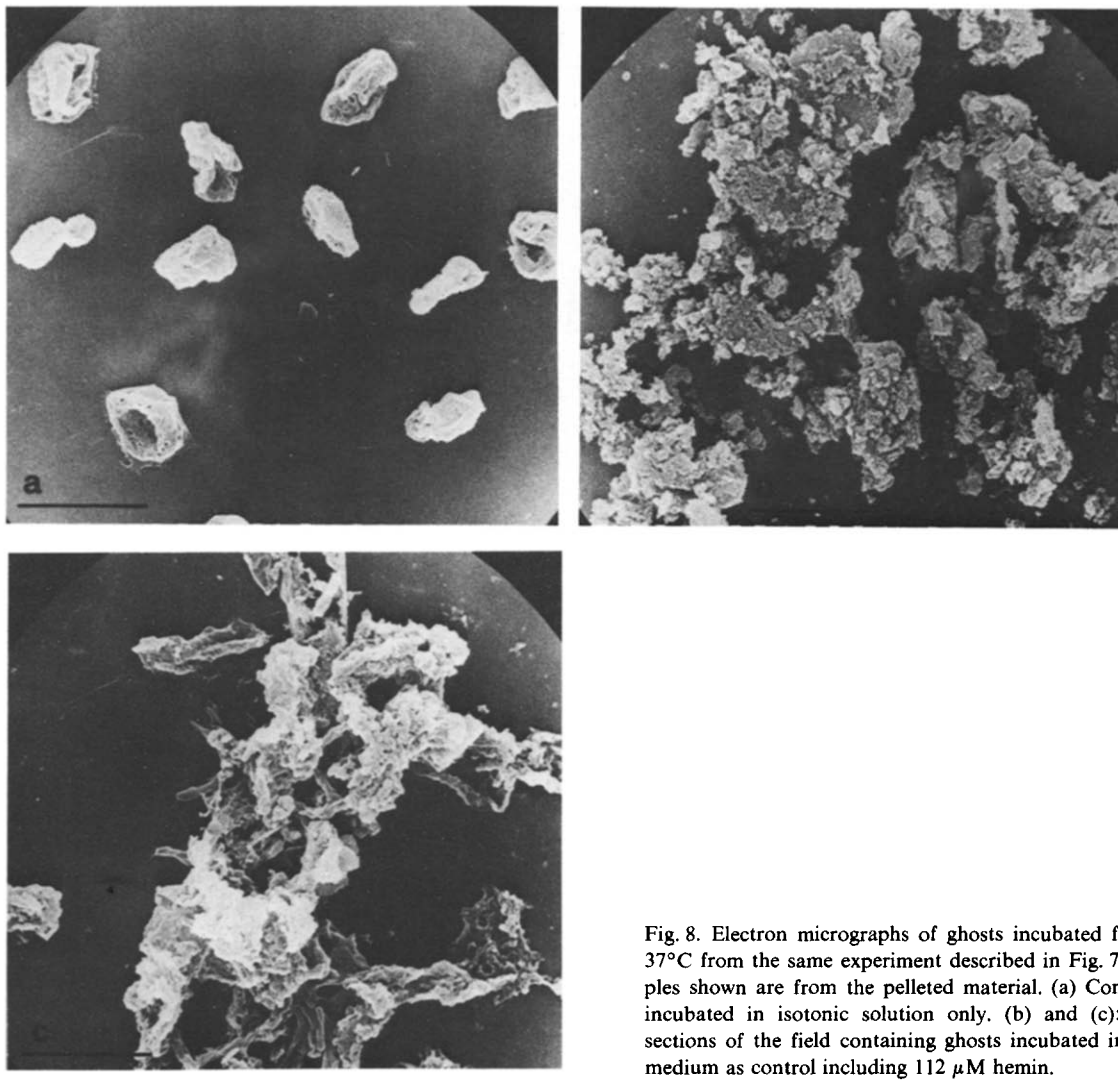

Fig. 8. Electron micrographs of ghosts incubated for 2.5 h at 37°C from the same experiment described in Fig. 7. The samples shown are from the pelleted material. (a) Control-ghosts incubated in isotonic solution only. (b) and (c): Different sections of the field containing ghosts incubated in the same medium as control including 112 μM hemin.

hemin to the membrane. Since under the experimental conditions of low pH and ionic strength hemoglobin at the concentrations of Fig. 5 is mostly bound [17], the greater quenching by hemin should be attributed to the existence of more binding sites for hemin in addition to a shorter average donor-acceptor distance. Anthroylstearic acid is embedded in the lipid core, while hemoglobin is surface bound [17], therefore, hemin being close to the probe must be membrane intercalated as well. Raising the ionic strength by 8 mM diminished the binding of hemoglobin so that very little quenching occurred (Fig. 5). On the other hand the amount of hemin bound to the membrane, as observed by the fluorescence intensity, was not influenced at all by the elevation of the ionic strength.

In Fig. 6 the quenching of the fluorescence intensity of hemin, hemoglobin and bilirubin is compared. In this case resealed ghosts are used. The ghosts were resealed by incubation at 37°C in isotonic solution pH 7.3. It can be seen that hemoglobin did not bind under these conditions, the small quenching observed should be attributed to trivial filter effects only. On the other hand, bilirubin and hemin were both able to interact with the membrane under these conditions as seen by

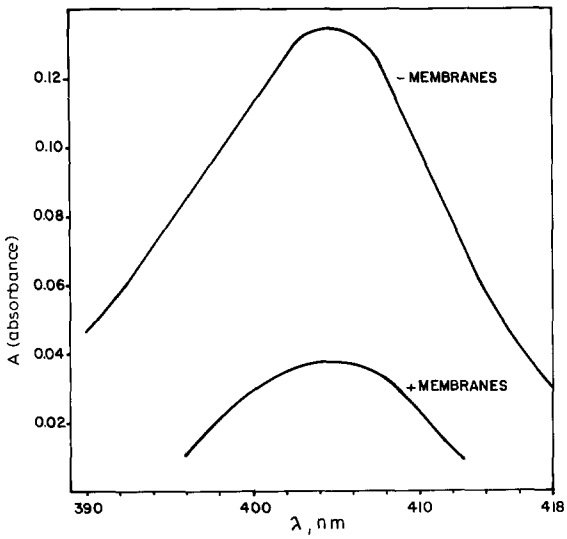

Fig. 9. Generation of methemoglobin by the interaction of hemin with globin. The appearance of methemoglobin is demonstrated by the spectral features of the Soret band. Spectra were taken after 10 min of equilibration of the reactants. Upper curve, Soret spectrum band of a mixture of 0.83 μM hemin and 1.25 μM globin in isotonic solution buffered by phosphate to pH of 7.3. Lower curve, Soret spectrum band of a mixture of 0.83 μM hemin, 1.25 μM globin and 1.0 mg/ml red blood cell membranes in isotonic pH 7.3 buffer. The amount of membranes was limited by the light scattering effect. The spectrum is a difference spectrum of the latter mixture and membrane alone in buffer.

their quenching effects. Bilirubin concentrations larger than 0.4 μM were not used since the fast quenching is followed by slow recovery of the fluorescence. This process observed by others as well was considered to be an aggregation phenomenon induced by bound lipid [24]. The fact that bilirubin causes less quenching in spite of its greater quenching potential can be attributed to its lower affinity to the membrane as compared to hemin.

Interaction of globin and hemin in the presence of red blood cell membranes

If free hemin is capable of fast interaction with the cell membrane, as the fluorescence binding experiments indicate, it is possible that red cell membranes and globin compete for hemin binding. Since the affinity of globin for hemin is extremely high [25], membranes can fail in competition with globin for hemin. In order to clarify this point globin was interacted with hemin in the absence and presence of erythrocyte membranes under isotonic pH 7.3 conditions and the formation of methemoglobin in both cases followed by the appearrance of the typical methemoglobin spectrum in the Soret band, as explained in Method. The results are shown in Fig. 9. Hemin was added in some excess to the globin in order to enable all globin to be expressed as methemoglobin in buffer solution. Under these conditions, as seen from Fig. 9 some methemoglobin was formed in the presence of the membranes. The same amount of MetHb was formed when the hemin was added to the membranes before the addition of globin. This information implies that some equilibrium was obtained where hemin was distributed between the globin and the red cell membranes. The ability of the membranes to compete for hemin is demonstrated by the fact that only one third of the amount of methemoglobin was formed in the presence of membranes as compared to the metHb formed in the buffer solution alone.

Discussion

Our results qualitatively confirm the observation made on mice red blood cells by Chau and Fitch [2] that hemin may serve as a hemolytic agent, also in agreement with their results, the amount of hemolysis observed in this study was found to be dependent on hemin concentration. However, the concentration dependency and the time-course of the hemolysis are different from those observed in mice. While in mice at 5 μM hemin 100% hemolysis occurred, in human erythrocyte 70 μM hemin were needed to achieve total hemolysis. Moreover, in mice red blood cell maximum hemolysis was achieved only after 1.5 h with a lag phase of 0.5–1 h, while in the human red blood cell hemolysis reached its maximal value within the first 15 min (15 min being the time limit of the technique). The data summarized in Fig. 7 indicate that the binding of hemin to the erythrocyte membranes is terminated within the same time range (0.5 h).

For better time resolution of the binding, the fluorescence quenching technique was introduced. These experiments showed that within one minute, the mixing time, the binding process of hemin to red blood cell membranes is terminated. Since it

was found that bilirubin also brought about hemolysis of red cells, it was decided to study the ability of bilirubin to interact with the erythrocyte membrane by the fluorescence technique as well. The results (Fig. 6) clearly show that bilirubin is able to bind to the membrane and the binding reaction is again fast, less than 1 min. The data in Figs. 5 and 6 demonstrate that the reaction of hemin is not effected by electrostatic interactions, since ionic strength did not influence the fluorescence quenching ability in both cases. The unavoidable conclusion is therefore, that the reactions of both hemin and bilirubin are basically hydrophobic. At this stage the interesting question would be where are these molecules bound in the membrane? For better understanding, we used the available information on binding of Hb to the red blood cell membrane. It was found previously that hemoglobin binds only to the inner surface of the red cell [26], therefore, at 37°C and isotonic conditions when the cells are resealed, it did not interact with the cell membrane (Fig. 5). On the other hand, when the interaction of hemin under the latter conditions is compared with that at 21°C hypotonic conditions, namely open ghosts, it can be seen that the fluorescence quenching is almost identical (Figs. 5 and 6). Previous data showed that hemin interacts readily with phosphatidylserine [3]. This component is known to be located in the inner surface of the RBC membrane [27]. In addition it was found by Tipping et al. [8] that hemin can interact with phospholipid components of the outer layer of the red cell membrane, like phosphatidyl choline. This available information suggests that hemin will bind to outer as well as inner red cell membrane components once it could reach them. The observation made in this study that hemin brought about similar quenching of the fluorescent probe by binding from the inside and outside or by binding from the outside alone strongly suggests that hemin is able to move freely through the membrane bilayer. This is in accordance with the conclusions made by Tipping et al. [8] working on phospholipid bilayers. However, Runquist and Loach claimed recently on the basis of their results that hemin is unable to traverse through the hydrophobic backbone of a phospholipid layer [28]. The contradictory conclusions could be the outcome of a different concentration range in our experiments and in the study mentioned above. It could be that at the nM concentrations range [28] of Runquist and Loach hemin prefers the water-membrane interface while when the concentration is increased to μM range as in our study, hemin is situated in the inner membrane core as well. In any case it seems that more precise information is needed to decide whether or not bound hemin is capable of diffusing across a bilayer.

The fluorescence binding experiments are limited to low bilirubin and hemin concentrations, since trivial quenching effects interfere with binding and analysis at high concentrations. Therefore, in order to estimate the amount of hemin that the red cell membrane is capable of binding the experiments summarized in Fig. 7 were designed. Estimation of the molar ratio of hemin to phospholipid shows that at saturation levels the number of hemin molecules in the membrane is in the order of phospholipid copies namely 10^8 per cell. Membrane proteins such as spectrin were also shown to be able to interact with hemin [10]. Since only 10^5 copies of this protein are available per cell [29], one would expect the bulk of hemin in loaded membranes to be lipid-associated. Beaven and Gratzer [10] showed that bilirubin will not bind at all to spectrin, yet we show here that it is a potent hemolytic agent. It therefore seems that the interaction of the lytic agents with membrane phospholipids is sufficient to cause hemolysis. Membrane integrity is the result of lipid and protein associations. Fig. 8, demonstrating the morphology of hemin-saturated membranes, clearly shows that the membrane is totally ruptured. Thus we conclude that all possible associations like lipid-lipid, lipid-protein and protein-protein are impaired. In accordance with this explanation is the electron microscopy of the Triton-treated ghosts which reveal structures reminiscent of the original membrane shape, the so-called Triton shells [30]. The latter are the result of the surviving protein-protein interactions of the cytoskeleton. In fact membrane damage by hemin or bilirubin begins much below saturation levels (one order of magnitude less) as demonstrated by the electron micrographs of the partially hemolysed red cells (see Fig. 4). It can also be seen in Fig. 4 that when hemolysis reached its maximum only part of the

cells were damaged. In other words, the degree of hemolysis calculated here is an average of the hemolysis resulting from cells that leaked totally and those which remained intact. This may not be surprising since the red cell population is known to be heterogenuous as a result of age variations [31].

The data of our study indicate that hemolytic effects of hemin as well as bilirubin occur faster than in mice. Also the dramatic temperature dependency of hemolysis which was reported to exist in the mice red cells [2], was not found in the human system (Fig. 3). On the other hand, the time course and temperature behaviour of hemolysis in the human cell resembles that reported for potassium in the mice [2]. It is possible that in the human erythrocyte hemin or bilirubin are capable of forming holes large enough to release hemoglobin while in mice, at a much lower concentration range, smaller holes are created and therefore hemoglobin will leak out only in a secondary event of cell rupture due to osmotic pressure differences. It is important to note here that at the concentrations needed to cause hemolysis in human red cells, hemin exists in the water phase as a dimer [32]. It may be possible to explain the larger lesions created by hemin in the human erythrocyte membrane on the basis of the existence of hemin aggregates rather than monomers in the membrane when its concentration is elevated ($>\mu$M). In correlation with such an explanation is the finding that bilirubin which tends to aggregate once bound to phospholipids [25], is capable of causing hemolysis at the same concentration range of hemin in spite of its lower binding affinity for the membrane. The slower hemolysis by bilirubin may be explained by slow aggregation of the latter in the membrane. That low concentrations of hemin are unable to cause hemolysis in the human erythrocyte as in mice may be attributed to differences in membrane composition known to exist between various species [33].

Where and when can hemin and bilirubin exert their hemolytic effects in vivo? Free bilirubin in the blood circulation is frequently found in newborns as the result of hemolysis. However, the existing free bilirubin could further bring about secondary events which prolong the hemolytic situation. In some severe cases of hepatitis lysis of erythrocytes is observed. This phenomenon could also be caused by free bilirubin present in the blood circulation. Free hemin is expected to be the outcome of events occurring within the red cell, since its main source, the hemoglobin, is situated in the red cell cytosol at an extremely high concentration of 20 mM. In Fig. 9 we demonstrate what could happen if free hemin exists within the red cell. The data show that the membrane can compete with globin for the oxidized heme namely hemin. While heme is strongly bound to protein backbone, once oxidized to form hemin, its binding to globin becomes looser [4]. Candidates for enhanced oxidation are free hemoglobin chains known to exist in thalassemia. Reducing agents in the membrane may prevent the latter event. It is interesting to note that MetHb reductase, the red cell enzyme responsible for reducing MetHb to Hb, was found to be associated with the inner surface of the red cell membrane [34]. The function of this enzyme in the membrane may be to keep hemoglobin in its reduced state, whereby preventing hemin dissociation. However, heme dissociation cannot be inhibited in this way when the causes of loose globin-heme association are mutations in the globin backbone. One example is Hb-Köln, in this case it is well known that hemolysis of erythrocytes occurs very frequently [35] and, moreover, that the globin mutation results in heme dissociation [5]. Hb-Köln is found to contain about 70% heme only and the fate of the released heme must be its accumulation in the membrane.

Hemin as well as bilirubin could be harmful to other membranes besides the red cell membrane. The toxicity of bilirubin to the membrane of the nervous system is well known [7]. The most typical case is kernicterus in the newborn. The fact that in mammals the hemoglobins are inserted into cells may be an advantage to the organism by restricting the damage exerted by hemin to the red cell membrane alone.

Acknowledgements

We thank Mr. A. Gabaso f nis excellent technical assistance, Mrs. A. Rozin for drawings and Mrs. S. Cojocaru for her expert assistance with the manuscript. This work was supported by

Research Funds from The Binational Science Foundation No. 1879/79.

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