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Accumulation and drainage of hemin in the red cell membrane

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The subject of hemin intercalation in red cell membranes and the correlation of the accumulated hemin level with the membrane pathology was studied. Methods which made use of dioxan and octan-2-ol mixtures to quantitate small amounts of hemin in membranes were developed. Applying these methods, hemin levels were measured in the cytoskeleton and the remaining lipid core of various red cell membranes. The amount of hemin, in both membrane fractions, was higher in pathological cells of sickel cell anemia and β-thalassemia as compared to normal circulating cells. Correlation exists between the amount of the membrane-accumulated hemin and the severity of the disease. The level of hemin in the membrane was found to be age dependent, old cells in circulation accumulating more hemin than young cells. The level of hemin in all cells tested was much lower than the amount found previously to cause immediate hemolysis when applied externally (Kirschner-Zilber, I., Rabizadeh, E. and Shaklai, N. (1982) Biochim. Biophys. Acta 690, 20–30). This was explained by the differences between the process leading to immediate lysis and membrane changes recognized as pathological by the in-vivo sequestration mechanism. In search of a physiological mechanism which may drain the cell membrane from the hazardeous hemin, albumin, the main serum protein, was found capable of serving as an efficient agent for extracting hemin trapped in red cell membranes. It is suggested that under normal conditions albumin extracts enough hemin to leave the erythrocyte with unharmful hemin amounts, however, under pathological conditions greater amounts accumulate leading to a shorter cell life span.

Introduction

The mature erythrocyte lacks some constituents available in other nucleated cells and as a result irrepairable mistakes accumulate in the cell determining its limited life time in circulation. It turns out that hemin, one of the major components of the erythrocyte is extremely hazardous when for some reason released from its natural anchor, the globin moiety. It has been shown that less than one percent of natural heme concentration is sufficient to distort completely the erythrocyte membrane [1,2]. In the latter studies hemin was introduced to the cell externally, however, recent studies in our laboratory revealed that hemin is able to destruct the cytoskeleton as well [3]. Thus, cytosolically released hemin may damage the cell membrane just by its harmfulness to the cytoskeletal proteins.

Under normal physiological conditions, the bond between heme and globin is very stable [4], hence the amount of free heme is expected to be negligible. However, in pathological or aged cells, some Hb is oxidized and denatured. In MetHb and even more so in the hemichrome oxidized states the bond between globin and hemin becomes much weaker, and therefore more free hemin is expected in equilibrium with globin [5,6]. Indeed in recent measurements determining free hemin in the red cell cytosol, elevation of hemin concentra-

tion in cells producing abnormal or oxidized Hb was reported [7,8]. The question that arises at this point is whether the increased amount in the cytosol reflects the entire hemin released from the Hb in these pathological red cells. This seems illogical since being a hydrophobic molecule, hemin may preferentially interact with hydrophobic membrane components neighboring the cytosol, as indeed was demonstrated [9–12]. The fate of hemin associated with phospholipids and cytoskeletal proteins could be accumulation in the membrane or, alternatively, a constant clearance, by transportation through the membrane to the outer surroundings of the red cell. The problem of hemin transport through biological membranes has already been raised in connection with the synthesis of various hemoproteins. Since final heme production takes place within the mitochondria and its target apoproteins are produced outside this organelle, it was suggested that heme must traverse the mitochondrial membrane to meet the protein moieties. Whether such a process indeed takes place and how, is yet unresolved [13]. Another system which possibly requires passage of heme through membrane compartments involves the process of clearing heme from the serum and passing it into its final destination, the liver cells. Receptor mediated heme uptake by liver cells was suggested as the mechanism of transport, but the details are again unclear [14].

The subject of hemin interrelation with the erythrocyte membrane, which may be crucial for the cell survival in circulation, has not yet been considered in the literature. The following report deals with the possible heme accumulation and transportation through the red cell membrane. Our first aim was to measure the level of hemin accumulated in vivo in circulating cells. To do so we concentrated our efforts on developing reliable methods for quantitation of minor amounts of hemin in membranes. These methods were then used to quantify hemin in membranes of normal, young or old red cells and also in those including abnormal hemoglobins. In order to correlate the amount of hemin measured in the membranes with that released by Hb during the red cell life span, we studied the ability of the system to drain excess hazardous hemin by use of physiologically available components.

Materials and Methods

Materials. Dansyl chloride, bovine serum albumin and hemin were purchased from Sigma and octan-2-ol from BDH. Dioxan was a product of Merck, Percol of Pharmacia and Urograffin of Schering. All other chemicals were of analytical grade. Fresh blood was obtained from healthy donors or from patients treated at Beilinson or Shiba medical centers. Outdated blood was obtained from the blood bank of Shiba medical center after 21 days of storage.

Hb preparation. Hb preparation was carried out as previously described [15] using $\epsilon^{415}_{mM} = 125$ for concentration determinations.

Hemin preparation. Hemin was dissolved in 20 mM NaOH, stirred well in the dark for at least 10 min, diluted with an appropriate volume of water to achieve a desired concentration of 2 mM hemin in 5 mM NaOH and the solution centrifuged to separate undissolved material. Hemin concentration was determined in 5 mM NaOH using an extinction coefficient $\epsilon_{mM} = 58.4$ at 385 nm [16].

Preparation of Hb-free ghosts (white membranes). Hb-free ghosts were prepared essentially according to Dodge et al. [17] as previously described [15] by hypotonic lysis 1 : 40 into 5 mM (pH 8) phosphate buffer. In the resealing experiments care was taken to shorten the time of preparation and reduce the volume of the last washes.

Separation of lipid core and cytoskeleton proteins. The membranes were freed from their cytoskeletal proteins by diluting 1 : 20 the Hb-free ghosts into 1 mM EDTA in 0.5 mM (pH 9) phosphate and incubating for 30 min at 37°C. The suspension was centrifuged at $40\,000 \times g$ for 90 min and the solution separated from pellet. This solution will be further referred to as the cytoskeleton fraction of the membrane. The pellet was further resuspended in the same volume of the EDTA-phosphate buffer and incubated overnight at 4°C for more efficient separation of residual cytoskeletal material and then centrifuged. In this manner residual cytoskeletal material in the pellet did not exceed 10% of the original as demonstrated electrophoretically. This final pellet will further be referred to as the lipid core. This pellet was diluted 1 : 25 (healthy donors) or 1 : 50 (patients) with isotonic phosphate buffer (pH 8), for the different

quantitative determinations. The first cytoskeleton fraction was used without further dilution.

Quantitation of membrane constituents. Hemin was determined in the lipid core and in the cytoskeletal protein fraction immediately after preparation. The determination was carried out essentially according to Morrison [18] by converting the hemin in the various fractions into its porphyrin derivative. This conversion is based on the use of oxalic acid as a reducing and acidifying agent. The porphyrin was then quantitated by measuring its fluorescence intensity and comparing it to known concentrations of Hb or hemin treated likewise, as standard. Quantitative determination of hemin in the lipid core was not directly possible and therefore needed further development. The details of the techniques are described in Results. Proteins in the lipid core and in the cytoskeletal protein fraction were determined according to Lowry et al. [19] using bovine serum albumin as standard. Calculation of membrane concentration was carried out either by protein measurements or alternatively, by phospholipid quantitation. The correlation between phospholipids and membrane concentration was performed as follows: phospholipids were quantitated in each preparation spectrophotometrically by determining the absorbance of a Fe(SCN)-phospholipid complex at 488 nm prepared according to Charles and Steward [20]. The absolute weight of a ghost preparation was determined by excessively dialyzing the ghosts against twice distilled water than lyophilizing to dryness namely, till a constant weight was reached. A standard curve of phospholipid content was then drawn against absolute weight of red cell ghosts. Two different standard curves were obtained, both linear, one for the red cell ghosts and the second for the lipid core alone. The slope in each case was calculated to be 1.16 A (absorbance units) per mg ghosts and 1.43 A per mg lipid core. The absorbance was measured at 488 nm and a light pathway of 1 cm was used for convenience.

Hemolysis of red cells by hemin. In light of previous findings [1,2], that red cells are lysed by externally added hemin, the sensitivity of different human red blood cells to externally added hemin was tested by incubating the red cells in its presence and measuring the extent of hemolysis in each case. The hemolysis experiments were carried out as follows: fresh blood samples were drawn either from the healthy donors or from patients suffering from sickle cell anemia or thalassemia. Out-dated normal blood was received from the blood bank after 21 days of storage. The samples were sedimented, washed twice with isotonic pH 7.3 buffer and resuspended to yield an 0.5% cell suspension in the same solution. The cells were then shaken in the dark at 37°C till temperature was reached at which time hemin was added to a final concentration of 40 μM (zero-time). After 30 min and 2 h, samples were drawn, the cells centrifuged at $1000 \times g$ for 3 min and Hb in the supernatant determined by measuring A at 415 nm. 100% hemolysis values were determined by measuring the Hb released after hypotonic dilution (1 : 30) and centrifugation of the lysed cells for 30 min at $40000 \times g$. The absorption of hemin did not contribute to that of Hb in the supernatant since firstly most of the hemin was bound to the red cell membranes and therefore sedimented with cells, and second, the molar absorption of hemin at 415 nm is negligible as compared to Hb. In one set of experiments normal red cells were incubated with 1% (150 μM) bovine serum albumin prior to the addition of hemin.

Separation of old and young cells. Fresh blood from healthy donors was passed once through α-cellulose paste (1 : 1 (w/w) mixture of α-cellulose and cellulose microcrystallin) in order to separate red blood cells from white cells. The red cells were collected by washing the cellulose three times with saline under the minimal water pressure vacuum which allows the cells to filter through slowly. The red cells were then spinned down for 5 min at 3000 rpm, mixed with the density gradient mixture according to Vettore et al. [21] and centrifuged, using a Sorvall RC-5B centrifuge, for 20 min at 15000 rpm (SS-34 rotor) for cell separation. The uppermost fraction (0.2–0.5 ml, enriched in young cells) and the lowest fraction (0.5–0.8 ml, enriched in the oldest red cells) were collected and washed 3–4 times to rid the suspension of the gradient mixture.

Preparation of resealed dansylated ghosts. Red cell ghosts were diluted 1 : 10 into a 5 mM sodium bicarbonate solution. Dansyl chloride from an acetone stock solution was then added to yield a final concentration of 0.17 mg/ml and the mixture

was incubated for 1 h on ice, stirring constantly. The ghosts were then centrifuged and resuspended in 5 mM phosphate (pH 8) buffer. Washing was repeated three times in order to remove any excess unbound dansyl chloride. At this point the ghosts were diluted 1 : 10 into an isotonic solution containing 2 mM $MgCl_2$ and 2 mM $CaCl_2$ and incubated with shaking at 37°C for 1 h to yield resealed ghosts.

Instruments. Fluorescence measurements were carried out using Hitachi-Perkin-Elmer model 44 B fluorimeter. For spectrophotometry a Cary 219 spectrophotometer was used. Centrifugations were carried out in a Sorval RC-5B or in a Backman 50L ultracentrifuge.

Results

A. Quantitative measurements of hemin in red blood cell membranes

Hemin is available in the membrane in small amounts. Thus, quantitation of hemin in the membrane calls for a sensitive technique. Radioactive measurements useful in such cases had to be ruled out in the present study since it concerns human samples. Light absorbtion spectroscopy was also found to be inadequate due to the extremely low amount of hemin in the membranes, resulting in a too low ratio of absorption to scatter signals. The most suitable technique found was fluorescence intensity measurements of hemin converted to porphyrin and thus was applied successfully to the cytoskeletal fraction, as described in Materials and Methods. Hemoglobin or free hemin both converted to porphyrin were used as standards. Hemin in solution could be used as a standard since its concentration determined by molar extinctions in NaOH was found to be as accurate as that of hemoglobin determined by molar extinction coefficients. It should be noted here that no naturally occurring porphyrin could be measured in the normal or pathological red cells studied, therefore all porphyrin fluorescence could be related to hemin.

In spite of the fact that fluorescence intensity was found to be the most sensitive technique, quantitation of hemin by this method in the membrane suspensions was still inaccurate due to the high membrane light scattering background. However, after converting hemin into porphyrin (see Materials and methods), we overcame this obstacle by the two useful methods described below.

(1) measurements in water/dioxan mixtures. In this method the non-linearity due to light scattering could be mimicked by various dioxan/water mixtures in which the fluorescence intensity of porphyrin was quenched due to porphyrin dimerization. As illustrated in Fig. 1, a good correlation

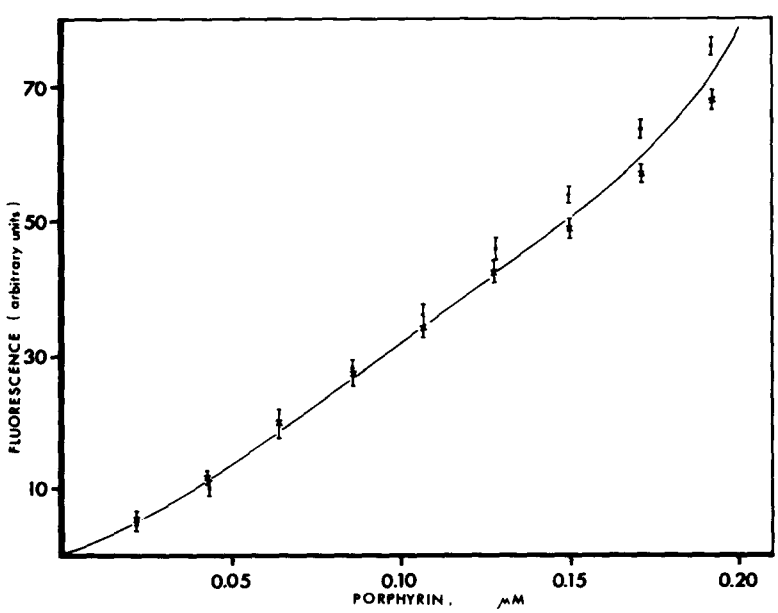

Fig. 1. Quantitative determination of hemin in red cell membranes using water/dioxan mixtures. Hemin was converted to porphyrin and its amount measured by fluorescence intensity. (●) Fluorescence intensity vs. porphyrin concentration in a 1 : 4 water/dioxan mixture only. (×) Fluorescence intensity vs. porphyrin concentration in an isotonic phosphate buffer (pH 8.0) in the presence of 0.05 mg/ml ghosts. Porphyrin was solubilized in an isotonic phosphate buffer (pH 8.0). Temp. 25°C; excitation wavelength 403 nm and emission wavelength 601 nm.

between the behaviour of porphyrin in a 20% dioxan solution and membrane suspension of 50 μg/ml lipid exists. A 20% dioxan solution was found as a suitable calibration curve for membrane concentrations in the range of 10–100 μg/ml lipids. This membrane concentration range included also enough hemin to yield measurable levels of porphyrin's fluorescence intensity, hence 20% dioxan calibration curves were used throughout the current study. However, other dioxan/water mixtures could fit different membrane concentrations: for example, porphyrin in a 33% dioxan solution was found to mimic a 150–250 μg/ml lipid (in membranes) suspension.

(II) Octan 2-ol/methanol mixtures. We further searched for alternative methods which would yield a linear correlation between fluorescence intensity and porphyrin concentration. Detergents known to solubilize membranes entirely were tested. Triton, reported to yield a linear dependency of porphyrin fluorescence intensity on its concentration [22], was tried out in the red cell membrane system of the current study. However, solubilization of the membrane by Triton did not result in linearization of the porphyrin fluorescence intensity, probably due to the light scattering resulting from the residual protein Triton shells [23]. Other detergents, such as SDS, were also examined and found unsuitable. The most satisfactory results were obtained using the semihydrophobic alcohol mixture of one part octan-2-ol and one part methanol. The fluorescence intensity of porphyrin was found to be linear in this mixture. Moreover, identical results were obtained when porphyrin alone was dissolved in this alcoholic solution or when membranes containing the same amount of porphyrin were dissolved in the same mixture (Fig. 2).

Several points should be noted summarizing the above techniques. (1) We have given the details of both methods, since the second was developed during the course of the study, namely, after measuring hemin levels by using the first method. However, the two methods when applied to the same samples gave identical results and were found to be equally convenient to work with and reliable. (2) Measurements of hemin in the red cell membrane have to be carried out immediately after ghosts preparation since longer storage periods

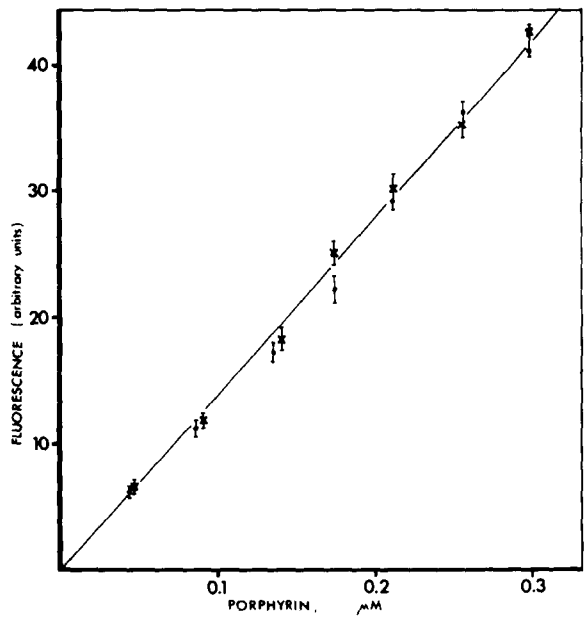

Fig. 2. Quantitative determination of hemin in red cell membranes solubilized in a 1:1 octan-2-ol:methanol mixture. (●) Fluorescence intensity vs. porphyrin concentration in the octan-2-ol:methanol mixture only. (×) Fluorescence intensity vs. porphyrin concentration in the presence of 0.05 mg/ml ghosts (added in a negligible water volume). Porphyrin was solubilized in isotonic phosphate buffer pH 8.0 increasing the percent of water content up to 10% of total volume only. Temp. 25°C, excitation wavelength 403 nm and emission wavelength 613 nm.

even at temperatures below 0°C resulted in a systematic time dependent reduction in the measurable hemin level. (3) Excitation and emission wavelengths applied in the different fluorescence measurements were chosen on the basis of the spectroscopic characteristics of porphyrin which depends also on the solvent.

B. Hemolysis of different red blood cells, induced by hemin

Table I demonstrates the results of hemolysis in the presence of hemin as obtained with normal cells in the absence or presence of albumin, with cells from out-dated blood and with cells from the following pathological cases: Heterozygous sickle cell anemia, β-thalassemia (major or intermedia), and sickle cell anemia-β-thalassemia. It can be seen that in cases where hemolysis did occur, the lowest extent was observed with normal cells (approx. 50%) and the highest with out-dated blood

TABLE I

HEMOLYSIS OF INTACT ERYTHROCYTES IN ISOTONIC MEDIA BY EXTERNALLY INTRODUCED HEMIN

0.5% cell suspension in pH 7.3 saline. Hemin concentration 40 μM, temp. 37°C. N, Normal cells; N + Alb, normal cells in the presence of 1% albumin; OD, outdated normal cells from blood bank; S, sickle cell anemia heterozygous cells; β-Th (i + m), cells from patients with β-thalassemia (intermedia and major); S/β-Th, cells from patients with sickle cell anemia and β-thalassemia. Figures presented are averages (ranges in parentheses).

Red cell type	Hemolysis (percent)	
	After 30 min	After 2 h
N	50 (42 –60)	54 (47 –65)
N + Alb	0.5 (0 – 1)	1 (0.5– 1.5)
OD	74 (63.5–77.5)	84 (72 –90)
S	58.5 (55.5–61.5)	62 (59 –65)
β-Th(i + m)	71.5 (65 –85.5)	77 (68 –87)
S/β-Th	67.5 (62.5–70.5)	67.5 (62.5– 70.5)

cells (approx. 80%). In all pathological red cells tested the extent of hemolysis fell within this range, namely, between 50 and 80%. The presence of 1% bovine serum albumin practically completely inhibited the lytic effect of hemin. Table I emphasizes an additional fact, almost maximum hemolysis was reached already after 30 min incubation with hemin, longer incubation times brought about only little additional changes. It should be noted here that the results in the cases of β-thalassemia major and intermedia were combined since their sensitivity to hemin was found to be similar, falling within the same range.

C. Hemin content in membranes of different red blood cells

The lytic effect of hemin described in the previous section raised the following questions; One, is hemin accumulating in the red cell membrane during its life span? Two, if so, is there any difference in the hemin content of the various red cells studied in the hemolysis experiments described above? Freshly drawn blood from normal subjects and from different hemoglobinopathic patients just prior to their next transfusion, was treated as described in Materials and Methods. The amount of hemin was then determined in

TABLE II

QUANTITATION OF RESIDUAL HEMIN IN THE MEMBRANE LIPID CORE OF VARIOUS RED BLOOD CELLS

N, Normal red cells (an average of 21 samples); S, heterozygous sickle cell anemia cells (an average of five samples); β-Th(i), β-thalassemia intermedia (five samples); β-Th(m), β-thalassemia major (nine samples); S/β-Th, patients carrying sickle cell anemia and β-thalassemia (five samples). Figures presented are averages (ranges in parentheses).

Red cell type	Hemin level	
	10^{-4} μmol/mg membranes	10^{-7} μmol/μg protein
N	2.3 (1.6– 3)	5.2 (3.6– 6.4)
S	10.2 (8.2–12.2)	21.2 (18.0–25.2)
β-Thi(i)	3.8 (3.5– 4.2)	7.6 (6.4– 8.0)
β-Th(m)	12.4 (6 –28)	26.4 (10.0–50.0)
S/β-Th	10.0 (5.8–16)	23.4 (10.2–30.2)

both fractions using the methods described above. Table II demonstrates the hemin content in the lipid core of normal cells, and cells from the following pathological cases: heterozygous sickle cell anemia, β-thalassemia major, β-thalassemia intermedia, and sickle cell β-thalassemia. It can be seen that in all pathological cases, except β-thalassemia intermedia, the average hemin content of the lipid core is 5–6-times greater than that of normal cells. In the lipid core of the β-thalassemia intermedia the average level of hemin was only twice that of normals. The amount of hemin was calculated in two different ways: per phospholipid translated into mg membranes (as described in Materials and Methods), or per protein. As can be seen from Table II, both determinations arrive at the same relatively results. Assuming that the phospholipid to protein ratio is the same for all cell types tested, each of these components can serve to standardize heme quantities in the various cells. The information in the literature claims that even in cases of homozygous sickle cell anemia, the lipid and protein content do not differ from normal [24]. In β-thalassemia major a twofold increase in lipids was reported [25]. Since the hemin was measured here on the basis of lipid, the increased amount of hemin found as compared to normal, will be even larger on cell basis. As explained in Materials and Methods, two extracts of cytoskeletal proteins were routinely performed to

yield a lipid core devoid of the cytoskeletal proteins spectrin and actin (protein 4.1 was not extracted since due to its small quantity it is expected to have little influence on the amount of hemin in the cytoskeleton). The first supernatant was found to contain most of the protein and hemin. Thus in determining the content of hemin in the cytoskeletal protein fraction only the results of the first extraction were considered. Table III demonstrates the hemin content in the EDTA extract containing the cytoskeletal proteins of the same red cell membranes shown in Table II. It can be seen that also here the average amount of hemin found in the cytoskeletal protein fraction of all pathological red blood cell was 3–7-times greater than that found in the same fraction of normal red cells.

It should be noted here that the average values of hemin content in the lipid core as well as the cytoskeletal protein fraction (Tables II and III) in some thalassemic cases is the outcome of widely dispersed data. On the other hand, in normal and β-thalassemia intermedia cells the dispersion of the results is much smaller.

On the basis of the amount of spectrin and actin and the lipids in the red cell we estimated (Tables II and III) that the average number of hemin molecules in membranes of normal cells is $4 \cdot 10^5$ per cell. In β-thalassemia major patients where the highest level was found, the number reached $(3–6) \cdot 10^6$ hemin molecules per cell (variations depend on the amount of lipid per cell considered). Despite the differences in normal and pathological cells and the wide scatter in the results, the relative amount of hemin in the two phases was found to be almost constant within experimental error, as demonstrated by the average levels given in Tables II and III. Moreover, each individual case showed a similar distribution of hemin between the cytoskeleton and the lipid core compartments. In most cases the cytoskeletal fraction was calculated to contain twice the amount of hemin found in the lipid core. Only β-thalassemia major cells included an average of three times more hemin in the cytoskeleton as compared to the lipid core.

TABLE III

QUANTITATION OF RESIDUAL HEMIN IN THE CYTOSKELETAL PROTEIN FRACTION

Abbreviations and number of samples are as in Table II.

Red cell type	10^{-7} μmol/μg protein
N	18 (8–28)
S	74.5 (46–100)
β-Th(i)	31 (20–41)
β-Th(m)	140.5 (90–250)
S/β-Th	68 (40–102)

It is well documented that the population of circulating red cells in various anemic cases is much younger than that of normal cells [26]. This phenomenon results from the high rate of sequestration of the damaged cells in the various anemias and the compensation process of faster production of new cells in the bone marrow. We therefore decided to investigate the correlation between normal red cell aging and hemin accumulation. For this purpose we chose cells aged in vivo, as well as blood bank aged red cells. Fresh normal erythrocytes were separated according to their density as described in Materials and Methods and hemin in each fraction determined. The results in μmoles of hemin per mg membranes in the lipid core of young and old cells as compared to out-dated cells can be summarized as follows: young cells (fresh blood), $(2.0 \pm 1.10) \cdot 10^{-4}$; old cells (fresh blood), $(4.8 \pm 2) \cdot 10^{-4}$; out-dated cells (21 days of blood storage), $(5.6 \pm 1.6) \cdot 10^{-4}$. The data demonstrate a definite increase with time, in the amount of hemin in the circulating cells. Moreover, preservation of red cells under blood bank conditions enhances hemin accumulation in the membrane lipid core even more.

D. Albumin-induced loss of hemin from red blood cell membranes

The typical light absorption characteristics of hemin can be advantageous in binding experiments involving free hemin or hemoproteins. Previously, we followed hemin or Hb binding to membranes by using their ability to quench the fluorescence intensity of a suitable membrane embedded probe, the fatty acid containing chromophore, anthroylstearic acid [15]. However, in the present set of binding experiments, albumin, known to bind fatty acids was found to perturb the fluorescence signal of anthroylstearate-labelled

membranes. The probe used was therefore the covalently bound dansyl which resulted in a stable fluorescence intensity.

Hemoglobin free ghosts were prepared as indicated in Materials and Methods from freshly drawn normal blood. The ghosts in a hypotonic solution (the so-called 'open ghosts') were immediately dansylated in the same media to allow exposure of cytosolic as well as external membrane amino groups of proteins and phospholipids to the dansyl probe. The labeled ghosts were immediately resealed (see Materials and Methods) and kept in isotonic resealing solution at 37°C for 1 h. When membranes were subjected to such dansylations, about half the fluorescence intensity could be found in the EDTA extracts, indicating binding to cytoskeletal proteins. In addition to the cytoskeletal proteins, the inner membrane bilayer itself contains most of the phospholipids with the active amino groups which are also suitable candidates for dansylations (phosphatidylserine and phosphatidylethanolamine). Hence we estimate that a major fraction of the dansyl fluorescence in such preparations resides in the inner bilayer components of the cell. Immediately after dansylation and resealing, hemin was added to a final concentration of 0.2 μM. This low concentration was chosen in order to eliminate any membrane changes known to occur in the presence of hemin [1]. Experiments were carried out at 37°C to provide maximal fluidity thereby allowing the hemin-membrane system to equilibrate. As demonstrated in Fig. 3, by following fluorescence intensity of the dansyl probe, equilibrium was reached after a few minutes and the system remained steady for at least an additional three hours. Addition of albumin caused an immediate elevation of the fluorescence.

About one-third of the quenched fluorescence (27%) was reversed within the mixing time, which is half a minute under the experimental conditions applied. An additional portion of the quenched fluorescence (44%) was further reversed but in a definitely slower mode (half time of 8 min). Within an additional 2 h no further changes in the fluorescence intensity could be observed. Therefore, residual quenched fluorescence was either irreversibly quenched or its release required a much longer time (in scale of days) therefore referred to as the

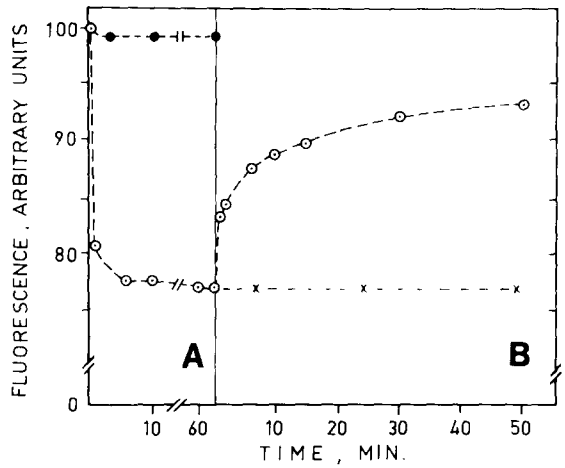

Fig. 3. Binding and efflux of hemin from red cell membranes. (A) Binding of hemin to resealed ghosts. Temp. 37°C; 0.05 mg/ml membranes; 0.2 μM hemin. Fluorescence intensity of dansyl-labeled membranes alone was set to 100%. The addition of hemin to the ghost suspension is considered as time zero of the reaction. Excitation wavelength 340 nm and emission wavelength at 500 nm. ●------●, Addition of hemin to ghosts suspended in 0.04% albumin; ○------○, addition of hemin to ghosts in the absence of albumin. (B) ×------×, An additional time extension of the hemin quenched ghosts in the absence of albumin; ○------○, Addition of 0.04% albumin to the ghosts which had been interacting with hemin for 1 h. All experimental conditions as in (A).

'extremely slow' phase. It should be noted here that the fraction of fluorescence quenched by each phase does not necessarily correlate with the fraction of effluxed hemin. As stated above, the dansyl probe is mainly concentrated on the inner surface of the membrane and therefore hemin associated with this membrane fraction is expected to have a greater quenching ability than externally situated hemin.

In the hemolysis experiments (Results, section B), it was found that introduction of albumin prior to the addition of hemin inhibited the hemin-induced hemolysis. The most direct analysis of these data imply that hemin was bound to albumin and as such could not bind to the membranes. We have therefore added hemin to dansylated ghosts which were already immersed in a buffer containing albumin and followed the changes in fluorescence intensity. As shown in Fig. 3A, no quenching of the fluorescence occurred upon addition of hemin, when albumin was already present.

Discussion

The red cell membrane can be divided grossly into two main compartments; the cytoskeleton composed of proteins only and the membrane lipid-core composed of hydrophobic mostly transmembrane proteins and the lipid bilayer. Since the hemin released from Hb into the cytosol will first topographically encounter the cytoskeleton, we originally assumed that the cytoskeleton can accomodate all the membrane associated hemin. However, determination of hemin levels in each membrane section separately (Tables II and III) clearly demonstrates that hemin in all red cell membranes tested has reached the lipid core as well. Moreover, as stated already in Results, hemin in the two membrane compartments has attained equilibrium since its partition showed a constant value. This distribution of hemin occurred despite the fact that our calculation of the amount accumulated even in the patients cells implied that spectrin was far from its maximal binding capacity [11]. We therefore concluded that hemin resides in the cell membrane of circulating cells in a dynamic equilibrium between the various membrane components.

All hemin in the red cell originates from Hb and thus could be either Hb bound or dissociated from globin, the so called 'free hemin'. The Hb bound hemin is expected to be dissociated from the membrane with spectrin and actin by EDTA. On the other hand, the 'free hemin' if not protein bound will be intercalated in the lipid core [9,28]. Therefore, the EDTA extract contains hemin still associated with Hb and part of the 'free hemin' bound to cytoskeletal proteins. The lipid core, in contrast, contains only 'free hemin'.

How much hemin can accumulate in the red cell membrane? The results as summarized in Tables I and II demonstrate the following: there is a definite difference between average amounts of hemin in normal blood cells and in cells from the various hemoglobinopathies examined. This is true for both the cytoskeleton and the lipid core. In cases where the large Hb precipitations formed are attached to the membrane, the distribution of hemin between the two membrane compartments discussed above may change due to the large amount of globin associated with the membrane. This is illustrated by the case of β-thalassemia major, where the surplus α-chains associate with the membrane in the form of inclusion bodies, and also relatively more Hb was found in the cytoskeletal fraction. The next conclusion is that in severe hemoglobinopathies like β-thalassemia major and sickle cell anemia, more hemin is incorporated into the membrane than in milder diseases like β-thalassemia intermedia. This result is of course best demonstrated when the same kind of mutation is considered, namely, β-thalassemia intermedia and major. These observations imply that a definite correlation exists between the severity of an Hb mutation disease and the amount of hemin accumulated in the red cell membrane.

The wide dispersion of hemin in cell membranes within each mutation category, as compared to normals (Tables II and III) calls for clarification. One trivial explanation would be that there are more fluctuations in the amount of Hb mutants settling on the membrane during hypotonic lysis [24,29]. However, as the time interval of the ghost preparation is not sufficient to allow the slower hemin dissociation to the lipid core, experimental variations are expected to result in enlarged and dispersed hemin, preferentially in the cytoskeleton. As stated above, this is not the case, since increased amounts in the cytoskeleton were followed by increased hemin in the lipid core. We therefore must conclude that heterogeneity is not the outcome of the preparation, but resides in the cells themselves. The heterogeneity of the patients' samples is not surprising in light of the variations in their clinical state even within the same hemoglobinopathy. In addition it should be noted that in spite of the care taken to get blood samples only just before transfusion, the frequency of blood transfusions varied according to the case and cooperation of the patient. Hence, it is expected that each individum carried a different fraction of normal cells when the blood sample was drawn.

Since there is a correlation between the amount of hemin in the membrane and the cell damage it is possible that excess membraneous hemin is related to the signals by which the cell is being recognised as a candidate for sequestration. Aged cells may possibly be marked for removal by hemin elevation. We therefore measured hemin levels in membranes of erythrocytes segregated into popu-

lations of progressive age. In light of previous data we relied on hemin accumulated in the lipid core in each case. The results imply that indeed the most aged cells have accumulated more hemin in their membranes. Blood bank out-dated cells which are also known to be filtered out of circulation faster [30] show an elevation of hemin in their membranes as well. It is of interest that the average value measured in normal individuals is nearer to the value found in younger cells. This is possibly the outcome of a nonlinear accumulation of hemin during the cell life span. The above findings stress even more the elevated amount of hemin in the hemoglobinopathies which are known to contain a higher level of young cells [26].

There is an apparent contradiction in the above conclusions which state on the one hand that normal cells accumulating a certain hemin level are sequestered and on the other hand pathological cells with higher hemin levels still exist in circulation. The main difference between normal and patients' cells resides in the longer life span of normal cells in circulation, namely, in the longer incubation time of their cell membrane with the intercalated hemin. The membrane accumulated hemin may not be only passive but may also interact with different membrane constituents generating products that are probably as hazardous to the cell membrane as hemin, if not more so. The fact mentioned in Results that the amount of hemin detected as porphyrin in the membranes decreased with time, is indicative of hemin reactivity in the cell membrane. It therefore seems that the time of hemin residency in the cell membrane plays an additional accelerating role in cell sequestration. This explains the ability of the younger pathological cells to accomodate larger amounts of hemin in their membranes before these membranes are recognized as damaged and therefore fit for sequestration.

Since, in this study, pathological cells were found to accumulate higher hemin levels than normal cells, it was postulated that the former will be lysed by less hemin added externally than the latter. However, the results in Table I demonstrate that the pathological cells lyse in the presence of a somewhat lower hemin concentration but the difference is not significant. The explanation probably resides in the fact that there is a vast gap of two orders of magnitude between the amount of hemin that has to be incorporated into the cells to cause total immediate lysis and that found in the membranes of all red cell types studied here. This difference may be explained by any or all of the following; (1) Lysis by externally added hemin is brought about by an entirely different mechanism than the physiological phenomenon in which the hemin comes from within the cell. (2) The hemin level detected in the RBC membranes in this study does not reflect the true total amount accumulated during the life span of the red cell, since the hemin is being gnawed at constantly by the different interactions with the membrane constituents, as discussed above. (3) Membrane changes recognized by the phagocytic system in the spleen may require much smaller amounts of hemin as compared to the concentrations needed for causing immediate total hemolysis in vitro.

If physiologically small amounts of hemin that slowly accumulate in the membrane are sufficient to bring about cell sequestration, the blood system must provide a way of defending itself from hemin accumulation. How can the cell be drained from the accumulating hemin? One possibility is a carrier requiring mechanism. At the moment we do not know of the existance of such a carrier in the red cell membrane. Another much simpler mechanism may involve uptake of hemin partitioned into the outer membrane layer by a heme-binding protein. The natural candidates to be involved in such hemin transport in vivo are the plasma proteins hemopexin and albumin that bind hemin most strongly. While it may be that hemopexin binds hemin somewhat more strongly than does albumin, the large ratio of concentrations of albumin to hemopexin (100 : 1) in all species examined, should make albumin the main carrier of hemin [31]. Previous studies have demonstrated that albumin binds specifically to the outer surface of the red cell membrane [32]. Association at the cell surface is apparently the underlying principle of clearance of some metabolites from the red cell membrane by albumin. It was observed that small molecules, such as fatty acids and lysophospholipids, which strongly bind to albumin, are selectively removed from the cell membrane by this protein [33]. We therefore designed the experiments demonstrated in Fig. 3 where hemin was

introduced to dansyl labeled ghosts as described in Results. As shown in Fig. 3A once the system reached steady state, the fluorescence signal remaining after quenching by hemin was extremely constant when monitored for about three hours. This implies that the random orientation of hemin and the dansyl probe remained the same during that time [34]. Since the fluorescent probe is covalently bound to the membrane, it may be concluded that hemin did not leave the membrane phase, or, in other words, no spontaneous efflux of hemin out of the membrane occurred. This equilibrium was immediately perturbed by addition of albumin to the cell suspension starting hemin efflux from the membrane. The albumin-induced reversal of fluorescence intensity (Fig. 3B) demonstrates a kinetically heterogeneous reaction composed of more than one phase. Hemin binds strongly and extremely fast to albumin [35]. This renders the diffusion of hemin to meet the surface-attached albumin to be the rate-limiting step in the interactions of membrane-intercalated hemin with albumin. We assume that the very fast phase involves hemin molecules, associated with the outer layer of the membrane phospholipids or other membrane surface components. It has been recently demonstrated by Cannon et al. [36] that such a fast phase exists also when hemin-included liposomes were interacted with albumin. Once the outer membrane surface has been differentially extracted by albumin, redistribution of the hemin between the inner and outer leaflets of the membrane bilayer is expected. Thus, slower phases actually measure the intramembrane flow rate of hemin towards the outer membrane layer of the red cell. Such a second slower rate phase was also described when albumin was interacted with hemin-included liposomes [36]. In contrast to the hemin efflux from liposomes [36], the process in the red cell membrane could not be analyzed by a two-step kinetic model. The very slow, not yet analyzed kinetic portion of the curve may correlate with the hemin associated with the cytoskeletal proteins.

In summary, this study demonstrates that hemin intercalated in the red cell membrane may be harmful to the cell's ability to survive in circulation. The serum, however, provides a self-defence machinery in the form of albumin, a protein which combines the unique properties of associating with cell surfaces and hemin binding. Under normal conditions, the efficiency of albumin is enough to leave the erythrocyte with amounts of hemin which are too low to become membrane hazards. However, in pathological situations, when free hemin is being transferred to the membrane at higher rates, this is not so and the accumulated hemin may lead to shortening of the red cell life span.

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