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Studies on the efflux of heme from biological membranes

Heng H. Liem [a], Noa Noy [b], Ursula Muller-Eberhard [a,*]

[a] *Department of Pediatrics, Pharmacology and Biochemistry, 1300 York Avenue, New York, NY 10021, USA*
[b] *Division of Nutritional Sciences, Cornell University, Ithaca, NY 14853, USA*

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Abstract

It is unknown how heme is distributed intracellularly from its site of synthesis in the mitochondria to other organelles. In previous work (Biochemistry 23, 3715, 1984) the transfer of heme from lipid bilayers to soluble proteins had been found to be independent of the recipient proteins' affinity for heme. Here, we investigated whether proteins are involved in the transfer of heme from biological membranes into aqueous media. We followed the release of ^{14}C-labeled heme, from mitochondria preloaded with the heme, to BSA and found that only about 28% of the heme was extracted on the first wash. After the third wash 35–50% of the heme that had been partitioned into the membranes was extracted. Fourth and fifth washes with BSA or a cytosolic heme-binding protein (HBP, also known as liver fatty acid binding protein) removed only insignificant amounts of ^{14}C-labeled heme. Similarly, a large portion of the preloaded ^{14}C-labeled heme could not be extracted from a variety of isolated membranes (inner and outer mitochondrial membranes, plasma membranes of liver cells, kidney cortex cells and erythrocyte membranes). By contrast, essentially all [^{14}C]palmitate preloaded in biological membranes and all ^{14}C-labeled heme preloaded in synthetic membranes was released to albumin (Biochemistry 23, 3715, 1984). These observations suggest that, in general, heme associates with membrane components which can be distinguished into two compartments. One compartment releases its heme spontaneously, while another compartment binds heme so tightly that a specific process has to be evoked for its release.

Key words: Heme; Mitochondrion; Biological membrane

1. Introduction

The mechanisms by which organic anions move throughout the cell are largely unknown. Soluble organic anions diffuse rapidly in aqueous solutions and lipophilic organic anions partition freely into membranes; yet both types of organic anions require specific mechanisms to be transfered across membranes and to reach sites of function or catabolism. The transfer of heme from artificial membranes to soluble proteins is found to be independent of the affinity of the recipient proteins for heme [1]. Likewise, the transfer of a variety of lipophilic molecules (fatty acids and bilirubin) from synthetic and biological membranes into aqueous media is not mediated by proteins [2–6]. Therefore, the transfer of heme, another lipophilic molecule, from biological membranes may or may not be protein mediated. Recent findings, on the efflux of newly synthesized heme from intact respiring mitochondria appeared to be facilitated [7] by an abundant heme-binding protein of liver cytosol (HBP) [8], identical with L-FABP [9]. The last step of heme synthesis occurs in the inner mitochondrial membrane, from which heme is distributed within the cell, e.g., to the endoplasmic reticulum (cytochrome *P*-450 formation) and peroxisomes (catalase assembly) [10]. It was suggested that HBP may have a role in the intracellular transport of heme [7]. A systematic approach to resolve these seemingly controversial findings, therefore, was initiated.

Abbrevations: ALA, δ-aminolevulinic acid; BSA, bovine serum albumin; EGTA, ethyleneglycol bis(β-aminoethylether)-*N,N'*-tetraacetic acid; HBP, heme binding protein; L-FABP, liver-fatty acid binding protein; IM, inner mitochondrial membranes; OM, outer mitochondrial membranes; Mops, 3-[*N*-morpholino]propanesulfonic acid; RT, room temperature; TLC, thin-layer chromatography.

* Corresponding author (present address): Department of Pediatrics/Hematology-Oncology, Cornell University Medical College, 525 East 68th Street, N-804 New York, NY 10021, USA. Fax: +1 (212) 7468458.

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The purpose of the present study was to inquire whether the properties of heme-binding of isolated biological membranes in general are similar or identical to those of respiring mitochondria [7] or to those of artificial membranes [1]. First, efflux studies with ^{14}C-labeled heme, preloaded into respiring mitochondria, were performed to determine how much of the heme could be transferred into the aqueous medium. Second, the efflux of ^{14}C-labeled heme, preloaded into isolated biological membranes, was determined in the presence of BSA or HBP after equilibrium had been reached. Third, the transfer of ^{14}C-labeled heme from membranes to HBP or BSA was compared with that of [^{14}C]palmitate, which freely partitions between membranes and soluble binding proteins [2,3]. Unlike palmitate, however, which transferred completely from mitochondrial membranes to BSA, heme transfer to BSA was only partially accomplished. All biological membranes examined showed a similar pattern of transfer of heme to protein in the medium: Part of the heme that partitioned into membranes was released into the medium and showed equilibrium kinetics, whereas another part of the heme was retained, in spite of repeated washings with protein containing medium, indicating that all biological membranes investigated contain heme retaining components.

2. Materials and methods

2.1. Chemicals

δ-[4-^{14}C]Amino levulinic acid (ALA, 40–60 mCi/mmol) and [1-^{14}C]palmitic acid (40–60 mCi/mmol) were purchased from Dupont - New England Nuclear, bovine serum albumin (A-6003) and human serum albumin (A-3782) from Sigma (St. Louis, MO). Chemicals for scintillation counting were from Amersham. All other chemicals were of analytical grade.

2.2. Animals

250–300 g male Sprague-Dawley rats from Hilltop (Scottdale, PA) were used. Food and drink were provided ad libitum.

2.3. Preparation of ^{14}C-labeled heme and heme binding protein

^{14}C-Labeled heme was prepared by incubation of [4-^{14}C]ALA with reticulocytes from chickens treated with phenylhydrazine; heme was crystallized by the method of Vogel [11]. The ^{14}C-labeled heme had a specific activity of $1.2 \cdot 10^7$ dpm per μmol. HBP was purified from rat liver cytosol as reported [8].

2.3. Mitochondria

Whole mitochondria were prepared as described previously [7]. Respiratory control index was measured, using succinate as substrate, and maintained a value greater than 4 throughout the 30 min of the experiments. For comparison of the heme transfer patterns from mitochondria with those of isolated membranes, we also used isolated mitochondria preloaded with labeled heme for 3 h; in those instances the respiratory control index was not measured.

2.4. Membranes

Unilamellar vesicles of phospholipids and dioleoylphosphatidylcholine were prepared as previously described [12]. Inner and outer mitochondrial membranes (IM and OM) were isolated by the swell-shrink-sonicate procedure [13]. The OM used in this study contained ≈ 10% IM based on the specific activity of succinate cytochrome-*c* reductase. The IM was contaminated with ≈ 10% OM based on the specific activity of monoamineoxidase. Since all experiments were performed in an incubation medium containing 80 mM KCl, 50 mM Mops, 5 mM potassium phosphate (pH 7.4) and 1 mM EGTA, the mitochondrial membrane fractions were washed with this medium (final wash) and centrifuged at 12000 × *g* for 60 min. The membrane pellets were resuspended in the same medium and stored at −70°C. Liver plasma membranes were isolated according to Leonard et al. [4]. Erythrocyte ghosts were prepared using a Millipore Pellicon Cassette system with an HVLP 0.45 μm filter [14]. Ghosts were washed twice with 0.15 M NaCl in 5 mM Na_2HPO_4 (pH 8.0), to remove spectrin and traces of hemoglobin, resuspended in 36 mM Na_2HPO_4 (pH 7.5), and stored at −70°C. Kidney cortex plasma membranes were isolated according to Thuneberg and Rostgaard [15].

2.5. Analyses

Concentrations of phospholipids in membranes were determined by the phosphorus content according to Dittmer and Wells [16]. Protein concentrations were determined by the Lowry method [17]. ^{14}C-Heme radioactivity was determined in a Tracor Analytic scintillation counter [18]. Quenching of the radioactivity was corrected by using the external standard ratio.

2.6. Experimental design

Studies with respiring mitochondria.

Whole mitochondria were 'pre-loaded' with ^{14}C-labeled heme by incubation of 5 mg mitochondria with 1.5 nmol ^{14}C-labeled heme in 1 ml incubation medium

at RT for 30 min, the longest incubation time for which the functional integrity (i.e., a respiratory control index greater than 4.0) of the mitochondria could be guaranteed. The incubation medium contained 80 mM KCl, 50 mM Mops, 5 mM potassium phosphate buffer (pH 7.4), 1 mM EGTA, 5 μM ADP and 50 mM succinate. Following the incubation, or 'pre-loading', the mitochondria were pelleted by centrifugation at 4°C and supernatants containing ^{14}C-labeled heme which were not associated with the mitochondria were discarded. The mitochondria were then resuspended in fresh incubation medium. Soluble binding proteins (16 μM BSA or 16 μM HBP) were added (or not added = control) and incubated for 30 min at RT to allow efflux of ^{14}C-labeled heme from the mitochondria to the proteins. Efflux was measured by separating mitochondria (pellet) from binding proteins (supernatant) by centrifugation at 4°C and counting for ^{14}C-labeled heme in both pellet and supernatant. The value obtained without protein added was subtracted from that obtained with protein added. Control or background levels ranged from 75 to 150 dpm. Observed counts after correction ranged from 150–3000 dpm in each of the fractions. Non-respiring mitochondria were also 'pre-loaded' with ^{14}C-labeled heme for 3 h. Efflux studies with these mitochondria showed the same pattern as with respiring mitochondria 'pre-loaded' for only 30 min (when equilibrium was not yet reached). All efflux studies were performed for 30 min.

Studies with isolated membranes.

These were carried out in a manner similar to experiments with whole mitochondria (see previous paragraph). Preloading with labelled ligands was achieved by incubation of 35 nmol lipid with 1.5 nmol ^{14}C-labeled heme or [^{14}C]palmitate per ml of medium for 3 h at RT under constant, gentle mixing. During the experiments, membrane fractions (OM and IM) were separated from free ligand or from binding proteins by centrifugation at 4°C at 12000 × g for 15 min, or 2 min for the other membranes (liver plasma membranes and erythrocyte membranes). These centrifugation steps sufficed to separate membranes from supernatants, since > 95% of the protein and phosphorus measured in the membrane suspension was recovered in the pellet. Since different types of membranes contain different phospholipid, cholesterol, and protein concentrations it is to be expected that different amounts of ^{14}C-labeled heme will partition into different types of membranes. The radioactivity associated with the membranes after the first incubation was considered as 100%; the dpm/35 nmol membrane lipids ranged from about 7000 dpm to 13000 dpm.

The stability of heme in supernatants and pellets was verified in several representative experiments that lasted up to 2 h after 'pre-loading'. At the end of the experiments heme was extracted into ethylacetate-acetic acid after addition of 100–200 μg of cold heme [18]. The recovery of the extracted ^{14}C-labeled heme was > 90%. More than 95% of this radioactivity migrated with heme when analyzed on TLC [19].

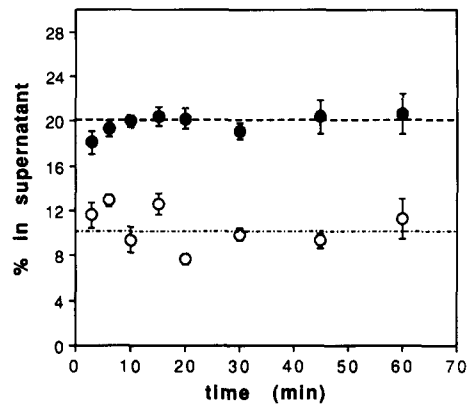

Fig. 1. Time course of heme transfer between mitochondrial outer membranes and BSA. Isolated outer mitochondrial membranes were 'pre-loaded' with ^{14}C-labeled heme for 3 h as described in Section 2. Membranes containing 35 nmol of lipids were mixed with either BSA or HBP (8 nmol) at a final volume of 1 ml. Incubations up to 60 min were stopped by centrifugation in an Eppendorf centrifuge. Supernatants and pellets containing BSA and membranes, respectively, were counted for the presence of ^{14}C-labeled heme. Data points are the mean ± S.E. of two to six experimental points as described in Section 2. Open and closed circles represent experiments with BSA and HBP, respectively.

All experiments except those reported in Table 5 were performed 2–6 times. However not all experiments were complete experiments: For instance, for Fig. 1, in one experiment all time points were determined at the same time and with the same batch of membranes 'pre-loaded' with ^{14}C-labeled heme (complete experiment); in other experiments efflux values were measured either at times 10, 30 and 60 min or at times 2, 5, 10 and 15 min (incomplete experiment) using a different batch of 'pre-loaded' membranes each time. The results of all these experiments, complete and incomplete, were combined and each time point called 'experimental point'.

3. Results

3.1. Kinetics of efflux of heme from isolated membranes to BSA and HBP

The optimal time of incubation for efflux was determined in a preliminary experiment. OM were 'pre-loaded' with ^{14}C-labeled heme (see Methods) and mixed with either BSA or HBP at RT. Fig. 1 shows the time course for the transfer of heme from the membranes to the proteins. A fraction of heme in the membranes transferred very rapidly to the added protein in the aqueous phase. All subsequent experiments could,

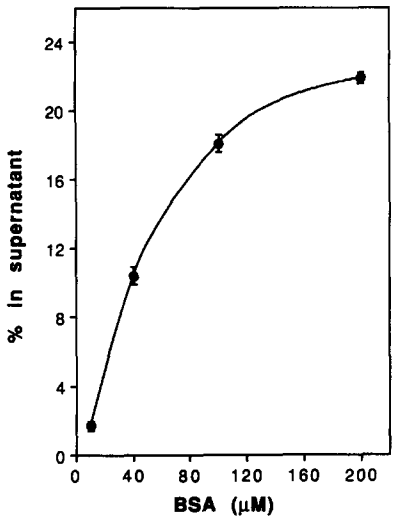

Fig. 2. Effect of BSA concentration on partitioning of heme between mitochondria and BSA. Mitochondria (5 mg/ml) were 'pre-loaded' with ^{14}C-labeled heme for 30 min and incubated with BSA for 30 min. BSA and mitochondria were separated by centrifugation and partitioning of heme between these mitochondria and BSA was determined as described in Section 2. Data points are the mean ± S.E. of two experiments in triplicate.

therefore, be conducted within 30 min (a time previously used for experiments with respiring mitochondria [7]).

3.2. Extraction of heme from respiring mitochondria to BSA

Mitochondria were 'pre-loaded' with ^{14}C-labeled heme. The percent of transfer to BSA of ^{14}C-labeled heme associated with these mitochondria is depicted in Fig. 2. The transfer was dependent on the BSA concentration and exceeded 20% at 160 μM BSA. Table 1 shows a heme distribution between mitochondria and BSA consistent with an equilibrium distribution between two phases; i.e., partitioning of heme between mitochondria and BSA was constant throughout the concentration range of BSA used. This indicates that the pool of heme observed in this experiment is free to partition between membrane and binding protein. The partitioning of heme was calculated as:

$K = ([\%\ \text{heme}]_{BSA}/\mu\text{mol BSA})/([\%\ \text{heme}]_{mito}/\text{mg mito protein})$ and had a value of 10.3 ± 1.2 ($n = 8$).

To determine the extent of heme that could be extracted from the mitochondria, mitochondria were preloaded with ^{14}C-labeled heme and incubated with 200 μM BSA for 30 min (this is considered 'pre-washing'). After removal of the supernatant the mitochondria were resuspended in different concentrations of BSA, incubated for an additional 30 min and centrifuged again. The radioactivity of heme in supernatants (containing BSA) and pellets (containing mitochondria) was measured. Only about 11% of the heme associated with these 'pre-washed' mitochondria was extracted into a solution containing 200 μM BSA (Table 2), in contrast to about 25% of the heme extracted during the initial wash (when mitochondria were not 'pre-washed') (Table 1).

Table 1
Partitioning of heme between mitochondria and BSA

BSA		(% heme)$_{BSA}$	(% heme)$_{mito}$	K
mg/ml	μM			
0.68	10	1.7±0.1	98.3	8.65
2.72	40	10.4±0.5	89.6	14.51
6.80	100	18.1±0.5	81.9	11.05
13.60	200	21.9±0.3	78.1	7.01

Mitochondria (5 mg/ml) were pre-loaded with ^{14}C-labeled heme and incubated with BSA for 30 min. BSA and mitochondria were separated by centrifugation and the concentration of heme in pellets and supernatants was determined as described in Section 2. K, the partitioning coefficient for heme between mitochondria and BSA is expressed as [(% heme)$_{BSA}$ /μmol BSA]/[(% heme)$_{mito}$ /mg mitochondrial protein]. Efflux values are the means ± S.E. of two experiments.

Table 2
Partitioning of heme from mitochondria pre-washed with BSA

BSA		(% heme)$_{BSA}$	(% heme)$_{mito}$	K
mg/ml	μM			
0.68	10	1.7± <0.1	98.3	8.84
2.72	40	4.7± 0.1	95.3	6.12
6.80	100	8.1± 0.8	91.9	4.08
13.60	200	11.3± 0.5	88.7	3.20

Mitochondria (5 mg/ml) were pre-loaded with ^{14}C-labeled heme and incubated with 200 μM BSA for 30 min. BSA and mitochondria were separated by centrifugation, and mitochondria were re-suspended in a solution containing fresh BSA. Following a 30 min incubation, the mixtures were centrifuged and the concentration of heme in pellets and supernatants was determined as described in Section 2. K, the partitioning coefficient for heme between mitochondria and BSA, is expressed as [(% heme)$_{BSA}$ /μmol BSA]/[(% heme)$_{mito}$ /mg mito protein]. Efflux values are the means ± S.E. of three experimental points.

3.3. Extraction of heme from mitochondria to HBP

The distribution of ^{14}C-labeled heme between HBP and mitochondria was measured before and after 'pre-wash' with BSA. The results in Table 3 show that extraction of heme from mitochondria was 10.4% with 10 μM HBP and 3.8% in mitochondria 'pre-washed' with 200 μM BSA. HBP, like BSA, did not totally extract ^{14}C-labeled heme from preloaded mitochondria. HBP, however, removed more heme in the first of a series of extractions than did BSA (10.4% removal with 10 μM HBP as compared to 1.7% removal with 10 μM BSA, Tables 3 and 1).

3.4. Extraction of heme from isolated mitochondrial membranes

The data in Tables 1–3 suggested that there is more than one mitochondrial heme compartment and that large differences exist between the availability of heme from these sites for efflux. Apparently, a fraction of heme rapidly partitions among all phases. Heme in another sizable compartment appears to be tightly associated with the membranes. Counts remaining in the membranes were shown to be still heme (see Section 2).

Since partitioning of heme between different compartments could be a specific property of respiring mitochondria we also investigated heme transfer to BSA from the two isolated mitochondrial membrane subfractions, (IM and OM). Isolated IM or OM preloaded with ^{14}C-labeled heme was subjected to sucessive incubations with 16uM BSA. The partitioning of heme between IM and OM and BSA during three consecutive extractions is shown in Table 4 (A and B). Partitioning of heme between mitochondrial membranes and BSA (% heme-membranes/%heme-BSA) was not constant; the affinity for heme of both membranes increased about 4-fold during the third *vs* the first extraction (Table 4). A large fraction of heme, (approximately 60%), was retained in both types of membranes in a compartment that did not freely partition into other phases. We did not continue to wash or incubate the 'pre-loaded' membranes in medium devoid of heme binding proteins because 'free' heme degrades and evaluation is only possible after equilibrium is reached, i.e., after 2–3 h.

Table 3
Partitioning of heme between mitochondria and HBP

HBP		$(\%\ \text{heme})_{HBP}$	$(\%\ \text{heme})_{mito}$	K
mg/ml	μM			
A Mitochondria to HBP				
0.14	10	10.4± 0.3	89.6	58.0
0.28	20	14.2± 0.2	85.8	41.4
B Mitochondria pre-washed with BSA to HBP				
0.14	10	3.8± < 0.1	96.2	19.8
0.28	20	5.8± 0.1	94.2	15.4

A Mitochondria (5 mg/ml) were pre-loaded with ^{14}C-labeled heme and incubated with HBP at the specified concentration. Efflux values are the means ± S.E. of two experiments in duplicate.
B Mitochondria (5 mg/ml) were pre-loaded with ^{14}C-labeled heme and incubated with 200 μM BSA for 30 min. BSA was removed by centrifugation, and mitochondria were re-suspended in a medium containing HBP (10 μM and 20 μM). Following a 30 min incubation, the mixtures were centrifuged and the concentration of ^{14}C-labeled heme in pellets and supernatants was determined. Efflux values are the means ± S.E. of three experimental points. K, the partitioning coefficient for heme between mitochondria and HBP is expressed as [(% heme)$_{HBP}$/μmol HBP]/[(% heme)$_{mito}$/mg mitochondrial protein].

Table 4
Extraction of heme from liver mitochondrial membrane fractions by BSA

Extraction	$(\%\ \text{heme})_{BSA}$	$(\%\ \text{heme})_{membrane}$
A Mitochondrial outer membranes		
1	34.5± 0.1	65.5
2	12.7± 0.1	87.3
3	8.0± 0.1	92.0
Heme retained in outer membranes following 3 extractions ≈ 53%		
B Mitochondrial inner membranes		
1	22.3± < 0.1	77.7
2	11.0± 0.1	89.0
3	6.5± < 0.1	93.5
Heme retained in inner membranes following three extractions ≈ 65%		

Membrane fractions (35 μM membrane lipids) were pre-loaded with ^{14}C-labeled heme and incubated with BSA (16 μM each step) for 30 min. Membranes were separated from the protein by centrifugation and the concentration of heme in the supernatants and the pellets was determined. Heme was extracted from the membranes by successive incubations with fresh medium containing BSA. Efflux values are means ± S.E. of three experimental points.

3.5. Extraction of heme from non-mitochondrial membranes

To investigate whether heme retention is a property of only mitochondrial membranes, heme was partitioned between BSA and plasma membranes of liver and kidney cortex cells or erythrocyte membranes. As shown in Table 5, partitioning of heme associated with all of these membranes into BSA decreased upon further extraction. This partitioning pattern indicates the existence of more than one pool of heme in each of the membranes studied. After three extractions with BSA, erythrocyte membranes retained the least amount of heme (≈ 30%).

3.6. Extraction of palmitate from mitochondria

To ascertain that the interaction of heme with the mitochondrial membranes was peculiar to heme, we also examined the efflux pattern of another hydrophobic substance, palmitate (1.5 μM). When membranes (35 μM lipids), pre-loaded with [^{14}C]palmitate, were incubated with BSA (16 μM), 94% of the palmitate partitioned from both mitochondrial membranes onto the protein. Two further incubations with BSA resulted in 97.4% ± 0.3 and 97.8% ± 0.2 extraction of palmitate for outer and inner mitochondrial membranes, respectively. These results confirm our previous observations [1] that palmitate freely partitions from membranes into other phases. Thus, a definite difference exists between the ability of the soluble

Table 5
Extraction of heme from non-mitochondrial membranes by BSA

Extraction	(% heme)$_{BSA}$	(% heme)$_{membrane}$	K
A Rat liver plasma membranes			
1	35.3	64.7	1.19
2	13.9	86.1	0.35
3	10.2	89.8	0.25
Heme retained in plasma membranes following three extractions ≈ 50%			
B Erythrocyte membranes			
1	58.3	41.7	3.06
2	18.7	81.3	0.5
3	14.4	85.6	0.37
Heme retained in erythrocyte membranes following three extractions ≈ 30%			
C Rat kidney cortex plasma membranes			
1	44.7	55.3	1.77
2	16.9	83.1	0.44
3	11.6	88.4	0.29
Heme retained in plasma membranes following three extractions ≈ 40%			

Membrane fractions (35 μM membrane lipids) were pre-loaded with ^{14}C-labeled heme and incubated with BSA (16 μM each step) for 30 min. Membranes were separated from the protein by centrifugation and the concentration of heme in supernatants and pellets was determined. Heme was extracted from the membranes by three successive incubations with fresh medium containing BSA. K, the partitioning coefficient of heme between membranes and BSA is expressed as: [(%heme)$_{BSA}$ / μM BSA]/[(%heme)$_{membrane}$ / μM membrane lipids]. Efflux values are the mean of single experiments performed in duplicate.

binding proteins to extract heme and palmitate from membranes.

4. Discussion

In a previous study it was found that ^{14}C-labeled heme from synthetic lipid bilayers transfers into an aqueous phase dependent on the differential efflux rate from two heme compartments [1, 21]. One compartment, associated with the external leaflet of the bilayer, transfers heme rapidly; a second compartment, associated with the internal leaflet, transfers it at a slower rate. Efflux from both compartments proceeds freely and is complete within about 30 min. The data presented here with membranes of different organs (Tables 4 and 5) indicates that, like the efflux from artificial membranes, the initial rate of efflux of heme from biological membranes is fast and already nearly complete within about 5 min (Fig. 1), and the amount of effluxed heme is dependent on the protein concentration in the medium, reaching a plateau at about 200 μM for BSA (Fig. 2). However, unlike the situation in synthetic bilayers [1], heme in biological membranes is retained to a significant extent (about 30–50%) in a compartment which is not easily accessible for transfer to the aqueous phase. This is shown by the high affinity of biological membranes for residual heme, i.e., that fraction of heme not extractable by either BSA or HBP (Tables 2–4). The initial kinetics of heme transfer from the isolated membranes, whether mitochondrial or of other origin, showed a rapid equilibration of heme between the membranes and both HBP or BSA. This observation indicates that all biological membranes (Table 5) contain different compartments of heme with varying degrees of binding affinities in equilibrium with proteins of the surrounding medium. It is unclear in which compartment endogenously labeled ^{14}C-labeled heme partitioned; this is under investigation. What can be stated at present is that the trends of efflux of total heme from isolated membranes to BSA or to HBP are the same, i.e., neither protein could extract all of the heme from the membranes. The observation that HBP causes greater extraction of heme from the membranes after only one incubation than does BSA can most simply be explained by the different affinities of these two proteins for this ligand (K_d 0.2 μM of HBP [9] as compared to $K_d > 2$ μM of BSA [22]) (Tables 1 and 3). The suggestion that HBP and BSA remove heme from different compartments would imply that heme, in membranes, is distributed in three pools: one from which heme moves to BSA, a second one that is somehow accessible to HBP, and a third pool which can not be exchanged under the experimental conditions. This scenario seems to us unlikely and we did, therefore, not speculate on a third heme pool.

There are two possible mechanisms by which heme could be partially retained in biological membranes. Membranes could contain lipids or proteins with such a high affinity for heme that it is only very slowly released. It has been reported that the rate of release of heme from artificial membranes strongly depends on the specific lipid composition [1,21]. That the lipid composition is also the major determinant of heme retention in biological membranes is, however, unlikely. The data on heme efflux (Tables 1–5) may be explained by a composite of several (or many) equilibrium affinities between phospholipids, proteins and lipoproteins and heme. Our data indicate that there is no significant difference between membranes, each showing a large fraction of heme tightly associated with them. Erythrocyte membranes have been shown to contain structural proteins (spectrin, actin and protein 4.1) which bind heme with high affinity [23]. A heme-binding protein has been suggested to occur in murine erythroleukemia cells [24] and in rat liver cells [25,26]. Different membranes may contain proteins with varying degrees of affinities for heme which would explain, for example, that membranes from mitochondria retained about 60% while those from erythrocytes (from which spectrin, one of the heme binding proteins, had

been removed) retained about 30% of the ^{14}C-labeled heme.

In summary, the release of heme from biological membranes is incomplete and thus differs from that observed for the efflux of heme from unilamellar membranes which completely release heme.

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