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STUDY OF HUMAN RED BLOOD CELL MEMBRANE USING SODIUM DEOXYCHOLATE

I. MECHANISM OF THE SOLUBILIZATION

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SUMMARY

1. If the deoxycholate concentration is raised, the ratio of solubilized proteins to phospholipids does not remain constant. The proteins are solubilized about two times faster than the phospholipids, but this solubilization is never complete. On the other hand, the solubilization of the phospholipids is proportional to the quantity of deoxycholate (1 μmole of solubilized phospholipid per 13 μmole of bile salt) and may be complete.

2. Sepharose-6B gel chromatography of solubilized membranes shows solubilization to correspond at the same time to a dissociation of the membrane lipoprotein complex into its two components and an association between the phospholipids and the deoxycholate, in the ratio of 1 lipid to 13 detergent. Molecular weight determination of these mixed complexes by chromatography on Sepharose-6B accounts for aggregate formation corresponding to the association of 10 phospholipid molecules per 130 molecules of deoxycholate (*i.e.* equivalent to about ten mixed complexes).

3. Sepharose chromatography finally shows the phospholipid–sodium deoxycholate complexes to be the same size, whatever the deoxycholate concentration. This finding confirms moreover the results of paragraph 1.

INTRODUCTION

It has become customary during the last few years to use detergent (sodium dodecyl sulfate) or bile salt (sodium deoxycholate) in order to solubilize various preparations of biological membranes. A solution containing the two main components of membranes, lipids and proteins is thus obtained in an aqueous medium. Many different authors have used this technique in order to study the plasma membrane structure.

Although one may first have thought (especially after ultracentrifugation experiments[1,2]) that this solubilization was accompanied by a division into identical lipoprotein subunits, it is now accepted for at least two types of plasma membranes (rat liver cells[3] and mycoplasma[4,5]) that a dissociation of the membrane into proteins and lipids occurs. There is, however, a difference of opinion when it comes to analyzing the structure of the formed precipitate after the detergent or bile salt has been removed by dialysis. Certain authors (*viz.* BONT *et al.*[3], and EMMELOT AND VAZ DIAS[6]) assert that dissociation persists after dialysis in an important portion of the solubilized

material (approx. 34–48% of the phospholipids according to the solubilizing agent used), whilst TERRY *et al.*[7], ENGELMAN AND MOROWITZ[8,9], RAZIN *et al.*[5] and ROTTEM *et al.*[10] show the formation of aggregates to have the same structural characteristics as the initial membranes (before solubilization) when dialysis is carried out in the presence of cations, such as Mg^{2+} (refs. 5 and 7–10), Ca^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+} or Na^{+} (ref. 10).

The main problem of membrane reaggregation is, however, to verify whether the structure obtained after removing the detergent is, in fact, that of the original membrane, *i.e.* whether one finds the same principal properties of the biological membranes, in particular, their enzymatic activities. One of these is extremely interesting in that its function is linked to membrane dissymmetry[11] and to phospholipid requirement[12] (Na^{+}-K^{+})-ATPase (EC 3.6.1.3), and thus necessitates a close resemblance between the reconstituted membrane structure and the original one. RAZIN *et al.*[5] showed that solubilization with dodecyl sulfate inactivates this enzyme in an irreversible manner. EMMELOT and co-workers[3] are unable to prove any kind of activity after solubilization (sodium deoxycholate, 1 %) and dialysis; however, some 10–15 % of the (Na^{+}-K^{+})-ATPase activity is retained after solubilization (sodium deoxycholate, 1 %) and centrifugation (105000 × *g* for 15 min)[13]. These results suggest that the use of dodecyl sulfate and deoxycholate in the study of plasma membranes is somewhat limited and should be subject to caution, as it does not allow reconstitution of the original membrane configuration.

However, we have shown that (Na^{+}-K^{+})-dependent ATPase activity[14,15] can be found in the precipitate obtained from solubilized human red blood cell membranes after removal of sodium deoxycholate by dialysis. Two factors are essential for this: firstly, the concentration in bile salt, and secondly, the presence of Na^{+} and K^{+} in the dialysis medium. These results show that the deoxycholate could be, nevertheless, a favoured agent for studying the structure and function of biological membranes. To settle this statement, we analyse the mechanism of action of sodium deoxycholate.

This work deals with the solubilization mechanism of erythrocyte membranes using deoxycholate, by specifying the nature of the solubilized particles. In a forthcoming paper, the analysis of the reaggregation of the solubilized particles after removal of deoxycholate will be presented.

MATERIALS AND METHODS

Preparation of red blood cell membranes

In order to obtain human blood red cell membrane preparations free of haemoglobin (which could prevent the continuation of the experiments), the technique of POST *et al.*[16] was used. A double hemolysis gave a sizable quantity of colour-free membranes: 48 ml of washed blood cells gave an average of 10–15 ml of membrane suspension with a content of about 10 mg/ml of protein. The phospholipid content of these preparations (μmoles per mg of protein) was 0.69 ± 0.01, calculated from the 28 samples used. The preparations were stored at 4°, pH 8.0, before using them; the storage time never exceeded one week.

Solubilization by sodium deoxycholate

A 241 mM sodium deoxycholate solution (Fluka, Buchs, Switzerland) was generally used, kept at room temperature, pH 8.1–8.3.

Different quantities of membrane suspensions and deoxycholate solutions were mixed in order to obtain the desired ratio of deoxycholate/phospholipid expressed in μmoles of deoxycholate per μmole of phospholipid*. The preparation was left to stand for 30 min at 0° and then centrifuged at 4° in a Spinco 40 rotor, 60000–80000 $\times$ g, for 30 min. The supernatant fluid was supposed to contain only the solubilized membrane material.

Chemical determinations

The proteins were measured according to the method of LOWRY *et al.*[17] (with beef serum-crystallized albumin as standard). It has been verified that the deoxycholate used during the experiments, even at higher concentrations (32 mM), did not interfere with the protein determination.

Measurement of lipid phosphorus was carried out according to the technique of BRADFORD *et al.*[18] for extraction and acid digestion; the inorganic phosphate formed was then measured using BARTLETT's method[29]. The deoxycholate present did not modify the results. Sodium deoxycholate was determined by spectrophotometry (technique derived from SINGER AND FITSCHEN[20]). Measurements were made at 310 mμ. Neither proteins nor phospholipids interfered.

^{32}P-Labelled phospholipids and radioactivity measurements

The labelling technique used was comparable to that described by PAYSANT *et al.*[21]. 50 ml of washed red cells were suspended in an equal volume of solution containing 0.5 mM histidine–imidazole (pH 7.2), 120 mM NaCl, 10 mM KCl and 4 mg glucose/ml. $H_3{}^{32}PO_4$ (in hydrochloric solution at 10 mC/ml), obtained from the Commissariat à l'Energie Atomique, Gif sur Yvette (France), was added to the solution before mixing (25 to 50 μC/ml incubation mixture). Incubation was carried out in a glass flask, gently shaken in a water bath at 37° for 6 or 14 h. At the end of this labelling phase, the suspension was centrifuged, and the cells were washed three times with about 50 ml of a radioisotope-free solution of the above composition in order to remove the excess $^{32}PO_4{}^{3-}$.

After the action of deoxycholate and chromatography of the solubilized membrane material, 0.2–0.5 ml of each fractions was dried on planchets under an ordinary incandescent lamp, and the radioactivity was measured (counts/min per ml). Counting was carried out in a conventional G.M. tube (Le Materiel Téléphonique, type 13 AP 7).

Chromatography on Sepharose-6B

The solubilized material was immediately chromatographed on a Sepharose-6B column (Pharmacia, Uppsala, Sweden). The gel was contained in a 45 cm $\times$ 2.5 cm column (Pharmacia), sometimes connected with a second identical column. The chromatography was carried out at 4°, after equilibration of the gel, with the same solution which was used for elution: 15 mM Tris–HCl (pH 8.0), 100 mM NaCl, 8–10 mM sodium deoxycholate according to the experiment. Protein, phospholipid and deoxycholate amounts were determined from the recovered fractions (about 5 ml, flow rate between 10 and 15 ml/h).

* The reason why this ratio was chosen in preference to deoxycholate/protein (μmole/mg) will be explained in the discussion.

RESULTS

Variations of solubilized protein and phospholipid quantities as a function of deoxycholate concentration

If the quantity of proteins and phospholipids in solution after bile salt action is measured, the following phenomena for 6 different preparations of red cell membranes are noticed. They are presented separately for proteins (Fig. 1) and phospholipids (Fig. 2).

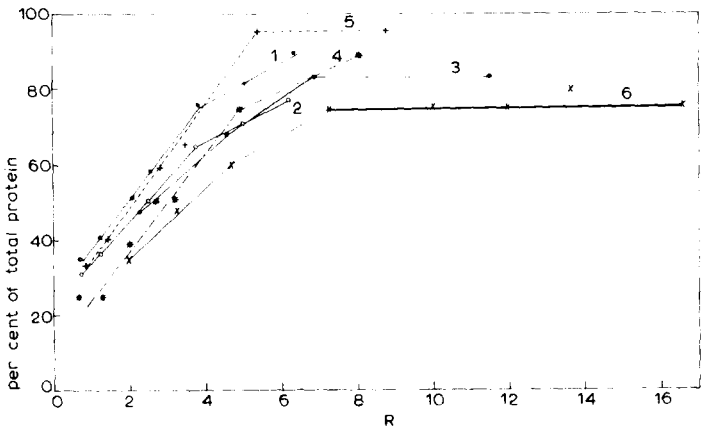

Fig. 1. Percentage solubilization of membrane proteins as a function of the ratio deoxycholate/phospholipid. This ratio R was calculated from deoxycholate and phospholipid concentration measured in the medium at the time of solubilization. The solubilized proteins were determined in the supernatant after 30 min of centrifugation at 80000 × *g*. Initial protein concentration in the different samples was as follows: Curve 1, 6.2 mg/ml; Curve 2, 5.9 mg/ml; Curve 3, 4.7 mg/ml; Curve 4, 4.3 mg/ml; Curve 5, 4.2 mg/ml; Curve 6, 3.7 mg/ml. These samples correspond to six different membrane preparations. (Curves drawn by eye).

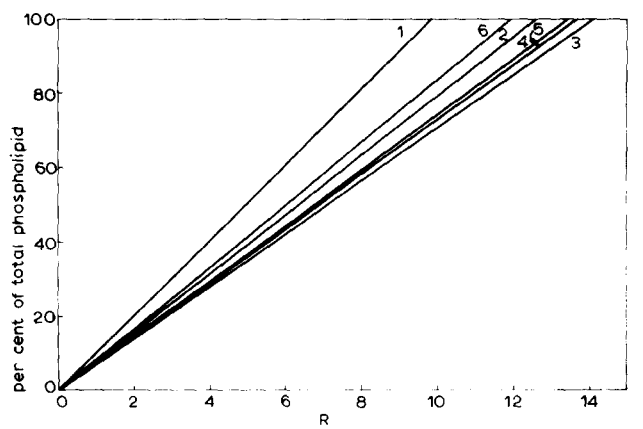

Fig. 2. Percentage solubilization of membrane phospholipids as a function of the deoxycholate/phospholipid ratio. These variations are represented by straight lines obtained by application of the least-squares method. The solubilized phospholipids were measured on the supernatant after 30 min centrifugation at 80000 × *g*. Initial phospholipids composition of the different samples (samples identical to those in Fig. 1) was as follows: Straight lines (1), 3.8 μmole/ml; (2), 3.9 μmole/ml; (3), 3 μmole/ml; (4), 3 μmole/ml; (5), 2.75 μmole/ml; (6), 2.7 μmole/ml.

TABLE I

SOLUBILIZATION OF MEMBRANE PROTEINS AND PHOSPHOLIPIDS AS A FUNCTION OF INITIAL DEOXYCHOLATE/PHOSPHOLIPID RATIO (R)

The left-hand columns give (*cf.* Figs. 1 and 2) the protein and phospholipid composition of the different membrane preparations before solubilization. The values of R corresponding to 50% of protein and phospholipid solubilization and maximum solubilization were obtained from the results of Figs. 1 and 2. Numbers given in parentheses correspond to the percentage of maximum solubilization.

Expt. No.	*Initial composition of the membrane preparation*			*Value of R corresponding to 50% of protein and phospholipid solubilization*		*Value of R corresponding to maximum solubilization*		*μmoles of deoxycholate necessary in order to solubilize 1 μmole of phospholipid*
	Protein (mg/ml)	*Phospholipid (μmoles/ml)*	*μmoles phospholipid/ mg protein*	*Protein*	*Phospholipid*	*Protein*	*Phospholipid*	
1	6.2	3.8	0.61	1.9	4.9	—	9.8 (100)	10
2	5.9	3.9	0.66	2.4	6.3	—	12.6	12.7
3	4.7	3.0	0.64	2.6	7.1	6.9(83)	14.1	14.3
4	4.3	3.0	0.70	2.8	6.7	—	13.4	13.7
5	4.2	2.75	0.66	2.1	6.8	5.3(95)	13.6	12.8
6	3.7	2.7	0.72	3.4	6.0	7.3(74)	11.9	12.5
Mean ± S.E.			0.67 ± 0.02					12.7 ± 0.05
P*				$P < 0.001$				

* Probability = comparison between the values of R corresponding to 50% protein and phospholipid solubilization.

1. It appears impossible to get by solubilization the same ratio between proteins and phospholipids as that found in the initial membranes. In order to dissolve 50 % of the initial amount of proteins, a value around 2.7 is needed for the ratio μmoles of deoxycholate per μmole of phospholipids, whereas a value of about 6.5 is necessary to dissolve the same percentage of phospholipids (Table I).

2. On the other hand, whatever the concentration of deoxycholate used may be (expressed by the preceding ratio value), protein solubilization is never complete. It lies between 75 and 95 %, whereas phospholipid solubilization can be complete if the mole to mole ratio is high enough (>10). When, however, the ratio corresponding to maximum protein solubilization is reached (approx. 85 %), 50 % of phospholipids are solubilized. It is to be noted that the curves in Fig. 1 never go through the origin, because the isolation of membranes leads to the presence of a certain amount of proteins in solution (0.6–1.5 mg/ml). In contrast, very few phospholipids exist in solution under the same conditions (0.1 to 0.3 μmole/ml), and therefore, the straight lines in Fig. 2 all go through the origin.

3. It is possible from the results of Fig. 2 to calculate the number of μmoles of deoxycholate necessary to solubilize one μmole of phospholipid. This ratio (μmoles deoxycholate per μmole phospholipid) can be obtained directly as the intersection of the straight lines with the ordinate at 100 % solubilized phospholipid. This number varies very little from one experiment to another, and its average value is around 12.7 ± 0.5 μmoles of deoxycholate per μmole of phospholipid. This number is very near to the number of moles of deoxycholate which are associated to form a micelle[22,23] beyond the critical micelle concentration (4–6 mM in absence of Na^+, 2–4 mM in the presence of 100 mM Na^+ (refs. 22, 23)). Deoxycholate concentrations used in these experiments are higher than the critical micelle concentration. Therefore, bile salt is in the micellar form, and one can thus assume that 1 micelle of deoxycholate is needed to solubilize 1 mole of phospholipid. These findings agree with the results of BENZONANA[23] who found 1 micelle of deoxycholate for 1 mole of oleic acid.

Nature of the particles in solution after solubilization

Chromatography in presence of deoxycholate. Fig. 3 presents the results obtained by chromatography on Sepharose-6B for a sample of detergent-solubilized red cell membrane preparation (3.3 mg protein/ml and 0.9 μmole phospholipid/ml) after removal of nonsoluble material by centrifugation. The deoxycholate concentration used for solubilization was 10.9 μmoles/ml, corresponding to a deoxycholate to phospholipid ratio of 25. The chromatography took place only after the gel had been equilibrated with the same solution of bile salt which was to be used afterwards for elution (8 to 10 μmoles deoxycholate/ml).

In five experiments carried out under the same conditions (mole to mole ratio = 2.5, 1.4, 2.6, 2.7, and 2.8) the proteins seemed to be clearly dissociated from the phospholipids. The first left-hand peak (Fig. 3) corresponds to the proteins associated with a small quantity of phospholipids (0.08–0.2 μmole of phospholipids per mg of protein) which were excluded from the gel and appeared in the void volume of the column. The second peak would mainly contain proteins as well as the following plateau. The final shoulder in Fig. 3 corresponds to traces of haemoglobin still fixed to the membranes and released by deoxycholate action. (The dotted curve corresponds to the absorbance at 540 mμ.)

Phospholipids, apart from the weak quantity excluded from the gel or that which remained as traces on proteins, were eluted as a single peak distinct from proteins. (The peak on the extreme right seems not to correspond to phospholipids by chemical determination (*cf.* MATERIALS AND METHODS) and will not be taken into consideration.)

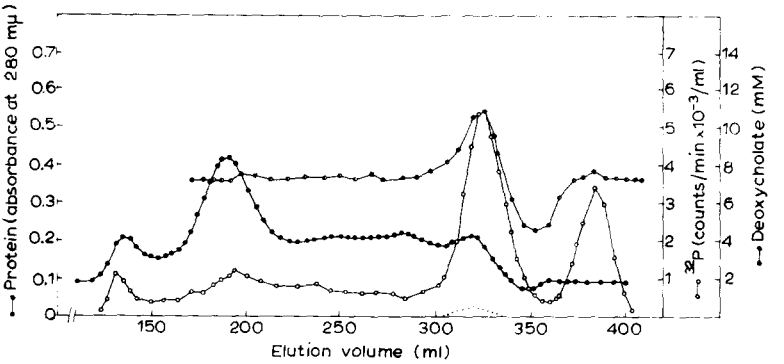

Fig. 3. Chromatography on Sepharose-6B of a sample of globular stromas solubilized by sodium deoxycholate (R = 2.5). The solubilized sample (9.4 ml) containing 3.3 mg/ml of protein, 0.9 μmole per ml of phospholipids, 10.9 μmole/ml of deoxycholate, was applied on a Sepharose gel contained in two identical columns (45 cm × 2.5 cm) connected together. The gel was equilibrated and then eluted with a solution of 15 mM Tris–HCl (pH 8.05), 100 mM Na^+, 10 mM deoxycholate. The elution volume was near to 5.5 ml/h. The experiment was carried out at 4°. Fractions were analysed for proteins, radioactivity and deoxycholate. The dotted curve corresponds to the absorbance at 540 mμ (haemoglobin).

The deoxycholate curve shows several peculiarities which permit an understanding of the action of the bile salt upon membranes. From the results of the first section, we would expect the phospholipid to complex deoxycholate in a stoichiometry of 12.7 (sodium deoxycholate/phospholipid). The superimposed peaks of phospholipid and deoxycholate appear in agreement with the existence of a complex between these two compounds. The trough which appears in the curve of sodium deoxycholate (Fig. 3) could be interpreted differently, according to the experimental conditions: (i) The chromatographed sample did not contain enough sodium deoxycholate for complexing the whole phospholipid, and a certain amount of sodium deoxycholate was sequestered from the medium. This explanation cannot be maintained, as the sample contains 10.9 μmoles sodium deoxycholate per 0.9 μmole phospholipid (12.1/1). This ratio (sodium deoxycholate/phospholipid) is almost the same as that obtained during four identical and independent experiments (right hand column of Table II, upper part). (ii) The sodium deoxycholate concentration in the sample was high enough for complex formation with the phospholipids but was less than the eluent concentration; this case has been excluded in our experiments. (iii) If the sodium deoxycholate concentration is almost the same for the eluent (10 μmoles/ml) and the sample (free sodium deoxycholate + bound sodium deoxycholate = 10.9 μmoles/ml), the trough can be explained as representing the amount of sodium deoxycholate from the eluent necessary to reestablish the equilibrium inside the sample because of the sodium deoxycholate–phospholipid complex formation. Thus it is possible to compare the areas of the peak and the trough for each experiment and measure the bound deoxycholate (Table II, upper part). In spite of the approximation of the method used, it can be ascertained

that there exists an association between the bile salt and phospholipids during solubilization. The number of μmoles of deoxycholate linked to 1 μmole of phospholipids (chemically determined) for each of the peaks obtained during the four identical experiments are in good agreement with those obtained previously (12.7 μmoles of deoxycholate per μmole of phospholipids), as shown in Table II.

Chromatography of membranes solubilized by increasing quantities of deoxycholate. Three identical samples of red cell membranes (4.5 ml containing 6.33 mg protein/ml) were treated with a concentrated solution of bile salt (241 mM) in order to furnish a

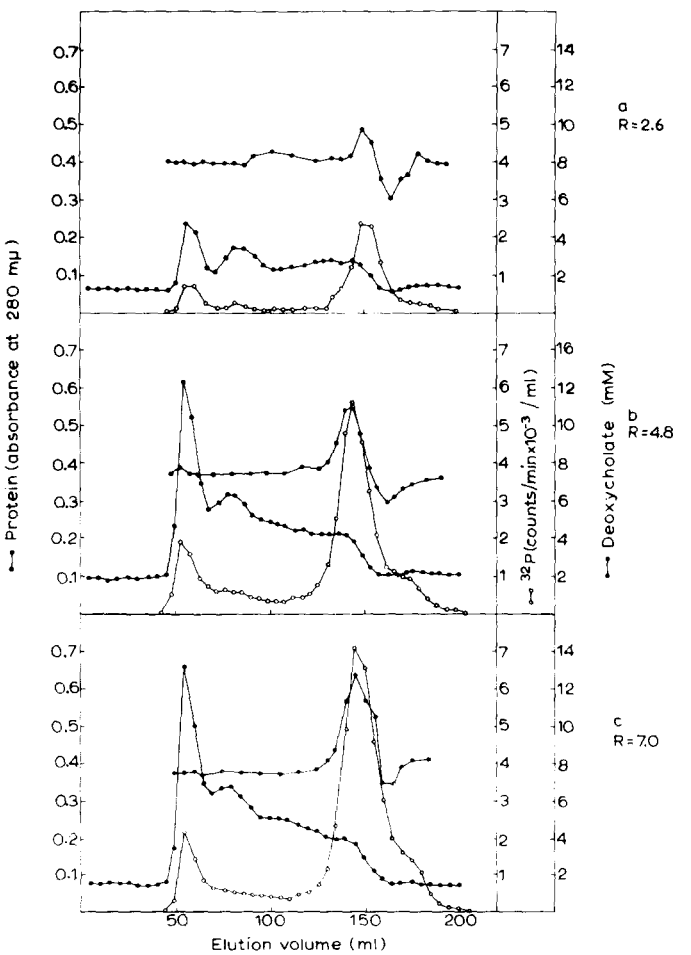

Fig. 4. Chromatography on Sepharose-6B of three samples of the same membrane preparation solubilized by different concentrations of deoxycholate: (a) R = 2.6 μmole of deoxycholate per μmole of membrane phospholipids; (b) R = 4.8; (c) R = 7.0. Protein (mg/ml), phospholipid (μmole/ml) and deoxycholate (μmole/ml) composition of the chromatographed samples was, respectively: 1.8, 0.7, 9.6 for sample (a); 3.8, 1.63, 16.9 for (b); and 3.92, 2.45, 24.1 for (c). 4.4 ml of each of the samples were applied on a column of gel (45 cm × 2.5 cm) equilibrated and eluted with a solution of 15 mM Tris–HCl (pH 8.0), 100 mM Na^+, 8 mM deoxycholate. The volume of elution varied between 8.4 and 10.4 ml/h according to the experiment. These were carried out at 4°. Measurements were performed as in Fig. 3.

TABLE II

ASSOCIATION BETWEEN PHOSPHOLIPIDS AND DEOXYCHOLATE AFTER MEMBRANE SOLUBILIZATION OF RED CELLS BY BILE SALT

Expts. 1–4 were carried out with four different membrane preparations, and experiments a–c, with the same preparation. After chromatography on a single Sepharose-6B column (45 cm × 2.5 cm) (Expts. 3 and 4) or two identical columns (Expts. 1, 2 and a–c), deoxycholate was measured (chemical determination) simultaneously with phospholipids (radioactivity measurement). The areas of peaks and troughs were evaluated by planimetry in arbitrary units. Phospholipids of the fractions corresponding to the main elution peaks were determined chemically at the same time. Numbers given in parentheses correspond to the trough area expressed in % of the peak area.

Expt. No.	*μmoles deoxycholate/ μmole of phospholipid used during solubilization*	*Peak area (arbitrary units)*	*Trough area (arbitrary units)*	*Concentration at the elution peak (μmoles/ml)*		*Ratio R after solubilization (measured at the elution peak)*
				Deoxycholate	*Phospholipid*	
1	2.5	154	141 (92)	3.6	0.32	11.3
2	1.4	305	281 (92)	4.6	0.25	18.5
3	2.6	40	45 (110)	2.0	0.16	12.5
4	2.6	99	72 (72)	3.4	0.29	11.7
Mean ± S.E.						13.0 ± 1.3
a	2.6	40	45	2.0	0.16	12.5
b	4.8	148	55	4.0	0.37	10.8
c	7.0	162	32	4.8	0.47	10.3
Mean ± S.E.						11.2 ± 0.7

mole to mole ratio (deoxycholate/phospholipid) successively equal to 2.6, 4.8 and 7.0. The final volume was adjusted to 8 ml with distilled water. The solubilized part was recovered after ultracentrifugation, and 5 ml of each were put into a column (45 cm × 2.5 cm) of Sepharose-6B. Proteins, phospholipids and deoxycholate were determined on the recovered fractions after elution with a solution containing 15 mM Tris–HCl (pH 8.0), 100 mM NaCl and 8 mM deoxycholate, which was also used to equilibrate the column. The results are shown in Fig. 4.

(a) The curves of the proteins and phospholipids confirm the results given in the first section. The quantity of solubilized protein, represented by the height of the two peaks and the plateau, increased from a deoxycholate/phospholipid (mole/mole) ratio of 2.6 to 4.8, but remained practically unchanged between ratios 4.8 and 7.0, as shown in Fig. 1. A constant increase in the quantity of solubilized phospholipids is noticed when the mole to mole ratio increased from 2.6 to 7.0, except perhaps for the fraction linked to the proteins excluded from the gel. This thus confirms the results given in Fig. 2.

(b) By increasing the quantity of deoxycholate used at the time of solubilization, the same number of peaks were found in the same places. Only the height and the area of the peaks were affected, and the second radioactivity peak (Fig. 3) appears here as a shoulder because the chromatography was run in a single column (45 cm × 2.5 cm). This could mean that an increase of the sodium deoxycholate concentration increased the amount of proteins and phospholipids in the solution without changing the size of the solubilized particles.

(c) The peak corresponding to the deoxycholate bound to phospholipids increased with the deoxycholate to phospholipid ratio. The ratios for each of these peaks (Table II, Expt. a, 12.5; b, 10.8; c, 10.3) are similar to those of the samples before chromatography (a, 13; b, 10; c, 10). Therefore, it is possible to assume that 1 micelle of deoxycholate is necessary to solubilize 1 mole of phospholipid and form a complex.

On the other hand, the trough area remained almost constant whatever the ratio. As the deoxycholate concentration of eluent (8 μmoles/ml) was less than that of the samples, the constant area of the trough depended on the sample volume, identical in the three experiments. If all deoxycholate of the sample was to be bound, the same quantity of bile salt from the medium was necessary for sample equilibrium.

Molecular weight determinations of the mixed complexes. We have to clarify one point concerning the deoxycholate– phospholipid association. As can be seen from Fig. 3, the mixed micelles emerged from the column at approximately the same time as the residual haemoglobin. If the molecular weight of haemoglobin is equal to 68000, that of a mixed complex (13 deoxycholate + 1 phospholipid) only equals $(414 \cdot 13) + 750 = 6182$. This noticeable difference would imply that mixed micelles are associated to form aggregates, or at least there are mixed particles where deoxycholate and phospholipids are in a ratio equal to 13.

The molecular weight of these aggregates was measured by chromatography on Sepharose-6B, which allows the determination of molecular weights between $1 \cdot 10^4$ and $1 \cdot 10^6$ (ref. 24). The substances necessary for the calibration of the Sepharose column (45 cm × 2.5 cm) were chosen so that the elution zone for haemoglobin, phospholipid and deoxycholate could be calibrated as precisely as possible (Dextran Blue 2000 of Pharmacia, mol. wt. = 2000000, excluded from the gel; lyophilised human haemoglobin, mol. wt. = 68000; trypsin of Boehringer TR 15330, mol. wt. = 28000; and a

polypeptidic pancreatic inhibitor Iniprol O of Lab. Choay, Paris, mol. wt. = 6500 ± 200). These substances were chromatographed in two runs. Iniprol was detected by the method of ERLANGER *et al.*[25].

Using the same column equilibrated with 15 mM Tris–HCl (pH 8.04), 100 mM Na^+, 8 mM deoxycholate, three solubilized samples (deoxycholate/phospholipid (mole/mole) = 3.06, 4.0 and 4.5) were separately eluted. Measurements of the volumes of elution for haemoglobin and the 'phospholipid–deoxycholate' complex allowed verification that haemoglobin did migrate as in the calibration experiment, and gave a molecular weight of around 62000 to the 'lipid–deoxycholate' complex, assuming that the behaviour of the particles was determined by their volume (or their molecular weight).

One can thus conclude that phospholipids and bile salt are associated into water-soluble aggregates composed of approximately 10 moles of phospholipids and 130 moles of bile salt; the exact arrangement of these molecules inside the complex cannot be given at this stage.

DISCUSSION

The present results permit the discussion of a possible mechanism for the solubilization of red cell membranes with sodium deoxycholate. Firstly, Figs. 3 and 4 safely allow a distinction to be made between the two hypotheses previously put forward[14,15]. Chromatography on Sepharose-6B clearly shows a dissociation of the membrane into its components due to the action of deoxycholate and not a dissociation into identical lipoprotein subunits. It is certain that a common peak for proteins and phospholipids does exist, but apart from its situation in the volume of exclusion where everything unfiltered by the gel is found (it could be therefore protein and lipid juxtaposed aggregates instead of lipoproteins); we notice that it corresponds to a phospholipid/protein ratio distinctly lower than that of the membrane suspension before solubilization (0.1–0.2, instead of 0.69). Therefore, a large quantity of the proteins, and mainly the phospholipids are separated under distinct peaks, which is in agreement with the results of ENGELMAN *et al.*[4] and BONT *et al.*[3] on other plasmatic membranes.

Secondly, the results of Tables I and II show that a close association exists between phospholipids and bile salt in a constant ratio of 13 moles of deoxycholate to 1 mole of phospholipid. HOFMANN AND SMALL[22] and BENZONANA[23] showed that these 13 moles are associated to form a micelle under the present conditions (0°–20°, pH 8.0–9.0, 100–150 mM Na^+). It could therefore be assumed that there exists an association of 1 micelle of deoxycholate for 1 mole of phospholipid; in fact, as is shown by the measurement of the molecular weight of the mixed micelles, phospholipid and detergent molecules are associated to form higher particles containing not less than 10 moles of phospholipid for 130 of deoxycholate. SMALL *et al.*[26] could specify the kind of binding between these two components by NMR. Therefore, if deoxycholate is mixed with a lecithin suspension the signal associated with the hydrophobic part of the deoxycholate molecule (methyl groups) becomes broadened, as well as the one associated with the alkyl chain of lecithin ($(CH_2)_n$). These results can be interpreted as a loss of mobility in that part of the deoxycholate molecule and in the aliphatic chains of lecithin by a common hydrophobic interaction.

On the other hand, the concentrations used here have not shown an association between the detergent and the proteins, and even if it does exist, it cannot be compared

to the association between deoxycholate and the phospholipids. This explains why the deoxycholate/phospholipid (mole/mole) ratio has been used to characterize a solubilized membrane suspension in preference to the deoxycholate/protein (mole/mg) ratio.

In these conditions, it is possible to suppose that the solubilization of the red cell membranes into a form which is nonsedimentable at 80000 $\times$ *g* for 30 min may be explained by the association between phospholipid and deoxycholate which gives a water-soluble complex[22].

It is necessary to point out, however, that the solubilization does not occur in the same way when the deoxycholate concentration is increased. As shown in Figs. 1 and 2, it was noticed that proteins solubilize faster than phospholipids, independently of the contact time (30 min giving the same results as 12 h). Thus the maximum protein solubilization (about 85 %) is reached for a deoxycholate/phospholipid mole to mole ratio corresponding to 50 % of solubilized phospholipids. It appears, as suggested by ENGELMAN *et al.*[4] that solubilization proceeds by a continual series of intermediary steps that can be shown by slowly increasing the quantity of deoxycholate inside the medium.

In spite of the different solubilization of proteins and phospholipids, the Sepharose chromatography showed no size changes of particles in the solution, as no significant difference of the protein and lipid peaks is noticed (Fig. 4).

The fact that proteins are solubilized faster when the quantity of deoxycholate present in the membrane preparation increases, should be added to what is presently known about the membrane structure of the red blood cell[31]. This can be schematically pictured as a mosaic of lipropotein subunits, in contact by their lateral sides, containing, firstly, two hydrophilic regions made up of protein on the opposite extremes on the outside of the membrane, and secondly, by a core made up of phospholipids and proteins.

As deoxycholate does not seem to form a complex with the proteins, or at least at the concentrations used during this work, it can be imagined that a micelle of deoxycholate is able to free several protein particles before binding with a phospholipid molecule. This also verifies the fact that solubilization by dissociation of the membrane into lipoprotein subunits does not occur. What takes place, in fact, is a deep transformation of the membrane structure by the detergent.

This solubilization, which leads to the $(Na^+\text{-}K^+)$-ATPase inhibition[28,29], is not an irreversible transformation of the membrane structure because it is possible under certain conditions to reconstitute a complex function such as that of the transport ATPase system by removal of the bile salt[14].

The study of the mechanism of the membrane reaggregation is under investigation. In connection with the study of the mechanism of inhibition and reactivation of the transport ATPase it could be shown that sodium deoxycholate plays an important role for the elucidation of the function of plasma membrane.

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