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DIFFERENTIAL SOLUBILIZATION OF PROTEINS, PHOSPHOLIPIDS, FREE AND ESTERIFIED CHOLESTEROL OF RAT LIVER CELLULAR MEMBRANES BY SODIUM DEOXYCHOLATE

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SUMMARY

1. Smooth microsomes, Golgi-rich fractions, and light and heavy plasmalemmal subfractions from rat liver were isolated and their purity assessed using enzymic, chemical and morphological criteria.

2. Membranes were prepared by Tris–EDTA washing combined with sonication treatment of the different subcellular fractions.

3. Washed membranes were submitted to differential solubilization with 0.26 % sodium deoxycholate. When the deoxycholate/phospholipid molar ratio (R) is raised, all the membranes showed a maximum protein solubilization occurring at $R \cong 2$. The higher the membrane neutral lipid to phospholipid molar ratio is, the lower the solubilized protein plateau lies.

4. Phospholipids are solubilized in slightly greater amounts than proteins and their solubilization is complete at $R = 14$–16.

5. For $R < 2$, sterols are solubilized in slightly greater amounts than phospholipids. At maximum protein solubilization, cholesterol and cholesterol esters completely differ in their behaviour. The whole membrane cholesterol goes into solution for $R = 14$–16 while the solubilization of esterified cholesterol is never complete. The higher the protein plateau is, the lower the cholesterol esters solubilization curve asymptote lies.

INTRODUCTION

Disruption of lipid–protein interactions requires substances that can compete for lipid binding sites on the proteins. Amphiphiles like sodium deoxycholate are suitable reagents [1–4].

High concentrations of deoxycholate remove all major lipids from proteins in membranes [5–9] and plasma lipoproteins [10], the bulk of lipids being incorporated into water-soluble mixed lipid–detergent micelles [11, 12, 7]. Using red blood cell membrane, Philippot [7] showed that phospholipid solubilization is proportional to the quantity of deoxycholate, in the ratio of 1 lipid to 13 detergent molecules. Lipophilic proteins may also form with deoxycholate water-soluble complexes but structural data on them are still lacking [1–4].

Attention has begun to focus on neutral lipids. Tanford [3] pointed out that difficulties may be encountered with amphiphiles during the separation of proteins from lipid-containing mixtures with a high cholesterol content. In addition, nexus-rich fractions have a very low phospholipid and glycolipid content compared with neutral lipids, including free and esterified cholesterol, and triglycerides [13–15], they were shown to be insoluble in deoxycholate solutions [16, 17].

Membranes are characterized by specific cholesterol/phospholipid and cholesterol ester/phospholipid ratios. The molar ratios vary considerably with higher values found in isolated plasma membranes compared to intracellular membranes [18, 19]. In order to examine the role of neutral lipids, especially of esterified cholesterol, in membrane structure and in the solubilization of membrane proteins by deoxycholate we studied the solubilizing effect of 0.26 % deoxycholate on four compositionally distinct washed rat liver membranes: smooth microsomal, Golgi, and light and heavy plasma membranes. This paper deals with a comparison of this solubilizing effect on proteins, phospholipids, cholesterol and cholesterol esters.

METHODS AND MATERIALS

Animals

Male Wistar rats (160 ± 10 g) were fed with standard diet and water ad libitum. They were not starved before sacrifice. The rats were decapitated and drained of blood, the livers were immediately perfused in situ with 0.9 % NaCl at 10 °C via the portal vein under a 1 m hydrostatic pressure. The wet weight of perfused liver was not significantly different from that of the unperfused tissue: 7.64 ± 0.34 g ($n = 30$) and 7.22 ± 0.19 g ($n = 16$), respectively. Therefore, no correction of yield was applied.

Preparation of subcellular fractions

Microsomes were prepared by the method of Moulé et al. [20] and the smooth microsomal fraction isolated essentially as described by Leskes et al. [21].

Golgi-rich fractions were prepared by a slight modification of the method of Leelavathi et al. [22]. Fifteen g of minced tissue were homogenized into 40 ml of the medium A (0.5 M sucrose, 5 mM $MgCl_2$, 0.1 M potassium phosphate buffer (pH 6.65) at 700 rev./min and 5 full strokes using a 100 ml methacrylate Potter homogenizer [23]. The pestle had a radial clearance of 0.5 mm [24]. The post-nuclear supernatant was processed as described by Leelavathi except that centrifugations were performed in a Spinco SW 25.2 rotor. Golgi-rich fractions were once washed with water [25]. Smooth microsomes also formed a band at the 1.1 M/1.25 M sucrose interface but, owing to very low yield and contamination by free ribosomes, they were discarded.

The plasma membrane fraction was prepared according to Evans [26, 27]. The rate-zonal centrifugation was performed in a Beckman 14 Ti rotor at 4000 rev./min for 50 min. Plasma membranes were collected between 35 and 39 % (w/w) sucrose and were then subfractionated as described by Evans [27].

Preparation of membranes by washing of subcellular fractions

Each crude membrane pellet (2.5–3 mg protein) was suspended by homogenization in 5 ml of 0.7 mM EDTA, 1 mM Tris–HCl solution (pH 7.5), diluted to

11 ml with the same medium, and centrifuged at 165 000 $\times g_{av}$ for 30 min in a Spinco 50 Ti rotor. This operation was twice repeated. The resulting pellet was then suspended in 3 ml of distilled water and sonicated five times at a setting of 1.5 (50 W) for 1 min under cooling with 1-min waiting cooling periods (Branson B-12 sonifier, fine probe tip, geometrical conditions according to Swensson et al. [28]). $MgCl_2$ was added to obtain a molarity of 10 mM [29, 30]. After centrifugation at 165 000 $\times g_{av}$ for 60 min, the pellet was washed twice more by suspension in 11 ml of the initial medium.

Solubilization of membranes by sodium deoxycholate

Sodium deoxycholate (Sigma) was pure on thin-layer chromatography [31] without prior purification. A 0.26 % deoxycholate, 0.25 M sucrose, 0.14 mM EDTA, 1 mM Tris–HCl solution (pH 7.8) [32, 33] was used in all studies. Different volumes of membrane suspensions in 0.25 M sucrose were pelleted and mixed by homogenization with different volumes of the deoxycholate solution in order to obtain the desired molar ratio R of deoxycholate/phospholipid. The preparation was left to stand for 60 min at 0 °C and then centrifuged in a Spinco 50 Ti rotor at 165 000 $\times g_{av}$ for 90 min [32]. The results were independent of the time of contact (60 min or 6 h).

Chemical analyses

Total lipids were extracted and purified as previously described [32]. Organic phosphorus values were obtained by the method of Macheboeuf et al. [34] or Bartlett [35]. Phospholipid was calculated by assuming 25 μg of phospholipid/μg of lipid phosphorus.

Fractionation into phospholipid and neutral lipid classes was carried out according to the technique of Borgström [36]. Neutral lipids were resolved by thin-layer chromatography on 500-μm layers of Silica Gel HR in a solvent system composed of pentane–diethyl ether–formic acid (80 : 20 : 1, v/v) [37] in order to achieve the separation of free and esterified cholesterol and of deoxycholate. Cholesterol and cholesterol esters were eluted with chloroform, and deoxycholate with methanol–chloroform (6 : 1, v/v). Free and esterified cholesterol were determined as described by Webster [38]. Deoxycholate was spectrophotometrically measured at 310 nm according to Singer et al. [39].

Protein was estimated by the method of Lowry et al. [40] with crystallized bovine serum albumin as a standard.

The RNA content of the subcellular fractions was determined according to Tsanev et al. [41] after extraction by the procedure of Schneider [42].

Enzyme assays

The crude membrane fractions were washed in the buffer required for the specific assay. The following activities were measured: glucose-6-phosphatase (D-glucose, 6-phosphate phosphohydrolase (EC 3.1.3.9)) [43], galactosyl transferase (UDPgalactose: *N*-acetylglucosamine galactosyl transferase (EC 2.4.1.-)) [44] and 5′-nucleotidase (AMP phosphohydrolase (EC 3.1.3.5.)) [45].

TABLE Ia AND Ib

CHEMICAL COMPOSITION OF RAT LIVER SUBCELLULAR FRACTIONS (a) AND MEMBRANES (b)

The values given are the averages ±S.E. The number of experiments is shown in parentheses. Protein and phospholipid are expressed in mg/g perfused liver, RNA in μg/mg protein. Cholesterol/phospholipid and cholesterol esters/phospholipid are molar ratios, assuming molecular weights of 386, 647 and 775 for cholesterol, esterified cholesterol and phospholipid, respectively.

	Protein	Phospholipid	$\frac{\text{Phospholipid}}{\text{Protein}}$	RNA
(a) Subcellular fractions				
Smooth microsomes	3.54±0.26 (4)	1.38±0.09 (3)	0.39±0.03	47±5 (3)
Golgi-rich fractions	0.360±0.015 (5)	0.150±0.010 (6)	0.42±0.03	13±3 (3)
Light plasma membrane subfraction	0.088±0.005 (2)	0.074±0.003 (2)	0.84±0.06	8±1 (2)
Heavy plasma membrane subfraction	0.024±0.002 (5)	0.016±0.003 (5)	0.67±0.08	38±7 (2)
	$\frac{\text{Phospholipid}}{\text{Protein}}$	$\frac{\text{Cholesterol}}{\text{Phospholipid}}$	$\frac{\text{Cholesterol esters}}{\text{Phospholipid}}$	
(b) Membranes*				
Smooth microsomal membrane	0.41±0.05 (3)	0.074±0.009 (2)	0.039±0.011 (2)	
Golgi membrane	0.66±0.08 (3)	0.143±0.013 (2)	0.062±0.009 (2)	
Light plasma membrane	0.82±0.09 (2)	0.460±0.027 (2)	0.095±0.012 (2)	
Heavy plasma membrane	0.67±0.08 (2)	0.590±0.031 (2)	0.114±0.014 (2)	

* Protein and phospholipid values (mg/g perfused liver) are calculated from washings as the difference between the subcellular fraction and the washing compositions.

TABLE II

MARKER ENZYME ACTIVITIES IN RAT LIVER SUBCELLULAR FRACTIONS

Values given are the means of three experiments ±S.E.

Assay	Smooth microsomes	Golgi-rich fractions	Unsubfractionated plasma membranes
Glucose-6-phosphatase*	5.00±0.24	1.00±0.14	0.42±0.08
5'-Nucleotidase*	1.30±0.12	1.98±0.22	12.86±0.94
Galactosyl transferase**	1.83±0.39	34.2±5.7	0.0

* Specific activity in μmoles P_i/20 min per mg protein at 37 °C.

** Specific activity expressed in nmoles of galactose transferred/h per mg protein at 37 °C.

RESULTS

Purity of the subcellular fractions

The chemical composition and marker enzyme activities of the smooth microsomal, Golgi-rich and plasmalemmal membrane fractions are summarized in Tables Ia and II.

Preparation of membranes by washing of subcellular fractions

The washing procedure described under Materials and Methods was designed for the Golgi-rich fraction, using precursors of plasma very-low-density lipoproteins as a morphological marker [46]. Slightly alkaline hypotonic shock was examined in combination with EDTA and sonication.

As observed by electron microscopy, treatment in 100 mM, 30 mM, 3 mM or 1 mM Tris–HCl (pH 7.5) appeared relatively more efficient as the molarity decreased.

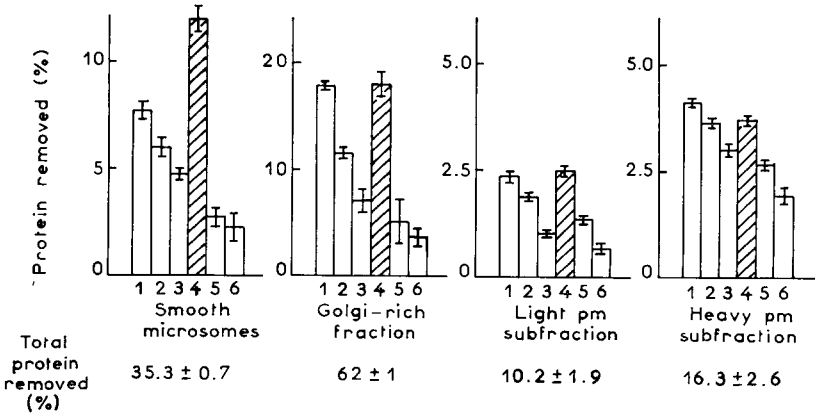

Fig. 1. Washing of subcellular fractions. Release of proteins. Percentages given are the means of three experiments ±S.E. The subcellular fractions were resuspended three times in 0.7 mM EDTA, 1 mM Tris–HCl, pH 7.5 (□ 1, 2, 3) and centrifuged at 165 000 × g_{av} for 30 min. The final pellets were dispersed in water, sonicated (■ 4) at 50 W for 5 × 1 min (Branson B-12 sonifier), added with 10 mM $MgCl_2$ and centrifuged at 165 000 × g_{av} for 60 min. The pellets were washed twice more (□ 5, 6) in the initial medium. pm is plasma membrane.

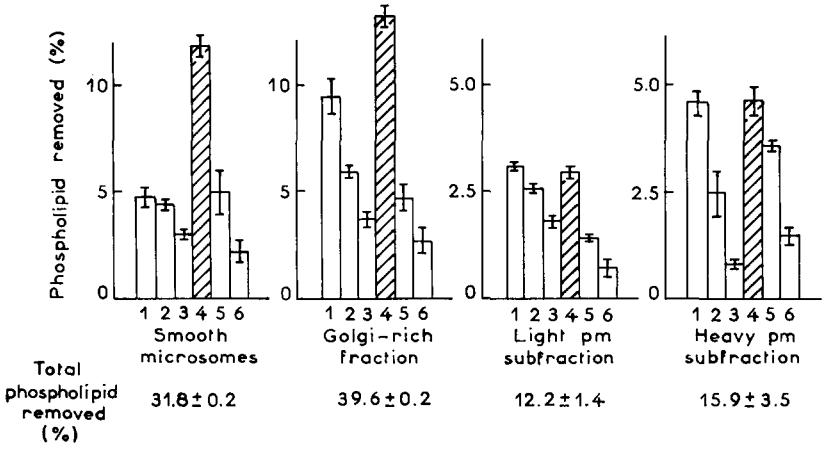

Fig. 2. Washing of subcellular fractions. Release of phospholipids. The percentages are the means of two experiments ±S.E. The experimental conditions are identical to those described in Fig. 1.

but there was still an abundance of very low density lipoproteins. Addition of 0.7 mM EDTA to 1 mM Tris–HCl (pH 7.5) and sonication for 5×1 min were followed by extensive fragmentation and releasing of Golgi elements. The same procedure was applied to smooth microsomes and plasmalemmal fractions. The relative and total amounts of proteins and phospholipids removed from the different subcellular fractions are shown in Figs 1 and 2. The gross chemical composition of washed membranes is presented in Table Ib.

Since low concentrations of deoxycholate (0.026 %) disrupted Golgi membranes as seen by the formation of a large lipoprotein layer after flotation, the treatment of Golgi-rich fraction proteins by 0.26 % deoxycholate was examined as a function of the deoxycholate/phospholipid molar ratio R (Fig. 3). Very low values of R could not be retained because they required too much material in our conditions. By extrapolation of the curve from Fig. 3 to $R = 0$ (see Discussion), one may get an order of

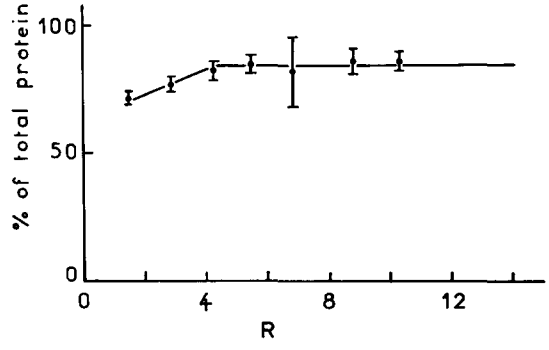

Fig. 3. Percentage solubilization of Golgi-rich fraction protein as a function of the molar ratio (R) deoxycholate/phospholipid. R was calculated from deoxycholate and the phospholipid concentrations measured in the suspension at the time of solubilization. The supernatant was obtained after deoxycholate action for 60 min and centrifugation at 165 000 × g_{av} for 90 min. Values given are the means of two experiments ±S.E.

magnitude of the quantity of Golgi extramembranous proteins of about 60 %. This result is in good agreement with the Tris–EDTA method. In addition, the washing of Golgi-rich fractions by a slight modification of Glaumann's technique [29] (resuspension in 0.15 M KCl, 10 mM EDTA following sonication was omitted and replaced by a new wash in 0.15 M Tris buffer (pH 8.0) since it gave us better morphological preservation of Golgi membranes) gave similar results on the basis of protein and phospholipid determinations (unpublished data).

Solubilization of washed membrane components by 0.26 % deoxycholate

The amounts of washed membrane proteins, phospholipids, cholesterol and cholesterol esters solubilized by 0.26 % deoxycholate treatment were measured as a function of the detergent/phospholipid molar ratio *R*. The percentage of deoxycholate in the supernatant was calculated from measurements of the detergent concentrations in homogenates, supernatants and pellets (twice washed in 0.25 M sucrose). Data are presented separately for phospholipids, cholesterol and deoxycholate (Fig. 4), and for proteins and cholesterol esters (Fig. 5). The following phenomena were observed:

(1) Whatever the molar ratio *R* value used may be, protein solubilization is never complete. In all membranes, the maximum protein solubilization occurs at $R \simeq 2$, the plateau lying around 61–63 % for smooth microsomal membranes, 53–55 % for Golgi membranes, 21–23 % for light plasma membranes and 15–17 % for heavy plasma membranes. One observes that the higher the native membrane free and esterified cholesterol/phospholipid molar ratio is, the lower the protein plateau lies.

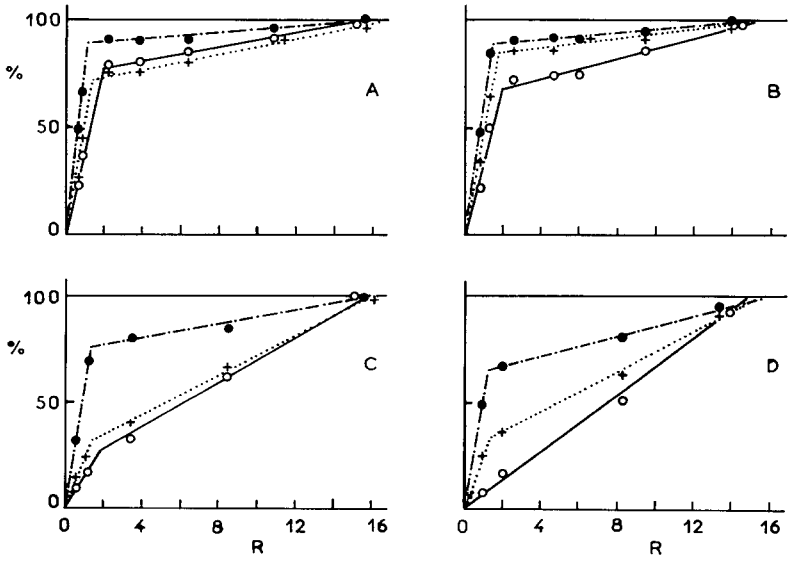

Fig. 4. Percentage solubilization of membrane phospholipids (○—○) and cholesterol (+ · · · +) as a function of the deoxycholate/phospholipid molar ratio (*R*). A, Smooth microsomal membranes; B, Golgi membranes; C, Light plasma membranes; D, Heavy plasma membranes. The percentage partition of deoxycholate, expressed as the weight ratio of deoxycholate in the supernatant to total deoxycholate added to the membrane, is also presented as a function of *R* (●- · · · -●). The experimental conditions are identical to those described in Fig. 3

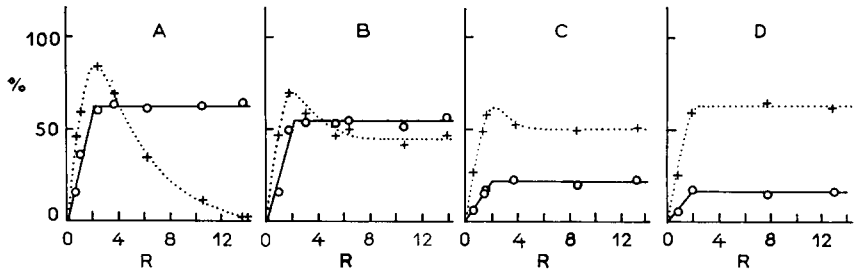

Fig. 5. Percentage solubilization of membrane proteins (○—○) and cholesterol esters (+ · · · +) as a function of the deoxycholate/phospholipid molar ratio (*R*). A, Smooth microsomal membranes; B, Golgi membranes; C, Light plasma membranes; D, Heavy plasma membranes. Experimental conditions are identical to those described in Fig. 3.

(2) A biphasic increase in the amount of solubilized phospholipids is observed when R varies from 0 to 14–16. The change of slope occurs at $R \cong 2$. Phospholipid solubilization is complete at $14 < R < 16$. Whatever the R value, phospholipids are solubilized in slightly greater amounts than proteins. With the exception of the heavy plasma membrane subfraction, it appears that the solubilization of membrane phospholipids is not proportional to the concentration of deoxycholate.

(3) When R is increased from 0 to 2, it appears that free and esterified cholesterol are solubilized in slightly greater amounts than phospholipids and proteins. With ratio values higher than 2, cholesterol and cholesterol esters differ markedly in their behaviour. Whatever the R value used may be, cholesterol esters solubilization is never complete, whereas cholesterol solubilization is complete at $14 < R < 16$. The higher the native membrane neutral lipids/phospholipid molar ratio is, the higher the cholesterol esters solubilization curve asymptote lies.

DISCUSSION

Purity of subcellular fractions

It is apparent from Table II that a 4–8 % contamination by Golgi elements and a 8–12 % contamination by plasma membranes occurred in the smooth microsomal preparations. Although the presence of 5'-nucleotidase in fragments of the endoplasmic reticulum from rat liver has been reported [47], the possibility of a contamination by plasma membranes cannot be excluded. The range of variation of total smooth microsomal cholesterol/phospholipid molar ratio values is considerable: our value (0.074) is low compared to 0.120 [48], 0.140 for smooth I microsomes (Fraction S_I) and 0.200 for smooth II microsomes (Fraction S_{II}) [48], 0.260 [49], and 0.271–0.287 [50]. Cholesterol esters/phospholipid molar ratios are much lower and our value (0.039) is in the range of data reported: 0.041 [49], 0.036 for Fraction S_I and 0.083 for Fraction S_{II} [51].

The Golgi-rich fraction seems to be contaminated with plasma membranes to an extent of 13–18 %. However, cytochemical tests on isolated Golgi fractions show that there is 5'-nucleotidase activity in morphologically identifiable Golgi elements [52, 53]. As observed by Leelavathi et al. [22], contamination with smooth endoplasmic reticulum is high, about 16–24 %. No cytochemical glucose-6-phosphatase

activity has been detected in the Golgi apparatus of hepatocytes in situ. The phospholipid/protein weight ratio (0.42 ± 0.03) is lower than the value reported by Bergeron et al. [53]: 0.5. This difference may be explained by the overloading of Golgi elements with very low density lipoproteins after alcohol administration and by fractionation of the Golgi complex into three subfractions, GF_1, GF_2 and GF_3 [46], 0.5 being relevant to the lighter subfraction GF_1.

The densities of the plasmalemmal subfractions are slightly different from Evans's values [27] (1.14 against 1.12 for light subfraction, 1.19 instead of 1.18 for heavy subfraction). Density may be influenced by the technique of homogenization as well as by dietary and other factors. The cholesterol/phospholipid molar ratio for total plasma membranes varies from 0.43 to 0.76 [18, 50]. As for the cholesterol esters/phospholipid molar ratio, 0.059 can be calculated from the data of Keenan et al. [18]. Our ratios (Table Ib) are in agreement with these values.

Preparation of membranes by washing of subcellular fractions

In our study, membranes were considered to be purified after application of the washing procedure designed for the Golgi-rich fraction.

The Golgi phospholipid/protein weight ratio increased from 0.42 to 0.66 (Table I). The biochemical characterization of the Golgi subfraction GF_1 isolated by Ehrenreich et al. [46] revealed that upon removal of the lipoprotein content by alkaline hypotonic shock followed by two passages through the French press, the ratio in Fraction GF_1 membranes became 0.36 [53]. Franke et al. [54] obtained a value of at least 0.5. Sodium deoxycholate at low molar ratios of detergent to phospholipid has been widely adopted as a method for purifying microsomal membranes [8, 55–57], and also for separating and identifying the content of vesicles [8, 58, 59]. One may thus justify the extrapolation to $R = 0$ of the curve of protein solubilization for the Golgi complex (Fig. 3). In addition, washing of the Golgi fractions by applying a modification of Glaumann's procedure [29] gave very similar results to those obtained by Tris–EDTA treatment. Thus, if one assumes that about 60–65 % of Golgi proteins are extramembranous and if one accepts the proposition of Ehrenreich et al. [46] that the weight ratio of phospholipid to protein in Golgi membranes is similar to that of microsomes (about 0.35), then 65 % of phospholipids should be extramembranous. This value is much higher than the one we determined (about 40 %) and would make questionable the concept that preferential extraction of non-membranous proteins is correlated with low phospholipid/protein weight ratios in washings.

As regards washed microsomal and plasma membranes, phospholipid/protein weight ratio values are not significantly different from those obtained before washing (Tables Ia and Ib). Whether the Tris–EDTA procedure may be applied to these membranes remains to be elucidated.

Solubilization of washed membrane components

Each detergent-treated membrane suspension was characterized [7] by the deoxycholate/phospholipid molar ratio R. Lipids removed from membranes are readily incorporated into micelles of deoxycholate. The deoxycholate molarity used in this study (6.3 mM) is higher than the critical micelle concentration (CMC) [7, 12]. The CMC is comparable to the threshold for protein solubilization and confor-

mational changes in spin-labelled human red cell membranes [60]. The choice of R does not exclude the binding of deoxycholate to membrane proteins.

By contrast with the erythrocyte membrane [7, 9], and with the exception of the heavy plasmalemmal subfraction, the solubilization of rat liver membrane phospholipids was not proportional to R. We found that this solubilization was complete at $14 < R < 16$, in accordance with Philippot's [7] value: $R = 13$. The differences in solubilization curves could be attributed to varying neutral lipid contents. It is observed that phospholipid solubilization is proportional to R only with neutral lipid-enriched membranes. The erythrocyte ghost cholesterol/polar lipid molar ratio is high (0.9–1.0) [61]. Heavy plasma membrane is also characterized by a high ratio value (Table Ib).

The characteristics of the solubility of all membrane components in 0.26 % deoxycholate appear to be relevant to the physico-chemical properties of the mixed lipid–deoxycholate micelles. Cholesterol solubility in model bile systems has been the subject of many investigations [62]. The degree of saturation of lecithin fatty acid chains is solely effective [63, 64]. Modifications of the cholesterol molecule largely affect its solubilization: cholesterol esters are much less soluble in the models than is cholesterol [63]. In addition, Helenius et al. [10], using plasma low-density lipoproteins and moderate concentrations of deoxycholate, observed that the bulk of cholesterol esters, and also of triglycerides, eluted with proteins in the void volume of a Sephadex G-200 column while phospholipids, cholesterol and some cholesterol esters eluted later in a separate peak. One may thus expect that, for $R < 2$, lipid solubilization would correspond to the optimal solubility of the different membrane lipid components in mixed micelles. For $R > 2$, the large affinity of deoxycholate micelles for phospholipid and cholesterol would favour the solubilization of phospholipid and cholesterol, while cholesterol esters, much less soluble in mixed micelles and in bile salt itself [63], would become partially insoluble.

A general explanation of the action of deoxycholate (and Triton X-100) is that deoxycholate monomers bind to hydrophobic areas in the regions occupied by lipids in the native membrane or plasma lipoprotein and that lipid removal would thus be an exchange of bound lipid for bound detergent on protein [1, 2]. In our experimental conditions, the presence of deoxycholate monomers cannot be excluded since the transition from the monomeric to the micellar state is less sharp with deoxycholate than usual [2]. One may hypothesize that esterified cholesterol could remain bound to proteins and thus limit their solubilization. It can be calculated from Table Ib and Fig. 5 that, at $R = 2$, about 7 μg of esterified cholesterol might be bound per mg of smooth microsomal membrane deoxycholate-insolubilized protein, and about 18, 32, and 31 μg/mg for Golgi, light plasma and heavy plasma membranes, respectively. Then the higher the amount of esterified cholesterol in the deoxycholate-insoluble membrane fractions, the lower the protein plateau lies. At $R = 14$–16, the whole membrane phospholipid and cholesterol content is in solution (Fig. 4). Values calculated from Table Ib and Fig. 5 become about 35, 42, 41 and 31 μg of esterified cholesterol which might remain bound per mg of smooth microsomal, Golgi, light and heavy plasma membrane-insolubilized protein. Differences observed at $R = 2$ and $R = 14$–16 might indicate that the level of the protein plateau is governed by several parameters, including protein–protein, protein–deoxycholate, protein–esterified cholesterol and probably protein–triglycerides hydrophobic interactions.

The precedent values calculated at $R = 14$–16 are not significantly different. This observation may suggest that 0.26 % deoxycholate-insolubilized proteins from the different cellular membranes under study could present some analogies.

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