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MECHANISM OF RELEASE OF INTEGRAL PROTEINS FROM RAT LIVER MICROSOMAL MEMBRANES

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The release of three integral enzymatic activities (NADH- and NADPH-cytochrome *c* reductase and 5′-nucleotidase) and total protein from washed rat liver microsomal membranes, upon simple incubation at 37°C in aqueous media, was investigated. Release does not depend on contaminating proteases and is enhanced by alkaline pH. Total protein and enzyme release is consistent with a loss of phospholipids which are not recovered in the soluble phase. Following incubation at pH 9.0 large amounts of free fatty acids were recovered in the soluble phase, accounting for a ratio of 1/1 (w/w) with released protein. This evidence, together with the data available about densities (1.07–1.08 g/ml) and molecular weights (1 700 000–700 000) of the released enzymes, suggests that they are solubilized from microsomal membranes in the form of mixed micelles mostly formed by free fatty acids and integral proteins, probably owing to the activity of endogenous phospholipases on membrane lipids. Release of total protein and enzymatic activities is decreased by Ca^{2+}, whose possible role in the phenomenon is discussed.

Introduction

Integral membrane proteins may generally be solubilized only by means which disrupt the structural integrity of the lipid bilayer in which they are embedded.

Agents used for solubilization include mainly detergents, namely amphipatic molecules which, when dispersed in aqueous solution, form micellar supramolecular aggregates, able, upon insertion into a membrane, to disaggregate the bilayer core and to organize mixed micelles in which integral proteins are contained [1–3]. The size of micelles and the number of proteins which they can accomodate depend on many factors (type of detergent, pH, ionic strength, etc.) [1–3] ; as a final result, however, integral membrane proteins exist in the soluble phase in a structure which simulates the native membrane environment, with a molecular weight small enough to allow the use of the most common analytical and preparative biochemical methods [2]. In the light of these considerations it is rather interesting to observe that small but significant amounts of integral enzymatic activities (NADH- and NADPH-cytochrome *c* reductase, EC 1.6.99.3 and EC 1.6.99.1, respectively, and 5′-nucleotidase, EC 3.1.3.5) may be recovered in the supernatant phase after centrifugation of membrane suspensions previously incubated in simple aqueous buffered media [4–6].

It must be remarked that, among the variety of substances having detergent properties, a number of compounds must be taken into account which are produced in biological membranes upon enzymatic degradation of membrane lipids by endogenous phospholipases [5,7–9]. As far as microsomal membranes are concerned, an enzymatic system exists, able, optimally upon Ca^{2+} activation, to catalyze extensive hydrolysis of fatty acid

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ester linkages in phosphatidylethanolamine and to a much lesser extent in phosphatidylcholine, main giving glyceryl-phosphoryl derivatives and free fatty acids as terminal products and lysoderivatives as intermediates [10–13]. It is well-known that lysoderivatives of phosphatidylcholine [8,10] as well as free fatty acids [14] possess detergent properties, although the latter compounds have been poorly studied from this point of view.

The possibility that the production of such endogenous detergents may promote the observed 'spontaneous' solubilization [4–6] of small percentages of NADH- and NADPH-cytochrome *c* reductase and 5'-nucleotidase is investigated.

Materials and Methods

Animals. Male albino rats weighing 150–180 g were starved 16 h before death and anesthesized by intraperitoneal injection of 0.3 ml sodium pentobarbital (60 mg/ml). Livers were perfused in situ by infusion of 100 ml of cold 0.25 M sucrose in the portal vein. For incorporation experiments rats were starved, anesthesized and perfused as before, and 10 h before perfusion received intraperitoneally 1 mCi of [2-^{3}H]glycerol (200 mCi/nmol, from New England Nuclear), dissolved in 1 ml distilled water per 100 g body wt.

Subcellular fractionation. Liver tissue was homogenized in 0.25 M sucrose and diluted with the same solution to a 25% (wet weight/volume) final concentration. Microsomes were isolated as described elsewhere [15]. All steps were performed at 0–4°C.

Tris/water/Tris washing of microsomal membranes. Isolated microsomal membranes were resuspended in 0.15 M Tris-HCl buffer pH 8.0 and centrifuged at 105 000 × *g* (the whole procedure carried out at 0–4°C) in order to remove non-membrane contaminating proteins which electrostatically adsorb to the external surface of the vesicles during fractionation. Microsomes were then resuspended in cold distilled water to a final concentration of 1 mg protein per ml, incubated 1 min at 37°C, quickly cooled at 0–4°C and again centrifuged at 105 000 × *g* for 1 h. This procedure leads to the rupture and resealing of the microsomal vesicles, allowing the shedding of the luminal content in the external milieu. However, since the removed luminal proteins may themselves electrostatically adsorb to the external surface of the vesicles, microsomes were once more resuspended in 0.15 M Tris-HCl buffer, pH 8.0, and centrifuged at 105 000 × *g* for 1 h (at 0–4°C).

The whole Tris/water/Tris washing procedure leads to the removal of about 50% protein, including ribosomal proteins, in comparison with the starting material [15].

Incubation of microsomal membranes. Tris/water/Tris-washed microsomes (60.0 mg protein) were incubated 30 min at 37°C in 10 ml of 0.125 M sucrose buffered with 0.2 M Tris-HCl when operating in the pH range 6.0–8.0, and with 0.2 M glycine-NaOH at pH 8.5 and 9.0. After incubation samples were centrifuged 150 min at 198 000 × *g*. Only the middle 5 ml of the supernatant (198 000 × *g* supernatant) were sucked off and analyzed, if not otherwise stated.

SDS-polyacrylamide gel electrophoresis. This was carried out on a linear slab gel gradient ranging from 7.5 to 15% acrylamide (Bio-Rad apparatus; resolving gel 1.5 mm thick, 8 × 14 cm; sample gel 3 × 14 cm). Aside from minor modifications, gel electrophoresis was performed according to Maizel [16]. To achieve a final concentration of 6.0 mg protein per ml, samples were precipitated with ice-cold trichloroacetic acid (10% final concentration), washed four times in distilled water to remove residual acid and finally dissolved in 10 mM Tris-HCl buffer, pH 6.9, containing 2% 2-mercaptoethanol and 3% SDS. Before electrophoresis samples were boiled 3 min. Trypsin inhibitors from soybean (M_r 21 500), bovine serum albumin (M_r 68 000) and α, β and β' subunits of RNA-polymerase from *E. coli* (M_r 39 000, 155 000 and 165 000, respectively) (Boehringer Mannheim) were used as molecular weight standards for the calibration of the SDS acrylamide gel. Slab gel was stained in 0.2% Coomassie brilliant blue dissolved in 5% methanol/15% acetic acid/water solution. Destaining was carried out in a 5% methanol/15% acetic acid/water solution. The gel was scanned at 550 nm using a Quick Scan (Helena Laboratories, Beaumont, TX).

Sepharose 6B gel filtration. After incubation of the washed microsomal membranes at pH 9.0, 12 ml of the 198 000 × *g* supernatant were pumped into a column (2.5 × 70 cm) containing Sepharose

6B (Pharmacia Inc. Uppsala, Sweden). The elution medium was 0.2 M glycine-NaOH buffer, pH 9.0, in 0.125 M sucrose, pumping rate 18 ml/h. Absorption at 280 nm of the effluent was recorded. Fractions of 6.5 ml were collected and analyzed.

Density-gradient centrifugation. The equilibrium densities of the enzymatic activities present in the 198000 × *g* supernatant, following incubation of washed microsomes at pH 9.0, were determined by 44- and 61-h centrifugations on a linear sucrose gradient ranging from $d = 1.04$ g/ml to $d = 1.23$ g/ml. Centrifugations were performed in an SW 27 rotor (Beckman L5-50 centrifuge) at 82000 × *g*. The gradients were fractionated by puncturing the bottom of the tubes, and 2.0-ml fractions were collected and analyzed.

Enzyme assay and chemical determinations. 5'-Nucleotidase activity was measured according to Touster et al. [17]. NADH- and NADPH-cytochrome *c* reductase activities were measured according to Dallner [18]. The extinction coefficient used for calculation was $18.5 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$. The enzymatic activities are expressed as munits (mU); 1 mU corresponds to 1 nmol NADH or NADPH oxidized per min. Proteins were determined according to Lowry et al. [19] using bovine serum albumin as standard. Phospholipids were extracted by the method of Bligh and Dyer [20]; all steps were performed under N_2 atmosphere. For the water-soluble phosphate and total lipid-phosphate determination, amounts of methanol/water and chloroform phases, respectively, were digested with $HClO_4$ and after cooling analyzed by the Barlett procedure as modified by Marinetti [21]. Determination of individual phospholipid species was performed by two-dimensional thin-layer chromatography, using silica gel G plates (Merck) previously activated at 120°C for 1 h. The solvents used were chloroform/methanol/water (60 : 25 : 4) for the first run and chloroform/methanol/acetic acid/water (90 : 40 : 12 : 2) for the second. The spots of separated phospholipids were visualized under I_2 vapour and identified by comparing their R_F values with those of pure phospholipids obtained by Fluka. The spots were scraped from the plates and extracted two times with 4 ml methanol. Extracts were assayed for phosphate. Radioactivity was measured in a Packard Liquid Scintillation Spectrometer model C2425, on both total phospholipid extracts and individual lipid species following TLC, by directly dissolving the methanol extracts in 10 ml Aquasol (New England Nuclear, Boston, MA). For fatty acid determination aliquots of total lipid extracts were methylated with BF_3 in methanol according to Morrison and Smith [22]. Fatty acid methyl esters were resolved and quantified employing a Perkin Elmer Sigma 1 gas-chromatograph equipped with two flame ionization detectors, operating at 210°C. The columns were spiral tubes (2 m × 4 mm i.d.) packed with 10% BSD chromosorb WHP (80–100 mesh).

Results and Discussion

Enzyme release in the presence of protease inhibitors

In a preliminary set of experiments the possibility that contaminating proteases could be involved

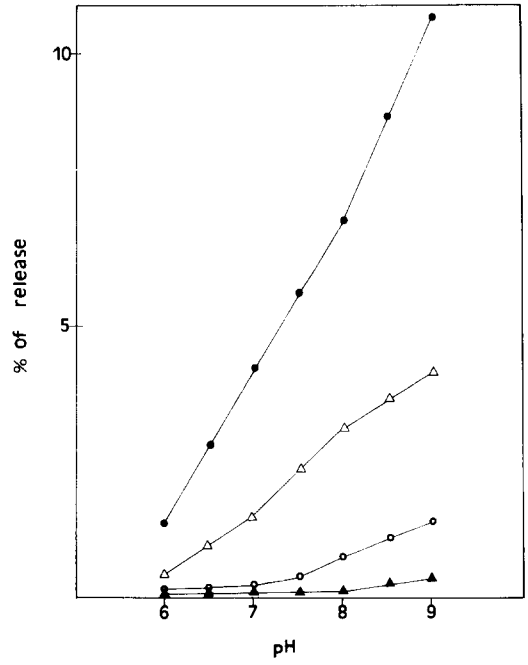

Fig. 1. Effect of pH on integral enzymatic activity and protein release from washed microsomes. Microsomal membranes were prepared, washed and incubated at different pH. Aliquots of the supernatant following centrifugation of the samples at 198000 × *g* for 150 min were assayed for total protein and enzymatic activities. Release is expressed as percentage of total incubated protein and enzymatic activities. Each value is the mean of five determinations. ●, protein; △, NADPH-cytochrome *c* reductase; ▲, NADH-cytochrome *c* reductase; ○, 5'-nucleotidase.

TABLE I

RELEASE OF INTEGRAL ENZYMATIC ACTIVITIES AND TOTAL PROTEIN IN THE PRESENCE OF PROTEASE INHIBITORS

Tris/water/Tris-washed microsomes (6.0 mg protein per ml) were incubated at 37°C, pH 7.0, for 30 min, in media supplemented with protease inhibitors (obtained from Fluka AG, Buchs, Switzerland). Protein and enzymatic activities were measured in the 198000 × *g* supernatant. Each value is the mean of two determinations.

	5′-Nucleotidase (mU/ml)	NADH cyt. *c* reductase (mU/ml)	NADPH cyt. *c* reductase (mU/ml)	Protein (mU/ml)
Control	3.74	0.30	1.20	0.26
Pepstatin (5 μg/ml)	3.87	0.34	1.33	0.25
Trypsin inhibitor (250 μg/ml)	3.78	0.30	1.20	0.25
Leupeptin ($1 \cdot 10^{-5}$ M)	3.80	0.31	1.25	0.26

in NADH- and NADPH-cytochrome *c* reductase release from microsomal membranes was tested.

The hydrophilic moieties of the two enzymes studied are in fact localized on the cytosolic side of the endoplasmic reticulum, and upon controlled proteolytic cleavage they can be separated from the hydrophobic portions and solubilized with retention of native conformation and enzymatic activities [23].

Contamination of microsomal membranes by proteases, especially lysosomal cathepsine, derived from damaged organelles during homogenization, could not be excluded even after Tris/water/Tris washing. For these reasons aliquots of Tris/water/Tris-washed microsomal membranes were incubated in the presence of protease inhibitors: trypsin inhibitor, leupeptin and pepstatin, which are known to inhibit lysosomal proteases [24].

The possibility that 5′-nucleotidase could also be released by protease action has been investigated, although this enzyme has never been reported to be solubilized by mild proteolytic digestion. Results (Table I) show that in no case is a decrease observed in the solubilization levels of total protein and enzymatic activities, speaking against an involvement of proteases in the release mechanism.

pH dependence of release

The ability of microsomal membranes to release integral enzymatic activities in the soluble phase has been tested over a 6.0–9.0 pH range. Results are shown in Fig. 1. It can be observed that increasing amounts of the three enzymatic activities studied as well as total protein are solubilized as the pH is raised in the incubation media, without any apparent optimum. It should be noted that a similar protein-solubilization pattern has been observed following incubation of unwashed micro-

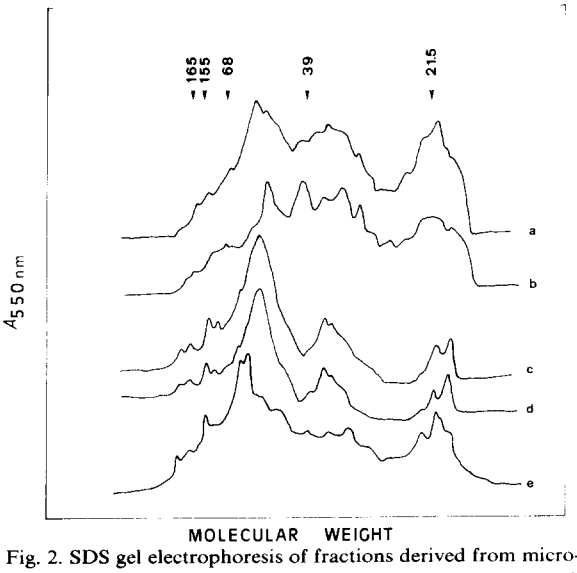

Fig. 2. SDS gel electrophoresis of fractions derived from microsomal membranes. Microsomal membranes were prepared, washed, incubated at pH 9.0 and centrifuged at 198000 × *g*. The polypeptide composition of the following fractions was analyzed: (a) unwashed microsomal membranes (approx. 300 μg protein), (b) material removed by Tris/water/Tris washing of microsomal membranes (approx. 180 μg protein), (c) washed microsomal membranes (approx. 120 μg protein), (d) pellet following 198000 × *g* centrifugation (approx. 110 μg protein) and (e) 198000 × *g* supernatant (approx. 60 μg protein). The arrows indicate standards with known molecular weights.

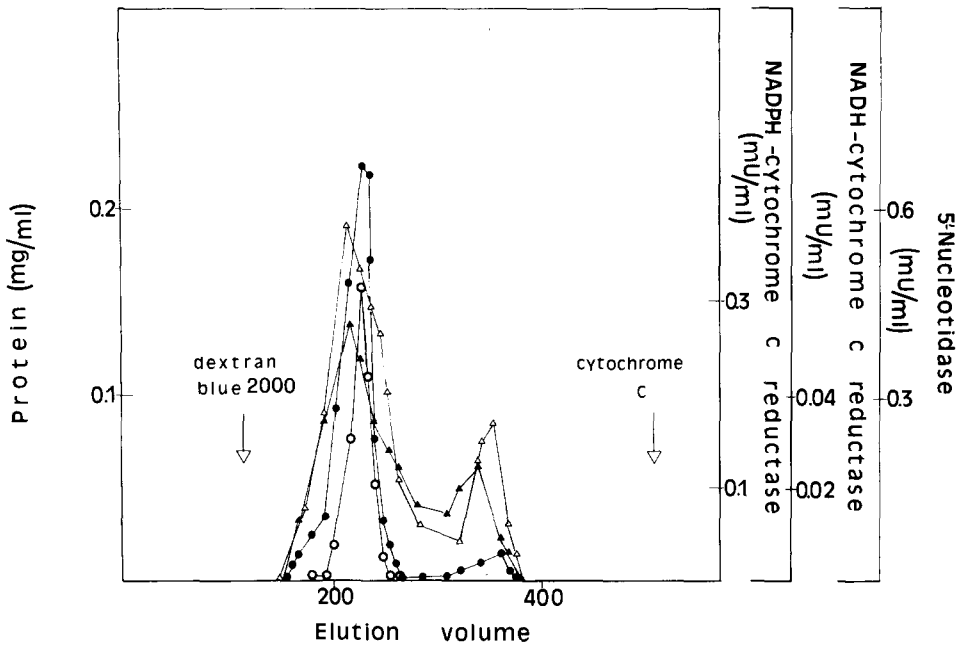

Fig. 3. Sepharose 6B gel filtration of the 198000 × *g* supernatant. Microsomal membranes were prepared, washed and incubated at pH 9.0. Aliquots of the supernatant following centrifugation of the samples at 198000 × *g* for 150 min were pumped into a gel filtration column. Protein (●) and enzymatic activities were measured in the fractions. △, NADPH-cytochrome *c* reductase; ▲, NADH-cytochrome *c* reductase; ○, 5'-nucleotidase.

somal membranes and has been thought to apply exclusively to adsorbed and peripheral proteins [15,25]. Both these kinds of protein are electrostatically bound to the membrane and their release would be enhanced by alkaline pH upon increasing discharge of the surface positive groups which normally allow electrostatic binding to the membrane [15].

It is evident that the release of the three studied enzymatic activities does not fit into this kind of explanation. A possible interpretation of the phenomenon will be provided further in the paper as data on molecular characteristics of the released enzymes are presented. It must be observed, in addition, that lysosomal protease involvement can once more be excluded, since the optimum for their activity lies in the acid field of pH [26].

SDS-polyacrylamide gel electrophoresis of released proteins

Distribution of the released polypeptides has been analyzed by SDS-polyacrylamide gel electrophoresis and compared with peptide profiles from other fractions. Gel scanning is shown in Fig. 2.

It can be seen that the major polypeptides released display a pattern which is rather similar to the one derived from Tris/water/Tris-washed microsomes, especially in the molecular weight region between 165000 and 39000. The bulk of contaminating non-membrane proteins removed from microsomal membranes by the Tris/water/Tris washing procedure is distributed in major peaks ranging in molecular weight between 68000 and 21000. In this area the released material displays only minor peaks, which in part may account for contaminating non-membrane polypeptides. In the lowest molecular weight area, comparison of electrophoretic patterns from microsomes before and after incubation and released proteins shows no presence of new peaks which could account for protease activity products.

Molecular weight and density determination of the released enzymes

The molecular characteristics of the released enzymatic activities were studied in order to have

preliminary information on the mechanism responsible for their solubilization.

Molecular weights and densities were determined by Sepharose 6B gel filtration and centrifugation on a linear sucrose density gradient. The range of molecular weights at which the solubilized enzymatic activities were eluted by filtration on Sepharose 6B is shown in Fig. 3. Protein and enzymatic activities were absent in the void volume. The bulk of protein present in the elution volume ranged in molecular weight between 1 700 000 and 700 000. NADH- and NADPH-cytochrome *c* reductase activities were eluted in a range of molecular weights from 1 650 000 to 700 000 with a main peak around 1 500 000 and a smaller one around 900 000. 5'-Nucleotidase displayed a sharp peak in the fractions corresponding to a molecular weight of approximately 1 400 000.

Although the molecular weights obtained for the three enzymatic activities studied are higher than those found for the isolated enzymes, since they were not excluded by the Sepharose 6B column, it may be concluded that they are not bound to membrane vesicles [4]. Both centrifugation times, namely 44 and 61 h, on a linear sucrose density gradient gave the same densities for the released enzymatic activities. The bulk of protein and enzymatic activities was detected in a density range between $d = 1.06$ g/ml and $d = 1.10$ g/ml with single peaks at $d = 1.07$ for 5'-nucleotidase and at $d = 1.08$ for reductase activities. It must be remarked that only very small amounts of protein

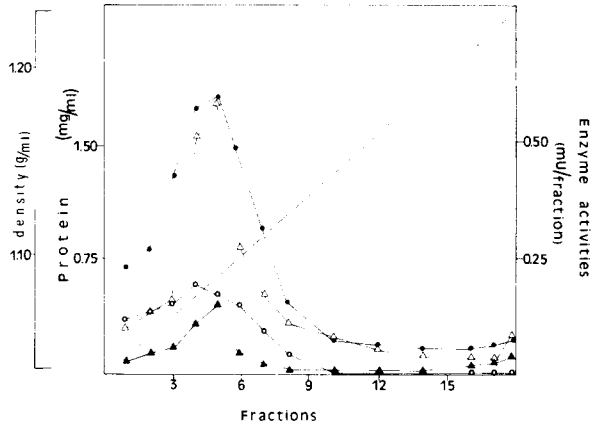

Fig. 4. Equilibrated density centrifugation of the 198 000 × *g* supernatant. Microsomal membranes were prepared, washed and incubated at pH 9.0. Aliquots of the supernatant following centrifugation of the samples at 198 000 × *g* for 150 min were centrifuged on a linear sucrose density gradient (from 1.04 to 1.23 g/ml) for 61 h. Density (— — —), protein (●) and enzymatic activities were measured in the fractions. Symbols are as in Fig. 3.

TABLE II

RELEASE OF TOTAL PROTEIN AND PHOSPHOLIPID FROM MICROSOMAL MEMBRANES DURING INCUBATION IN MILD ALKALINE CONDITIONS

Microsomal membranes were washed and incubated (6.0 mg protein per ml) at pH 9.0. Protein and phospholipid content was measured in the fractions obtained after centrifugation for 150 min at 198 000 × *g*. Each value is the mean of three determinations.

	Protein (mg/ml)	Phospholipid (mg/ml)	Protein/phospholipid
Microsomes before incubation	5.41	2.92	1.86
Microsomes after incubation	4.88	2.60	1.87
Supernatant after incubation	0.52	0.06	8.81

TABLE III

DISTRIBUTION OF [^{3}H]GLYCEROL, PO_4^{-3} AND FATTY ACIDS BETWEEN CHLOROFORM AND METHANOL-WATER PHASES FOLLOWING BLIGH AND DYER EXTRACTION [20] OF THE 198 000 × *g* SUPERNATANT

Rats were injected intraperitoneally with 1 mCi of [^{3}H]glycerol/100 g body weight, and livers were perfused 10 h later. Microsomes, showing a radioactivity of 20 000 cpm/mg protein, were washed and incubated (6.0 mg protein/ml) at pH 9.0. Aliquots of the supernatant after 150 min at 198 000 × *g* centrifugation were processed according to Bligh and Dyer [20]. Radioactivity, PO_4^{-3} and total fatty acids were determined in the fractions. Each value is the mean of three determinations.

	[^{3}H]Glycerol (cpm × 10^{-3} per mg protein)	PO_4^{-3} (μmol per mg protein)	Fatty acids (mg per mg protein)
Chloroform phase	0.171	0.141	0.997
Methanol-water phase	1.185	0.868	—

and reductase activities were detected in a thin pellet at the bottom of the tubes (Fig. 4).

Lipoprotein nature of the released enzymes

The equilibrium densities observed for the three released enzymatic activities upon centrifugation on a linear sucrose density gradient suggest that they must be somehow associated with lipids.

Lipid phosphorus and protein content have been measured in washed microsomes before and after incubation and in the resulting 198 000 × *g* supernatant. As shown in Table II, about 0.12 mg phospholipid per mg protein has been detected in the 198 000 × *g* supernatant, which may not account for the observed densities. It must be noted, however, that there is an 11% phospholipid loss, comparing microsomes before and after incubation, which is not recovered in the supernatant phase.

Table III shows the partitions of glycerol, phosphate and fatty acids, extracted according to the procedure of Bligh and Dyer [20] from the 198 000 × *g* supernatant, between the chloroform and the methanol-aqueous phases. It can be observed that phosphate and glycerol are present in very large quantities in the aqueous phase, while large amounts of free fatty acids are present in the chloroformic phase. Such amounts account for a ratio of 1 mg fatty acid per mg protein in the 198 000 × *g* supernatant. Molecular species and relative percentages of free fatty acids are presented in Table IV.

On the basis of the presented data the most likely hypothesis to explain the release of the integral enzymatic activities must take into account a detergent action of free fatty acids and the formation of mixed micelles inside which integral membrane proteins are inserted.

The question arises as to whether the observed ratio of 1 mg free fatty acid per mg protein accounts for the density of 1.07–1.08 g/ml in the released complexes. Such a density seems likely, assuming for proteins a mean density of 1.32 g/ml [27] and considering that the mean density of the reported free fatty acids is 0.86 g/ml [28]. It should be observed that the enhancement of integral enzyme release by alkaline pH may support this hypothesis. It is in fact well-known that, when anionic detergents are used to solubilize membrane proteins, the charged form of the hydrophilic groups is necessary for maximal effectiveness [1]. Free fatty acids fall into this category of detergents [2]. Since their carboxyl groups have pK_a values around 4.0–5.0 and are fully ionized only at pH ⩾ 11.0 [29], it is reasonable that their solubilizing properties increase with the rise of pH from 6.0 to 9.0

TABLE IV

GAS-CHROMATOGRAPHIC ANALYSIS OF FATTY ACIDS IN THE 198 000 × *g* SUPERNATANT

Microsomes were washed and incubated (6.0 mg protein/ml) at pH 9.0. Aliquots of the supernatant after centrifugation for 150 min at 198 000 × *g* were processed according to Bligh and Dyer [20]. Each value is the mean of two determinations.

Compounds	No. of C atoms and double bonds	Amount (μg/mg protein)
Mirystic acid	14:0	19.8
Palmitic acid	16:0	256.7
Palmitoleic acid	16:1	61.1
Stearic acid	18:0	99.8
Oleic acid	18:1	202.1
Linoleic acid	18:2	220.4
Linolenic acid	18:3	8.6
Gadoleic acid	20:1	22.9
Arachidonic acid	20:4	50.0
—	22:6	56.0

TABLE V

CHANGES IN INDIVIDUAL PHOSPHOLIPID SPECIES IN THE 198 000 × *g* PELLET FOLLOWING INCUBATION OF MICROSOMAL MEMBRANES

1 mCi of [^{3}H]glycerol per 100 g body weight was injected into rats, and livers were perfused 10 h later; microsomes were prepared and incubated (6.0 mg protein per ml) at pH 9.0. Phospholipids (from 2 mg microsomal protein) were extracted, isolated and quantified. Values are expressed as counts/min per phospholipid fraction per mg protein. Each value is the mean of two determinations.

Phospholipids	Before incubation	After incubation
Phosphatidylcholine	2867	2590
Phosphatidylethanolamine	1613	1443
Phosphatidylserine + phosphatidylinositol	361	355
Lysophosphatidylcholine	0	40

Possible role of phospholipases in the release mechanism

Production of free fatty acids may occur in microsomal membranes by the action of endogenous phospholipases on membrane phospholipids. The system is maximally effective upon 5–10 mM Ca^{2+} activation at pH 8.5–9.0 [10–12].

Incubation of microsomal membranes in these conditions for 1 h results in the hydrolysis of about 50% phosphatidylethanolamine and 15% phosphatidylcholine, mainly into glycerylphosphoryl derivatives and free fatty acids [11]. Phosphatidylethanolamine and phosphatidylcholine hydrolysis, however, are known also to occur, although to a lesser extent, independently of pH, in the absence of Ca^{2+} [11].

In Table V changes in the main individual phospholipid species, following incubation of microsomal membranes in our experimental conditions, are presented.

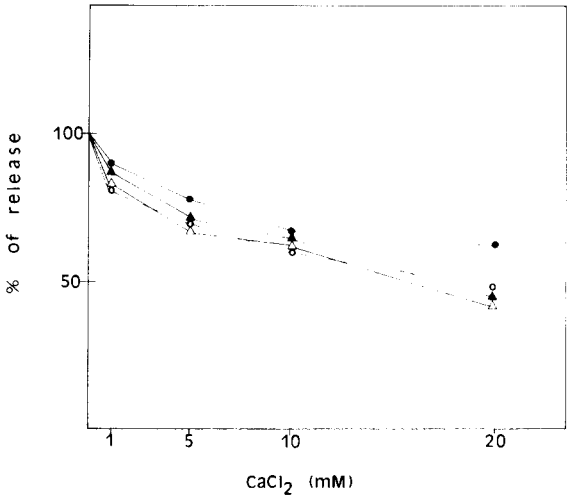

Fig. 5. Effect of Ca^{2+} on the release of protein and enzymatic activities from washed membranes. Microsomal membranes were prepared, washed and incubated (6.0 mg protein per ml) at pH 9.0 in the presence of various concentrations of Ca^{2+}. Protein and enzymatic activities were measured in the supernatant following centrifugation of the samples at $198000 \times g$ for 150 min. Values are given as percentages of a control incubated without Ca^{2+}. In the control release was 0.62 ± 0.05 mg/ml (mean $\pm$ S.D.) for protein, 9.80 ± 2.80 mU/ml for 5'-nucleotidase and respectively 1.07 ± 0.05 and 4.17 ± 0.50 mU/ml for NADH- and NADPH-cytochrome *c* reductase activities. In the presence of 10 mM Ca^{2+} the corresponding data were: 0.40 ± 0.06 mg/ml for protein and 6.60 ± 1.80, 0.63 ± 0.04 and 3.18 ± 0.03 mU/ml for the reported enzymatic activities. Each value is the mean of four determinations. Symbols are as in Fig. 3.

To test whether upon phospholipase activation protein and enzymatic activities are released to a greater extent, aliquots of microsomal membranes were incubated at pH 9.0 in the presence of 1–20 mM Ca^{2+}. Results (Fig. 5) show a progressive inhibition of release as Ca^{2+} concentration is raised in the incubation media. At 10 mM Ca^{2+} a significant reduction of release is observed, being about 35% ($P < 0.01$ as assessed by Student's test) for both protein and enzymatic activities, in comparison with a control incubated without Ca^{2+}, although extensive hydrolysis of phospholipids occurs (Table VI) as described by many authors [11,12,30].

Results are consistent with previous findings showing that extensive hydrolysis of membrane phospholipids by Ca^{2+} activated phospholipases does not lead per se to extensive solubilization of membrane proteins [30,31].

It is well-known that the detergent effect of many substances on biological membranes requires appropriate physicochemical conditions of the environment to be effective [1]: it seems likely that calcium ions, although responsible for massive

TABLE VI

PHOSPHOLIPID HYDROLYSIS FOLLOWING INCUBATION OF MICROSOMAL MEMBRANES IN THE PRESENCE OR ABSENCE OF CALCIUM IONS

Microsomal membranes were washed and incubated (6.0 mg protein per ml) for 30 min at pH 9.0. Aliquots of the incubation mixture were processed according to Bligh and Dyer [20]: Lipid-P refers to PO_4^{-3} found in the chlorophorm phase, whereas Free-P refers to PO_4^{-3} found in the methanol-water phase. The percentage of hydrolysis was calculated on the basis of the value found in the Lipid-P fraction of microsomes before incubation. Each value is the mean of three determinations.

	Lipid-P (μmol/mg protein)	Free-P (μmol/mg protein)	Hydrolysis (%)
Microsomes before incubation	0.604	0.023	3.8
Microsomes incubated without calcium	0.550	0.064	10.6
Microsomes incubated with calcium (10 mM)	0.502	0.119	19.7

production of detergent-like substances in the membrane, upon activation of endogenous phospholipases, also determine unfavorable conditions for solubilization.

Calcium ions interact very strongly with negatively charged phospholipids and free fatty acids, as documented by many biophysical and biochemical studies performed either on sonicated dispersions of phospholipids or on isolated membrane fractions from various cell organelles [29,32,33].

Calcium ion interaction with membrane lipids mainly results in a reduction of their motional freedom and in an appreciable general decrease in membrane fluidity [32].

Concerning the inhibition of release it can be hypothesized that calcium ions, interacting with the charged carboxyl groups of free fatty acids, form cross-links which reduce the repulsive forces between the hydrophilic moieties necessary to micelle formation.

Conclusions

Hydrolysis of membrane phospholipids, probably due to endogenous phospholipases, results in the production of substances (free fatty acids and lysophosphatidyl compounds) capable of damaging membrane integrity on the basis of their solubilizing properties [8,9,14].

Homeostatic enzymatic mechanisms exist, however, in the cell which can modulate the accumulation of such compounds. These mechanisms mainly include acyl-transferase activities [34], which are stimulated by the presence of the terminal products of phospholipid hydrolysis and phospholipase activities, which may be dependent on Ca^{2+} concentration [11].

In the latter case it should be pointed out that, although Ca^{2+} stimulates phospholipase activities, our data show that it also stabilizes lipid bilayers, even when large amounts of detergent-like substances are present, providing in this way a further mechanism in preventing membrane disaggregation.

Results suggest, however, that accumulation in membrane of free fatty acids may result in the solubilization of small quantities of integral membrane proteins if favourable environmental conditions exist.

The question arises as to whether such a mechanism of solubilization is also effective in vivo, leading to the release of small amounts of integral membrane proteins into the cell cytosol. Such a release, if operating in vivo, could provide an important tool for the explanation of different turnover rates reported for closely associated membrane proteins [35]. In this case authophagy, namely sequestering of large portions of membrane into lysosomes and their subsequent degradation into low molecular weight units, can not in fact be invoked [36]. Alternatively, a model has been proposed according to which membrane proteins first dissociate from the membrane matrix before undergoing degradation by normal intracellular process [35].

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