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Differential permeabilization of membranes by saponin treatment of isolated rat hepatocytes

Release of secretory proteins

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Monolayer cultures of rat hepatocytes were treated with increasing concentrations of saponin (prepared from *Gypsophila* plants) for 30 min at 6 °C. Differential permeabilization of the intracellular membranes could be demonstrated: at 0.040 mg of saponin/ml the plasma membrane was permeabilized, as assessed by the release of 50 % of the total cellular amount of lactate dehydrogenase, and at 0.20 mg/ml the endoplasmic reticulum was permeabilized, as measured by the release of 50 % of pulse-^{35}S-labelled albumin. The Golgi complex was permeabilized at an intermediate saponin concentration, as indicated by the release of homogeneously ^{35}S-labelled albumin; about half the intracellular albumin is located in this organelle. At 1.0 up to 5.0 mg of saponin/ml 90–95 % of the radioactively labelled albumin was released. Even at 5.0 mg/ml less than 10 % of the membrane of the endoplasmic reticulum was solubilized, as judged by the degree of release of a membrane-bound enzyme specific for this organelle. These results demonstrate the usefulness of saponin as a tool for investigating the interior of different intracellular compartments.

INTRODUCTION

The analysis of intracellular processes often requires the disruption of the cellular membranes. In some cases this can be done simply by the addition of an excess of detergent, which leads to complete solubilization of the membranes. In other cases it may be necessary, at least in part, to retain the integrity of the membranes, which is achieved if they are permeabilized. Treatment with bacterial toxins (Ahnert-Hilger *et al.*, 1985; McEwen & Arion, 1985), the polyene antibiotic filipin (Jorgensen & Nordlie, 1980; Gankema *et al.*, 1981), or saponins (at low concentrations) (Fiskum *et al.*, 1980; Hirata & Koga, 1982; Burgess *et al.*, 1983) has been widely used to permeabilize the plasma membrane of different cells. Saponins have also been used (at high concentrations) in immunocytochemistry to permeabilize intracellular membranes (Ohtsuki *et al.*, 1978; Willingham *et al.*, 1978; Saraste & Hedman, 1983) and to permeabilize membrane vesicles derived from intracellular membranes (Castle & Palade, 1978; Rottier *et al.*, 1984).

More than a decade ago Redman & Cherian (1972) examined whether newly synthesized secretory proteins are bound within the lumen of the ER. They prepared microsomal fractions from rat liver and made the microsomal membranes permeable to proteins by treatment with a low concentration of deoxycholate. They found that virtually all of the luminal albumin was released, whereas the other (glycosylated) secretory proteins remained associated with the microsomes. However, in later studies of the same kind, it has been reported that both albumin and other major secretory proteins are released on permeabilization of the microsomes (Rask *et al.*, 1983; Morgan & Peters, 1985; Boström *et al.*, 1986).

On the basis of kinetic studies, it has been suggested that secretory proteins bind transiently with different affinities to luminal components of the transport apparatus, apparently in the ER (Lodish *et al.*, 1983; Fries *et al.*, 1984). We were interested in obtaining direct evidence for such binding in isolated hepatocytes, which we are using as a model system for protein secretion (Fries *et al.*, 1984; Fries & Lindström, 1986). As an introduction to these studies, we wished to perform experiments similar to those done by Redman & Cherian (1972). However, to avoid the possible effects of homogenization, we wished to do our experiments with whole cells. Furthermore, to eliminate the possibility of solubilization of the cellular membranes, we wished to effect permeabilization with a saponin rather than with a detergent. For this purpose we chose a saponin prepared from *Gypsophila* plants, which, according to previous immunocytochemical work, has a mild effect on membranes (Saraste & Hedman, 1983).

Previous studies had indicated that higher saponin concentrations are required to effect permeabilization of internal membranes than for the plasma membrane (Fiskum *et al.*, 1980), but quantitative data were lacking. Since differential permeabilization of the cellular membranes could be a useful tool for the study of the secretory process, one of the objectives of this study was to determine the conditions at which this could be achieved. We also report that total permeabilization of the hepatocytes results in the release of all major secretory proteins, leaving only a small, but possibly significant, amount associated with the cells.

Abbreviations used: ER, endoplasmic reticulum; GC, Golgi complex; CMC, critical micelle concentration; PBS, phosphate-buffered saline; LDH, lactate dehydrogenase.

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MATERIALS AND METHODS

Materials

Culture media and newborn-calf serum were purchased from Labassco, Stockholm, Sweden, dexamethasone was from Sigma Chemical Co., St. Louis, MO, U.S.A., and insulin from Novo Industries, Copenhagen, Denmark. Collagenase was obtained from Sigma, saponin (white pure) from E. Merck, Darmstadt, Germany, and [^{35}S]methionine (> 800 Ci/mmol) from Amersham International, Amersham, Bucks., U.K. Rabbit antibodies against human serum albumin and haptoglobin were obtained from Dakopatts, Glostrup, Denmark; these antibodies cross-react with the corresponding rat proteins (Fries & Lindström, 1986). Rabbit antibodies against rat serum were obtained from the same source. Bovine serum albumin was obtained from Boehringer, Mannheim, Germany.

Cell culture

Hepatocytes were isolated essentially as described by Seglen (1976) with some modifications (Smedsrød & Pertoft, 1986). In short, male Sprague–Dawley rats weighing 175–200 g were starved overnight and then anaesthetized with diethyl ether. After perfusion with collagenase, the released liver cells were fractionated by differential centrifugation, and the parenchymal cells were isolated by centrifugation through a cushion of colloidal silica. To each dish (35 mm diameter, precoated with 20 μg of fibronectin), 10^6 cells in 3 ml of Dulbecco's modified Eagle medium (Morton, 1970) further modified as described by Fries & Lindström (1986) were added. The cells were cultured for 20–24 h before use, with a change of medium after 3–4 h.

Saponin treatment and solubilization

A 1 ml portion of PBS (123 mM-NaCl, 8 mM-Na_2HPO_4 and 3 mM-KH_2PO_4, pH 7.4) containing the appropriate concentration of saponin was added to each dish, and the dishes were rocked for 30 min at 6 °C. The media were then removed, and 300 μl of 50 mM-Tris/HCl (pH 8.0)/0.15 M-NaCl, containing 10 mg of Triton X-100/ml, 10 μl of Trasylol (Bayer, Leverkusen, Germany)/ml and 5 mM-EDTA were added to the dishes. The cells were scraped off the plates and centrifuged for 5 min at 15000 g. The supernatants and the media were kept at −70 °C.

Enzyme assays

Lactate dehydrogenase (LDH) was assayed as described by Reeves & Fimognari (1966). For these experiments the saponin solutions were supplemented with 2 mg of bovine serum albumin/ml. Phosphodiesterase was assayed in microtitre plates by using the reaction mixture described by Aronson & Touster (1974). The reaction was stopped by the addition of NaOH, and the A_{400} was measured. For these determinations EDTA was omitted from the solubilization buffer. NADPH–cytochrome c reductase was measured as described by Omura & Takesue (1970). Triton X-100 was included in all enzyme assays.

Labelling with [^{35}S]methionine

Each dish was rinsed twice with buffer 3 (Rubin *et al.*, 1977), and to each was added 900 μl of Eagle's minimum essential medium (Morton, 1970) buffered with 25 mM-Hepes/HCl (pH 7.4) lacking methionine and $NaHCO_3$. The dishes were rocked for 40 min, and 5–10 μCi of [^{35}S]methionine in 100 μl of the same medium was added. After another 10 min of incubation, the dishes were placed on ice and rinsed twice with PBS.

Quantification of ^{35}S-albumin

The cells were extracted with detergent as described above, and antibodies to albumin and haptoglobin were added in excess to samples of the cell extracts and the media. After incubation for 3–4 h, immunocomplexes were collected and analysed by polyacrylamide-gel electrophoresis. The gels were soaked in 2,5-diphenyloxazole, dried, and put on a photographic film. The relative amounts of ^{35}S-labelled proteins were determined by densitometric scanning (Fries & Lindström, 1986).

Subcellular fractionation

The cells were cultured as described above, except that 3.0×10^6 cells in 5.0 ml of medium were added to dishes (diameter 60 mm) which had been coated with 60 μg of fibronectin. The cells in each dish were labelled for 60 min with 1.0 ml of Eagle's minimum essential medium containing 10 μM-methionine and 100 μCi of [^{35}S]methionine. The cells were then homogenized, and part of the post-mitochondrial supernatant (500 μl) was made 1.8 M in sucrose (final volume 1.6 ml). This solution was layered between 1.0 ml of 2.25 M-sucrose and 13 ml of a linear gradient of 1.0–1.55 M-sucrose in a centrifuge tube. Finally 0.50 ml of 0.25 M-sucrose was added. (All sucrose solutions were in water.) After centrifugation for 15 h at 85000 $g_{av.}$, nine fractions were collected. Each fraction (1.5 ml) was divided into two equal parts, to one of which was added PBS to 4.5 ml and to the other PBS containing saponin (2 mg/ml). After about 15 min the solutions were centrifuged at 150000 $g_{av.}$ for 60 min. The supernatants were aspirated off, and 200 μl of the solution (above) for solubilizing the cells was added. After thorough mixing, the samples were withdrawn from the centrifuge tubes, frozen in liquid N_2 and kept at −70 °C. NADPH–cytochrome c reductase and radioactive albumin were determined as described above. Galactosyltransferase was assayed essentially as described by Rothman & Fries (1981).

Electron microscopy

After treatment with different concentrations of saponin, the cells were rinsed twice with PBS at room temperature and fixed for 15 min in 4% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M-sodium cacodylate buffer (pH 7.4). The cells were then incubated for another 15 min with 10 mM-glycine in PBS, rinsed with PBS and post-fixed for 60 min with OsO_4 in 50 mM-phosphate buffer (pH 7.2). The cells were then dehydrated and 'rinsed' with a mixture (1:1) of ethanol/unpolymerized plastic (Epon 812). Finally 1 ml of the monomeric plastic was added and the samples were incubated overnight at room temperature and subsequently at 60 °C for 72 h. Thin sections (50–70 nm) were cut with an LKB Ultratome V and then stained with uranyl acetate and lead citrate.

RESULTS

Critical micelle concentration (CMC)

Saponins, like detergents, are amphiphiles which, when added to water, will occur both at the air/water

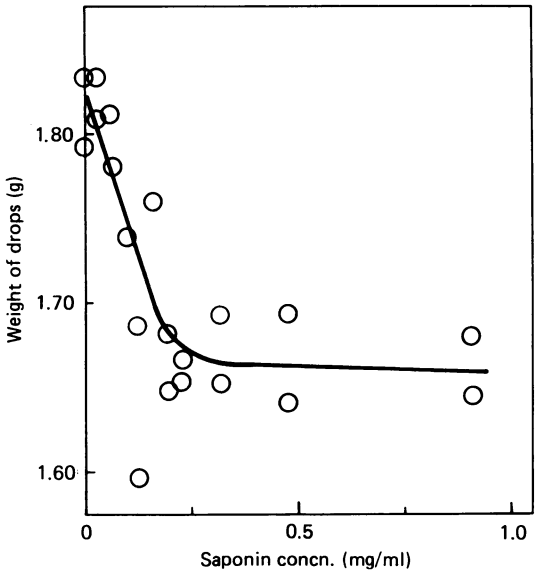

Fig. 1. Determination of CMC of saponin

Samples (40 drops) from each of a series of solutions containing increasing concentrations of saponin were collected in test tubes by means of a peristaltic pump and a fraction collector, and were then weighed. The weight of the drops is approximately proportional to the surface tension of the liquid. The surface tension will decrease with increasing saponin concentration up to its CMC, in this case 0.2–0.3 mg/ml. Results are measurements from two experiments performed at 6 °C.

interface and in the bulk liquid. As more amphiphile is added to the solution, more molecules will occur at the air/water interface and the surface tension will decrease; above a certain concentration, the CMC, most additional amphiphile will form aggregates (micelles) and further decrease in surface tension will be small (for a review see Helenius & Simons, 1975). The CMC can be determined from measurements of the surface tension of solutions containing increasing concentrations of amphiphile. The surface tension can be gauged by the weight of the drops of these solutions (Bull, 1964). Fig. 1 shows that the weight of drops of saponin solutions decreased markedly with increasing saponin concentration up to about 0.2–0.3 mg/ml, demonstrating that the saponin had a CMC in this concentration range. We are not aware of any CMC determination for the saponin used in this study, but the CMC value that we have obtained is close to that determined for sapoalbin (Vochten *et al.*, 1969), which is apparently of similar structure (see the Discussion section).

Permeabilization of the plasma membrane

LDH is widely used as a marker for soluble cytosolic proteins, and release of this enzyme from cells as an indication of permeabilization of the plasma membrane. Fig. 2 shows that treatment of hepatocytes with saponin causes the release of LDH; after 30 min in the presence of 0.025–0.050 mg of saponin/ml only 50% of the total LDH activity was recovered with the cells, and above 0.10 mg/ml about 10% was recorded. Since the incubation medium was not quantitatively removed from the dishes in these experiments, the amount of enzyme

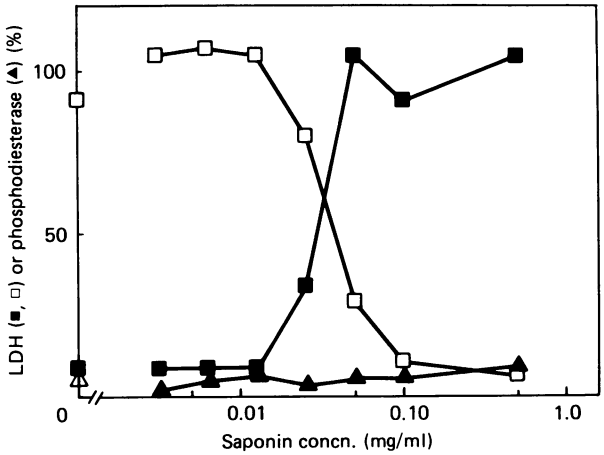

Fig. 2. Permeabilization of the plasma membrane of hepatocytes by saponin

Monolayer cultures of hepatocytes were incubated for 30 min at 6 °C with different concentrations of saponin. The LDH activities of the solubilized cells (□) and the media (■) were then determined. As a measure of the solubilization of the plasma membrane by the saponin treatment, the phosphodiesterase activity in the medium was determined, and is shown as a percentage of the total activity in untreated cells.

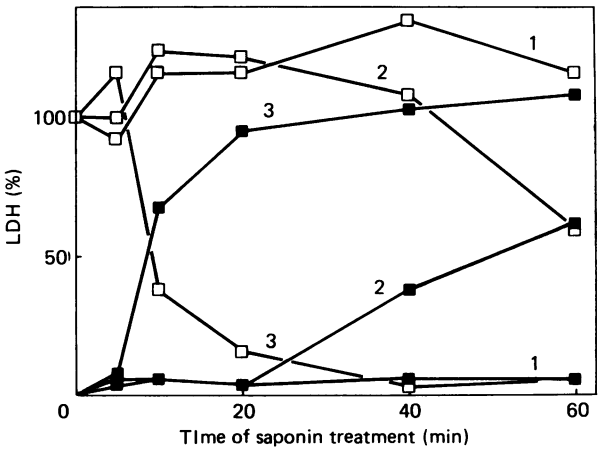

Fig. 3. Time course for saponin-induced release of LDH

Monolayer cultures of hepatocytes were incubated for different times with different concentrations of saponin. The LDH activity of the solubilized cells (□) and the media (■) was then determined; the curves 1, 2 and 3 represent the time courses at 0, 0.025 and 0.100 mg of saponin/ml respectively.

actually associated with the cells was even lower. Up to 0.50 mg of saponin/ml, less than 10% of a marker enzyme for the plasma membrane was released; indicating that integral membrane proteins were not released by the saponin treatment. The time course for the release of LDH was strongly dependent on the saponin concentration (Fig. 3). In the presence of 0.025 mg of saponin/ml (curve 2), some 30% of the total cellular activity could be found in the medium after 40 min, and 50% after about 60 min. At 0.100 mg of saponin/ml (curve 3), practically all release occurred within 20 min.

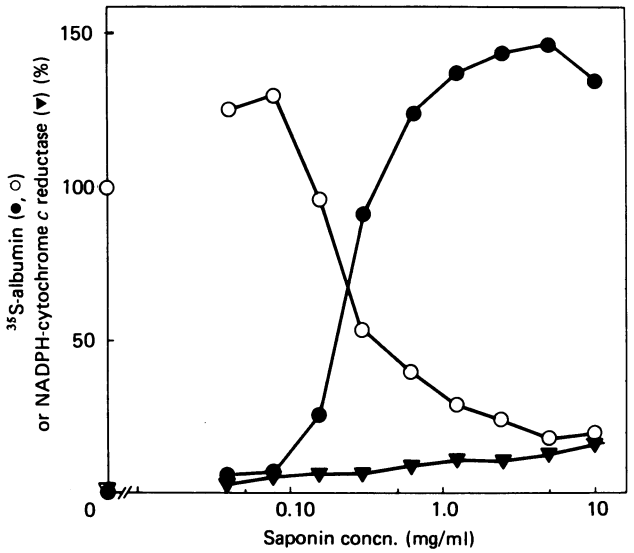

Fig. 4. Permeabilization of endoplasmic reticulum by saponin

Monolayer cultures of hepatocytes were labelled with [^{35}S]methionine for 10 min at 37 °C and were then treated with different concentrations of saponin for 30 min at 6 °C. After solubilization with detergent, the amount of ^{35}S-albumin in the cells (○) and in the media (●) was determined by immunoprecipitation, followed by gel electrophoresis, autoradiography and densitometry. The solubilization of ER membranes by the incubation with saponin was assessed by measuring the NADPH–cytochrome *c* reductase activity in the incubation medium, shown as a percentage of the total activity in untreated cells.

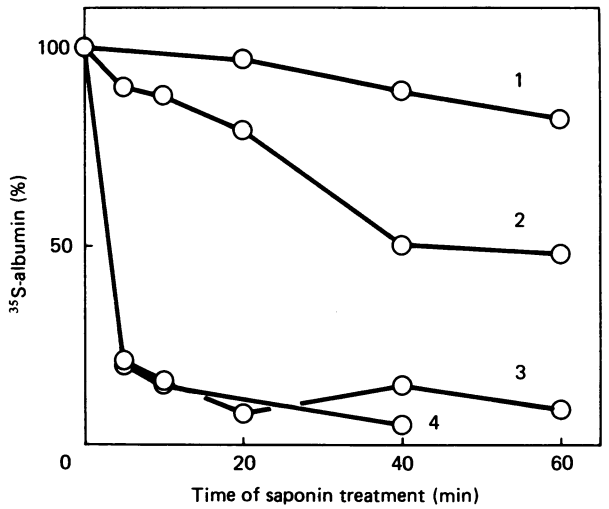

Fig. 5. Time course for the release of pulse-labelled albumin

Cells were labelled for 10 min with [^{35}S]methionine and treated with different concentrations of saponin at 6 °C. The amount of ^{35}S-albumin in the cells was determined after different times of incubation as described in Fig. 4. The curves 1, 2, 3 and 4 denote the time courses for 0, 0.125, 1.0 and 5.0 mg of saponin/ml respectively.

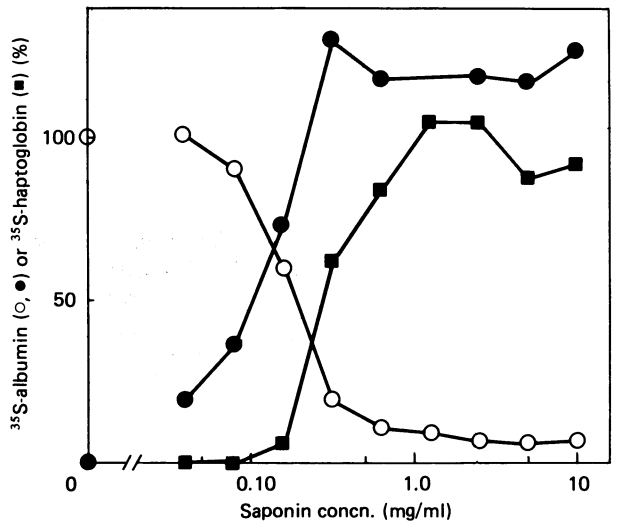

Fig. 6. Release of total albumin and haptoglobin by saponin

Monolayer cultures of hepatocytes were labelled with [^{35}S]methionine for 60 min and were then incubated for 30 min at 6 °C with different concentrations of saponin. The amount of ^{35}S-albumin in the cells (○) and incubation media (●) was then determined by immunoprecipitation as described in Fig. 4. ^{35}S-haptoglobin was quantified in an analogous fashion; the results for media only are shown.

Interestingly, LDH was released with a time lag, which was 20 min at 0.025 mg/ml and 5 min at 0.100 mg/ml.

Permeabilization of endoplasmic reticulum

Half of the newly synthesized albumin is transported from the ER to the GC in 15 min at 37 °C; at temperatures below 16 °C transport has virtually stopped (Fries & Lindström, 1986). Release of pulse-labelled albumin from cells by the incubation with saponin at low temperatures will, therefore, be an indication of the permeabilization of the ER. Fig. 4 shows that half the total amount of the pulse-labelled albumin was released after 30 min incubation with 0.125–0.250 mg of saponin/ml. At the highest concentration tested (10 mg/ml), some 10% of the albumin was recovered with the cells. Measurement of NADPH–cytochrome *c* reductase activity showed that only 10% of this membrane-bound and ER-specific enzyme was released from the cells at the highest saponin concentration. Likewise, less than 10% of the cellular phospholipids labelled during 60 min incubation with [^{32}P]P_i were extracted by the saponin treatment (results not shown). The release of pulse-labelled haptoglobin by saponin showed a similar, if not identical, concentration-dependence (results not shown).

We also determined the saponin concentration required for the permeabilization of ER microsomes (kindly provided by Dr J. Riesenfeld). At a final protein concentration of about 8 mg/ml, 50% release of albumin, as assessed with an enzyme-linked immunoadsorbent assay, was obtained at 0.4 mg of saponin/ml (M. Wassler & E. Fries, unpublished work). This result shows that the amount of bound saponin required for permeabilization is less than 5% of the weight of these membranes (on a protein basis). In the experiments with whole cells the total protein concentration was only about 1 mg/ml, implying that under these conditions the free saponin concentration was close to the total concentration.

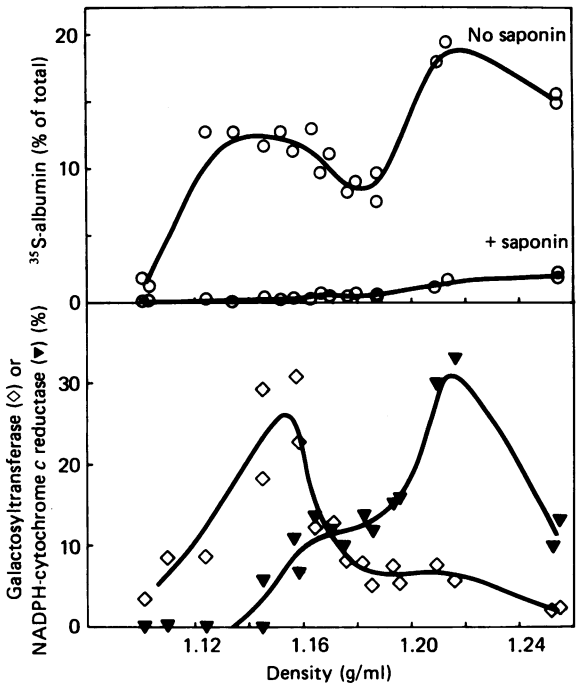

Fig. 7. Subcellular localization of albumin remaining in hepatocytes after saponin treatment

The cells in two culture dishes were labelled for 60 min with [^{35}S]methionine, homogenized, and the postmitochondrial supernatant was subjected to equilibrium density centrifugation. To one half of each fraction, saponin-containing buffer was added to give a final saponin concentration of 1 mg/ml. Buffer only was added to the other half. The membranes were pelleted and solubilized, and the amount of ^{35}S-albumin in each fraction was determined by quantitative immunoprecipitation. The lower panel shows the distribution of an ER and a GC marker, NADPH–cytochrome *c* reductase and galactosyltransferase respectively, in fractions obtained with unlabelled cells. The results from two gradients run in parallel are shown.

The rate at which pulse-labelled albumin was released on treatment with different saponin concentrations was also determined (Fig. 5). At 0.125 mg/ml (curve 2) the release was slow and continued for at least 60 min. At the two higher concentrations tested, almost complete release occurred within 5 min. There was no evidence of a lag period in the release of this protein. The same observations were made with haptoglobin (results not shown).

Release of total albumin

The half-life of pulse-labelled albumin in isolated hepatocytes is about 30 min (Fries *et al.*, 1984). Therefore, continuous labelling of the cells for 60 min will produce virtually homogeneous labelling of intracellular albumin. Cells labelled for this period of time were used to measure the effect of saponin on the membranes of all organelles containing albumin. As shown in Fig. 6, about 0.10 mg of saponin/ml was required to effect 50% release of ^{35}S-albumin, i.e. a concentration intermediate between those required to permeabilize the ER and the plasma membrane. Since the GC and the ER each contain about half the intracellular amount of albumin (Fries *et al.*, 1984; Fig. 7 of the present paper), these results demonstrate that permeabilization of the GC requires a lower concentration of saponin than for the ER. As had been found with pulse-labelled albumin, a small fraction (5–10%) of the homogeneously labelled albumin was not released even at the highest saponin concentration.

In the same experiment the release of haptoglobin was also determined. Release of 50% of this protein required about 0.20 mg of saponin/ml (see Fig. 6), a concentration similar to that required for the release of albumin from the ER. This result is consistent with the idea that the major intracellular part of slowly transported proteins are in the ER (Fries *et al.*, 1984); haptoglobin is externalized at about half the rate of that for albumin. After treatment with the highest saponin concentrations, 5–10% of the labelled haptoglobin was recovered with the cells (results not shown).

Subcellular fractionation

The previous experiment showed that part of the intracellular albumin was still associated with the cells even after prolonged treatment with high concentrations of saponin. To get an indication of the localization of the bound protein, cells labelled for 60 min were homogenized and the postmitochondrial supernatant was subjected to equilibrium-density-centrifugation to separate ER- and GC-derived membrane vesicles. Then saponin in PBS was added to one half of each fraction, and to the other half only PBS was added. Washing of membranes with a solution that has an ionic strength similar to that of PBS has been shown to remove most of the secretory proteins that bind non-specifically during homogenization of pancreatic cells (Scheele *et al.*, 1978). The membranes were then pelleted by centrifugation, and the amount of ^{35}S-albumin in each fraction was determined (Fig. 7). In the absence of saponin treatment, the GC and ER regions of the gradient (fractions with densities lower and higher than 1.17 g/ml respectively) contained equal amounts of radioactively labelled albumin. After treatment, a total of about 7% of the labelled albumin was recovered, most of which (83%) was in the ER region. The distribution of this protein did not follow the pattern of total protein, which was essentially identical with that of the ER marker (results not shown), indicating that the remaining albumin was not simply adsorbed to the bulk of membranes.

Removal of secretory proteins by saponin

Permeabilization of membrane vesicles to release the secretory proteins may be used as a purification step in the isolation of membranes (Castle & Palade, 1978). The most widely used procedure for this purpose seems to be the treatment of the membranes with a low concentration of detergent (Kreibich & Sabatini, 1974). A disadvantage of this technique is that the amount of detergent added must be carefully chosen, otherwise solubilization may ensue or permeabilization will be incomplete. As is clear from the results described above, this problem is not encountered with the *Gypsophila* saponin.

To assess the extent of release of different secretory proteins, other than albumin and haptoglobin, hepatocytes (labelled for 2 h) were incubated with saponin, and the content of secretory proteins before and after treatment was determined with an antiserum against rat serum proteins. As shown by the autoradiogram of the resulting electrophoresis gel (Fig. 8), little of the major secretory proteins remained in the cells after saponin

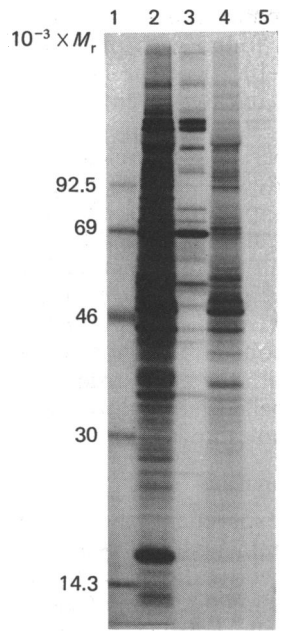

Fig. 8. Release of secretory proteins by saponin

Two monolayer cultures of hepatocytes were labelled for 120 min with [^{35}S]methionine, and one of the cultures was subsequently treated with saponin (1.0 mg/ml) for 30 min. The cells in both dishes were then rinsed twice with buffer 3 and solubilized. One portion of each cell sample was treated with trichloroacetic acid, and another with antibodies against serum proteins. Shown in an autoradiogram of the acid precipitates of the control cells (lane 2) and the saponin-treated cells (lane 4) and of the respective immune precipitates (lanes 3 and 5) after separation by gel electrophoresis. The M_r values ($\times 10^{-3}$) of ^{14}C-labelled proteins (purchased from Amersham International) run in lane 1 are shown to the left.

treatment. (In a control experiment we found that the protein patterns obtained from immune and acid precipitates of the medium of the labelled cells appeared identical, demonstrating that the antiserum contained antibodies against all major secretory proteins.)

Electron microscopy

The biochemical characterization described above indicated that the saponin-induced permeabilization occurred with little solubilization of the membranes. To determine the effect of the saponin on the morphology of the intracellular membranes, we treated the hepatocytes with saponin at different concentrations and studied the cells by electron microscopy. At 0.10 mg of saponin/ml (Fig. 9*b*), the cytosol was translucent, in agreement with the biochemical finding that most of the soluble enzyme LDH was released at this concentration (Fig. 2). Furthermore, the ER was dilated and the nuclear chromatin more evenly distributed within the nucleus than in the untreated cells (Fig. 9*a*). At 0.50 mg/ml (Fig. 9*c*) the inner membrane of the mitochondria was contracted, resembling the morphology described by Schnaitman & Greenawalt (1968) for isolated mitochondria. At 1.0 mg of saponin/ml (Fig. 9*d*) the mitochondria had partially lost their outer membrane, were irregularly shaped and apparently larger than normal. The electron density of their interior was low, but clearly higher than that of the surroundings, indicating that the inner membrane was intact. Even at the highest saponin concentration the membranes of all organelles appeared as distinct lines, albeit discontinuous, confirming the biochemical finding that little solubilization of the membranes had occurred.

DISCUSSION

Saponins are naturally occurring glycosides, predominantly of plant origin, characterized by their foaming and haemolytic activities (for a review see Tschesche & Wulff, 1973). The aglycan part of the saponin molecule (the sapogenin) may be a triterpene or a steroid, to which one or two sugar chains are bound. The structures of more than 400 saponins have been determined, and they show a great deal of variation in both the aglycan and the glycan moieties. This structural variation is also manifested in quantitative differences in their interactions with cell membranes (Schlösser & Wulff, 1969; Tschesche & Wulff, 1973). The saponin used in the present study was, according to the manufacturer, prepared from roots of *Gypsophila* plants, with the major sapogenin having an elementary composition of $C_{30}H_{46}O_4$. A saponin with an aglycan of this composition has been isolated from different *Gypsophila* species and its structure determined (Kochetkov *et al.*, 1963, 1964; Khorlin *et al.*, 1963). This saponin, gypsoside, is an acidic terpene glycoside with nine sugar residues in two branched chains. T.l.c. of the commercial product yielded two major migrating components (Dalsgaard, 1970; G. Hallberg, M. Wassler & E. Fries, unpublished work) which had R_F values possibly identical with those described for purified gypsoside; gypsoside exists in two isomeric forms in equilibrium which separate upon t.l.c. (Kochetkov, 1964).

The lytic effect of saponins on different cells, notably erythrocytes, is well documented and the concentration required for lysis has been determined for a number of saponins (Tschesche & Wulff, 1973; Schlösser & Wulff, 1969). The membrane-perturbing effect of saponins seems to be due chiefly to their property to form complexes with cholesterol (Schlösser & Wulff, 1969). Bangham & Horne (1962) and Glauert *et al.* (1962) have demonstrated that a negatively stained mixture of cholesterol and saponin exhibits hexagonally arranged rings, which have an inner diameter of about 8 nm. The same pattern can be seen on the surface of saponin-treated cells (Dourmashkin *et al.*, 1962). Glauert *et al.* (1962) have suggested that the rings represent complexes between saponin and cholesterol, with the centre containing the carbohydrate moiety of the saponin molecules. Surprisingly, there seem to be no membrane discontinuities in these structures, and it is possible that the release of intracellular proteins occurs through larger defects (Seeman *et al.*, 1973). Indeed, analysis by scanning electron microscopy has shown that saponin-treated cells have holes in their surface membrane, ranging in size from 100 nm to 1 μm (Brooks & Carmichael, 1983). This finding supports the suggestion that the rupture of the plasma membrane caused by many lytic agents is due to colloid osmotic swelling (Bashford *et al.*, 1986).

We have observed that the release of a soluble cytosolic protein (LDH) on addition of saponin occurs with a clear lag phase, which is particularly evident at low saponin concentrations. This phenomenon has also been

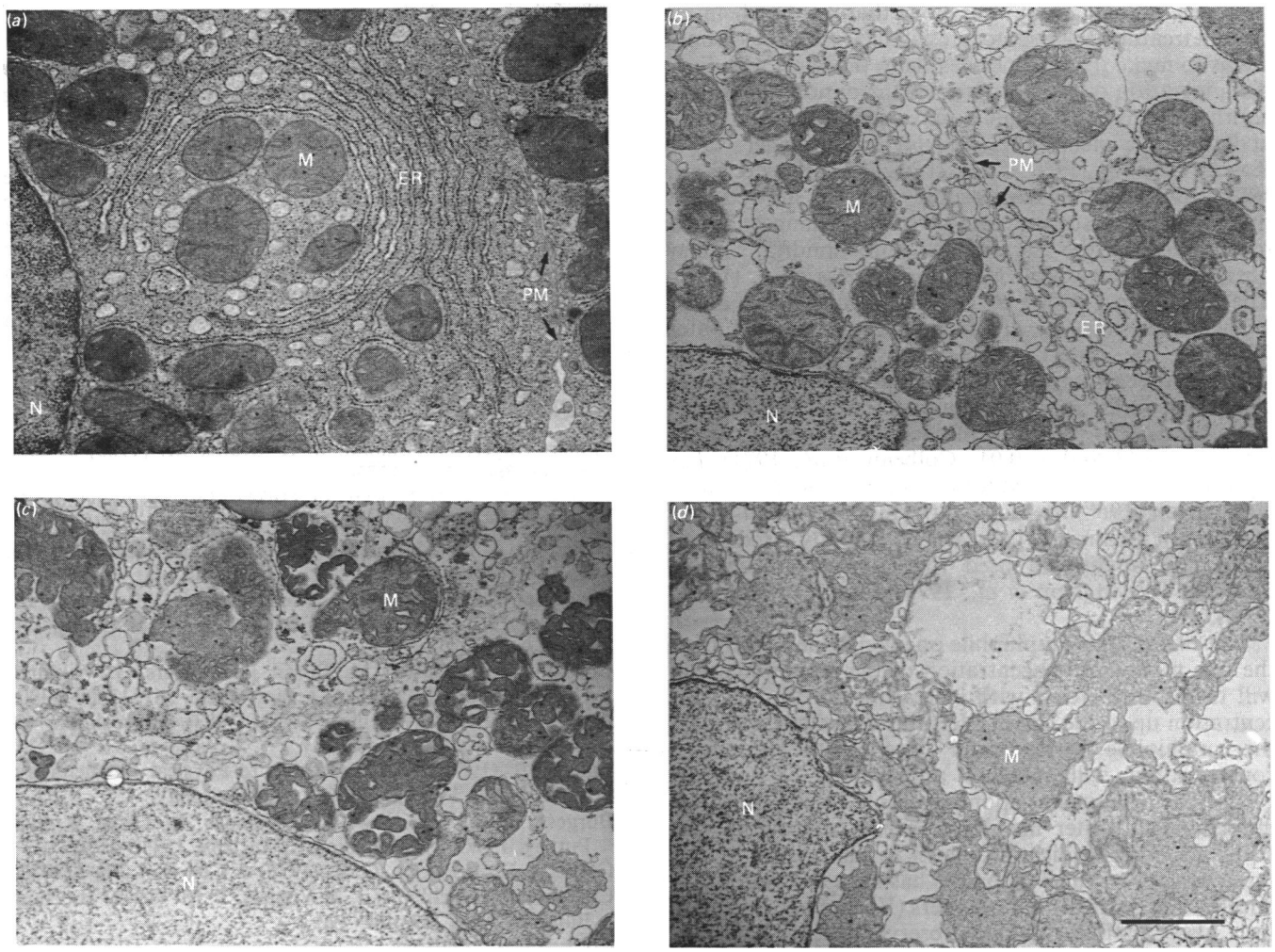

Fig. 9. Electron micrographs of saponin-treated cells

Monolayer cultures of hepatocytes were treated for 30 min at 6 °C with different concentrations of saponin and were then processed for electron microscopy. The saponin concentrations used were (*a*) 0, (*b*) 0.10, (*c*) 0.50 and (*d*) 1.0 mg/ml. Abbreviations: N, nucleus; PM, plasma membrane; M, mitochondrion. Note the dilation of the ER and the mitochondria that occurs on saponin treatment. The bar represents 1 μm.

observed with hepatocytes treated with filipin, another cholesterol-interacting compund (Gankema *et al.*, 1981). The reason for this delay is not clear; possibly it could reflect the time required for the formation of the lesions in the plasma membrane through which the proteins are released. In this context it is noteworthy that saponin treatment has been used in short-term experiments to make the plasma membrane of hepatocytes permeable only to low-M_r molecules (Burgess *et al.*, 1983; Lapetina *et al.*, 1984; Guigni *et al.*, 1985). In agreement with these studies, we have found that ATP is released at lower saponin concentrations than is LDH (M. Wassler & E. Fries, unpublished work).

Previous studies have demonstrated that saponin treatment will make not only the plasma membrane but also intracellular membranes permeable to proteins (Ohtsuki *et al.*, 1978; Castle & Palade, 1978; Rottier *et al.*, 1984; Morgan & Peters, 1985). There is, however, little quantitative data in the literature on the interaction of any specific saponin with different intracellular membranes in one and the same cell type. With the saponin used in this study we have shown that a 5-fold higher concentration is required to permeabilize the ER than the plasma membrane in hepatocytes; this difference in sensitivity to saponin can be accounted for by the different cholesterol contents of the membranes of these organelles, the respective cholesterol/phospholipid molar ratios being 0.06 and 0.76 (Colbeau *et al.*, 1971). Furthermore, we have found that the lysosomal membranes are permeabilized at a concentration about 50% higher than that required for the release of cytosolic proteins (M. Wassler & E. Fries, unpublished work), and the Golgi membranes are permeabilized at a concentration intermediate between those required for the permeabilization of the plasma membrane and the ER. The cholesterol/phospholipid molar ratios in the membranes of these organelles are 0.52 (Henning *et al.*, 1970) and 0.27 (Fleischer & Kervina, 1974) respectively. The differential sensitivity of cellular membranes to permeabilization by a saponin has been used as a tool to localize enzymes intracellularly (Janski & Cornell, 1980; Gordon *et al.*, 1985; Kelner *et al.*, 1986).

In the present study we show that by biochemical criteria treatment of hepatocytes with *Gypsophila* saponin seems to make the cells permeable to proteins, with little distintegration of the membranes. In contrast, digitonin, another widely used saponin, may cause solubilization of at least some membranes (Devlin & Lehninger, 1958; Rosenqvist *et al.*, 1980; Cook *et al.*, 1983; Katz & Wals, 1984). Our electron-microscopic analysis showed that the saponin treatment caused major changes in the morphology of the ER and the mitochondria. Similar observations have been made by Burgess *et al.* (1983). The major effect on the mitochondria was seen at about 1.0 mg of saponin/ml, at which concentration the outer (but not the inner) membrane was disrupted. The differential sensitivity of the mitochondrial membranes to saponins, which can be accounted for by their different cholesterol/phospholipid contents (the respective molar ratios are 0.13 and < 0.01; Colbeau *et al.*, 1971), has previously been described for digitonin by Schnaitman & Greenawalt (1968). Because of the morphological effects of saponin treatment, cells are first lightly fixed when these compounds are used for immunocytochemical purposes (Willingham *et al.*, 1978; Saraste & Hedman, 1983).

The effects of an amphiphile on membranes depend on the free monomer concentration of the amphiphile and will therefore increase with increasing amphiphile concentration up to the CMC (Helenius & Simons, 1975). If the amphiphile forms small micelles, the monomer concentration will increase appreciably beyond the CMC. The *Gypsophila* saponin seems to interact with the mitochondrial outer membrane at concentrations well above its CMC, indicating that it forms small micelles; the apparently similar sapoalbin has been shown to form micelles containing five monomers (Vochten *et al.*, 1969). It is also possible that the observed effects could be caused by minor components in the saponin preparation. It should also be noted that saponin from the same source has been reported to interact with an artifical membrane lacking cholesterol (Gögelein & Huby, 1984).

As mentioned in the Introduction, recent studies indicate that secretory proteins bind differentially to structural components of the transport machinery. One of the purposes of this investigation was to demonstrate such bindng in hepatocytes. We assumed that it should be possible to study this by permeabilizing the cells, which would allow the release of the free secretory proteins, leaving the bound proteins associated with the cells. Using this approach, we found that after treatment of hepatocytes with high saponin concentrations only a small fraction of albumin (and other major secretory proteins) remains associated with the cells, apparently in the ER or some other organelle of high density. Possibly this albumin is bound to specific structures within the secretory machinery. However, it is also possible that this residual protein occurs in soluble form in compartments which are not susceptible to permeabilization by the saponin.

Preliminary results indicate that some proteins are bound to a greater degree after permeabilization than is albumin, as has been previously reported to be the case for apolipoprotein B (Boström *et al.*, 1986). However, we did not find any evidence for a correlation between the rate of transport and the degree of binding. It is possible that the conditions during the permeabilization induced dissociation and that the bound albumin that we have studied is unspecifically bound. To facilitate further analysis, conditions need to be found under which secretory proteins will accumulate intracellularly, leading to an increased degree of binding of the proteins.

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