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Investigation of a possible correlation between rates of secretion and microsomal membrane association of plasma proteins synthesized by rat liver

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The rates of secretion of complement C3, haptoglobin and plasminogen have been determined after pulse labelling with [^{3}H]leucine, and compared to the secretion of prothrombin, albumin and transferrin investigated previously (Kvalvaag, A.H., Tollersrud, O.K. and Helgeland, L. (1988) Biochim. Biophys. Acta 937, 319–327). To study membrane association, rough microsomes were treated with increasing concentrations of saponin, sodium deoxycholate or Triton X-100. All six proteins were quantitated in the soluble and membrane fraction by enzyme immunoassays. At concentrations of saponin from 0.08% to 0.32%, each secretory protein showed a characteristic distribution, almost identical to that obtained with 0.05% sodium deoxycholate or 0.08% Triton X-100. Albumin and transferrin with half-times for secretion ($t_{1/2}$) 30 and 75 min, respectively, are both almost exclusively found in the luminal fraction (> 95%). Prothrombin and plasminogen, which both show an intermediate $t_{1/2}$ (approx. 55 min), are partially associated with the membranes, as only about 60% was released. Haptoglobin and complement C3 also show some association with the membranes (80–85% released). C3 is secreted at the same rate as prothrombin and plasminogen ($t_{1/2} = 55$ min), whereas haptoglobin is secreted more rapidly ($t_{1/2} = 40$ min). Accordingly, no correlation between kinetics of secretion and membrane association was demonstrated.

Introduction

Endoplasmic reticulum is the site of synthesis of a variety of proteins, including both stationary proteins, and proteins destined for export, among them secretory proteins. It is well known that the rates of secretion vary significantly between different proteins, and that the rate-limiting step primarily is the exit from endoplasmic reticulum [1–5]. Whether there exist specific transport signals incorporated in the protein structure [6], or whether there are only retention signals [7], has been a matter of discussion during the last years [8–10]. Transient binding of specific secretory proteins to non-exported, soluble components like BiP would cause retention of some proteins relative to others [11]. Specific or nonspecific interactions with membrane components might also result in retention of some proteins. On the other hand, relatively weak associations between selected secretory proteins and membrane components in the transport vesicles as they bud from the rough endoplasmic reticulum, might result in accelerated transport of specific proteins [9]. It is interesting that the propeptide seems to be essential for the rapid transport of albumin [12].

Furthermore, there has to be some kind of quality control in the endoplasmic reticulum, as incompletely folded or abnormal proteins accumulate in the endoplasmic reticulum, some of them associated with BiP [13–16].

Associations between secretory proteins and stationary components in the endoplasmic reticulum might thus have different purpose, and might involve either soluble or membrane-bound components of the endoplasmic reticulum. In this work, the time courses of secretion of various rat plasma proteins were studied in vivo. The membrane association of the proteins in liver rough microsomes was also investigated. The microsomes were treated with increasing concentrations of the anionic detergent sodium deoxycholate, the nonionic detergent Triton X-100, and a saponin, an acidic

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terpene glycoside being able to permeabilize cellular membranes without disintegrating the membrane structure [17]. Significant variation between different proteins is observed, and the implications for different models of protein sorting are discussed.

Materials and Methods

Materials

L-[4,5-^{3}H]Leucine (130–190 Ci/mmol) were purchased from Amersham International; Soluene and Hionic Fluor from Packard; TH-1 (D-cyclohexylglycyl-L-alanyl-L-arginine-*p*-nitroanilide dihydroacetate) and PL-2 (D-alanyl-L-cyclohexylalanyl-L-lysine-*p*-nitroanilide dihydroacetate) from Nycomed AS, Norway. Rabbit anti-rat albumin IgG and rabbit anti-rat transferrin IgG were obtained from United States Biochemical Corp.; goat anti-rat C3 from Cappel. D-Biotinyl-ε-aminocaproic acid *N*-hydroxy-succinimide ester and Streptavidin-alkaline phosphatase conjugate were obtained from BRL (Bethesda Research Lab.); *p*-nitrophenyl phosphate from KPL (Kirkegaard & Perry Lab. Inc.); and microtiterplates from Costar. *Echis carinatus* venom, urokinase and deoxycholic acid from Sigma, Triton X-100 from Calbiochem and saponin (white pure) from Merck, Germany.

Animals

Male Wistar rats, 240–260 g, from Möllegaard, Havrup, Denmark, were used. The rats were fasted for 18 h prior to subcellular fractionation experiments.

Pulse labelling of secretory proteins

[^{3}H]Leucine was injected into a cannula in the femoral vein during pentobarbital anaesthesia. At the indicated time intervals, 300 μl blood was collected via the cannula, as described previously [1]. For studies of haptoglobin, [^{3}H]leucine was injected in the femoral vein, and blood collected by heart puncture.

Preparation of rough microsomes

Rough microsomes were prepared essentially as described by Eriksson and Glaumann [18]. 30 g of liver were homogenized in 0.35 M sucrose, and the 20% homogenate centrifuged $10000 \times g$ for 20 min. The supernatant was collected, and a second $10000 \times g$ supernatant was prepared from the first pellet resuspended in 0.35 M sucrose. The combined post-mitochondrial supernatant was layered on a double sucrose cushion of 1.3 M sucrose/15 mM CsCl, and 0.6 M sucrose/15 mM CsCl, in 38 ml tubes, and centrifuged at $104000 \times g$ for 3 h 20 min in a Beckman SW28 rotor. Rough microsomes aggregated during passage through the intermediate CsCl-containing layer, and were pelleted. Adsorbed protein was removed from the surface of the microsomes by resuspension in a weakly basic Tris-buffer as described (19).

Preparation of rough microsomal subfractions

Rough microsomes were suspended in ice-cold LSB (50 mM Tris-HCl/50 mM KCl/5 mM $MgCl_2$) at a concentration of 2–3 mg/ml. Solutions of saponin, sodium deoxycholate or Triton X-100 10 times the final concentration, were added dropwise during mixing. After 30 min on ice, the sample was layered onto a 0.25 M sucrose cushion, and centrifuged. The supernatant contained the released proteins. The membrane fraction was solubilized by treatment with 2.5% (w/v) Triton X-100, and centrifuged, as described previously [1].

Purification of proteins for preparation of antiserum

Plasminogen was purified from oxalated rat plasma with 0.03 mg aprotinin/ml, on Lysine Sepharose 4B, essentially as described by Sodetz et al. [20]. Before application, the plasma was diluted 1 : 1 with equilibration buffer (0.3 M phosphate buffer/0.03 M EDTA (pH 7.9)). The column was washed with equilibration buffer, and with the same solvent added 0.2 M NaCl. Plasminogen was eluted by 0.1 M phosphate buffer/0.1 M ε-aminocaproic acid (pH 7.9), ultrafiltrated through a Diaflo membrane YM10 for change into 0.9% NaCl, and stored at −20°C. The purity of the protein was verified by SDS-polyacrylamide gel electrophoresis [21], before antiserum was raised in rabbits.

Haptoglobin was purified by a two step procedure described by Wassdal et al. [22], which included an initial chromatography of rat plasma on Blue Sepharose CL 6B, followed by affinity chromatography on rabbit hemoglobin Sepharose 4B. Both columns were equilibrated with PBS (1.47 mM KH_2PO_4/2.68 mM KCl/6.46 mM $Na_2HPO_4 \cdot 2H_2O$/0.137 M NaCl (pH 7.4)), and the hemoglobin Sepharose was eluted with 6 M guanidine hydrochloride. The purified haptoglobin was immediately dialyzed against 20 mM Tris-HCl/50 mM NaCl (pH 7.4), ultrafiltrated and treated further as plasminogen (see above).

Prothrombin was purified by adsorption of oxalated rat plasma on barium sulphate, followed by anion-exchange chromatography on a Mono Q column in an imidazole-HCl buffer, as described by Myrset and Helgeland [23]. Minor contaminations were removed by preparative polyacrylamide gel electrophoresis (5%), and antiserum was raised in rabbit.

Assay of protein

Protein was determined in acid-precipitable material according to Lowry et al. [24], using bovine serum albumin as standard. To avoid interference by detergents, precipitates were thoroughly extracted by ace-

tone and washed by 6% trichloroacetic acid/0.5% phosphotungstic acid.

Immunosorbent assay

Specific proteins were quantitated in the submicrosomal fractions using an enzyme immunoassay (ELISA) with a biotin-streptavidin system. IgG fractions was biotinylated by addition of 0.2 ml biotinyl-succinimide ester (1 mg/ml dimethylsulphoxide) to 1 ml IgG (1 mg/ml 0.1 M sodium carbonate buffer at pH 8.0) at room temperature. After 2 h the sample was dialyzed against PBS overnight, and stored at −20 °C. The microtiterplate was coated with antibody (IgG-fraction) at a concentration of 2–5 μg/ml in 0.05 M sodium carbonate buffer (pH 9.5), at 4°C overnight, before the sample diluted in PBS-Tween (0.05%, v/v), was added. Between each addition the wells were washed five times with PBS-Tween. The antibody-antigen reactions proceeded for 1 h at 37°C. Biotinylated antibody was added at a concentration of 1–3 μg/ml in PBS-Tween. Alkaline phosphatase conjugated with streptavidin diluted in PBS-Tween was added, and after 15 min at room temperature, the plate was finally washed, and *p*-nitrophenylphosphate, 1 mg/ml in 1 M diethanolamine buffer/0.5 mM $MgCl_2$ (pH 9.8) was allowed to react for 30–60 min at 37°C. A_{410} was measured in a Dynatech MR 700 microplate reader. Each sample was added in a volume of 100 μl, and standard was pooled plasma from six normal rats. The standard curves were in the range of $1 \cdot 10^{-10}$–$1 \cdot 10^{-9}$ g of the specific proteins.

Amidolytic assays

Prothrombin was quantitated amidolytically by using the chromogenic substrate TH-1, after activation by *Echis carinatus* venom, as described [1]. Plasminogen was quantitated by using the chromogenic substrate PL-2. The sample was acidified by HCl and neutralized with Trizma base, prior to activation by urokinase and addition of substrate in a microtiterplate assay (Kulseth, M.A. unpublished results).

Immunoprecipitation

Immunoprecipitation of albumin, plasminogen and complement C3 was performed essentially as described [1], with an addition of aprotinin to 10 μg/ml. Immunoprecipitation of haptoglobin was carried out in 8 mM sodium-barbital-HCl/0.15 M NaCl/15 mM K-oxalate/2 mM leucine (pH 8.2) with aprotinin (4 μg/ml). After incubation overnight at 4°C, the immunoprecipitates were washed twice with the same buffer containing 0.4 M NaCl, solubilized and counted as described previously.

Results

To determine the kinetics of secretion of complement C3, haptoglobin and plasminogen [^{3}H]leucine was injected into the femoral vein, and blood samples collected at time intervals of 10–20 min, as described in Materials and Methods. The average time course of secretion of total labelled plasma protein for the different animals was determined (Fig. 1, Ref. 1). Corresponding curves from individual animals were compared to this average time course, and when necessary normalized by multiplying the values on the time axis by the factor needed to obtain coincidence. The results for specific proteins were multiplied with this same factor to ensure reproducibility of the experimental data.

Haptoglobin forms a complex with hemoglobin released from erythrocytes during hemolysis, and the haptoglobin-hemoglobin complex is subsequently degraded [25]. The concentration of plasma haptoglobin was reduced significantly in experiments with repeated injections and collections through a cannula in the femoral vein, probably due to slight hemolysis (results not shown). With haptoglobin therefore, only one plasma sample was obtained from each rat (Materials and Methods). The incorporation of [^{3}H]leucine into protein varied between different animals, and had to be normalized. The ratio between incorporation into total plasma protein in a specific animal and the corresponding average value was therefore calculated, and the value for incorporation into haptoglobin was divided by this factor. This procedure proved to be valid, as the kinetics of secretion of albumin determined in the same plasma samples, coincided with that obtained previously [1].

Fig. 1 summarizes the results for incorporation of [^{3}H]leucine into complement C3, plasminogen, haptoglobin and albumin in plasma. The corresponding curves of prothrombin and transferrin [1] are included for comparison. The intermediate time course of secretion of prothrombin as compared to albumin and transferrin, is closely similar to that of plasminogen and C3 with a half-time of secretion ($t_{1/2}$) of approx. 55 min. An intermediate $t_{1/2}$ for C3 has previously been reported in rat hepatoma cells [3] and for plasminogen in human hepatoma cells [5].

Studies of possible relationship between rates of secretion and membrane association in rough endoplasmic reticulum, have led to different conclusions [1,17,26–28]. Strous and Van Kerkhof [28] suggested that protein–lipid interactions are involved in the retention of 'slow moving' secretory proteins.

To investigate this possibility in our system, rough microsomes were treated with increasing concentrations of saponin (0.02–0.32%, w/v). The specific secretory proteins were quantitated in the released and the

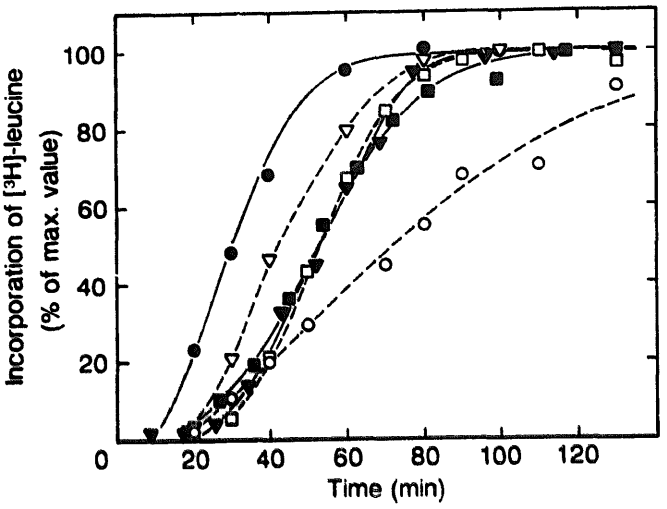

Fig. 1. Rates of secretion of plasma proteins. 200 μCi [^{3}H]leucine was injected in the femoral vein at time 0. Blood was collected and the proteins immunoprecipitated according to Materials and Methods. For haptoglobin, each point is the mean value of three to five animals, for albumin from two animals. For the other proteins, the values are the mean from two to three experiments with combined plasma from two rats, differing by less than 5%. The results were calculated as cpm/mg protein, normalized as described in Results, and expressed as % of maximum value. Albumin (●), haptoglobin (▽), complement C3 (▼) and plasminogen (■). Prothrombin (□) and transferrin (○) (from Ref. 1) are included for comparison.

membrane associated material, and the results are represented as percentage released of total, Fig. 2. There was no detectable interference in the ELISA by the detergents in the dilutions used in the assay. In the amidolytic assay for prothrombin, the detergents increased the background, and had to be corrected for.

It is generally accepted that albumin exists soluble in the lumen of endoplasmic reticulum, and it has frequently been used as a marker for the luminal content [29]. Albumin is almost totally released at concentrations of saponin above 0.08%, indicating total permeabilization of the membrane at this concentration. This is further confirmed by the fact that the percentage released of each of the six secretory proteins, remains at a plateau level at concentrations of saponin from 0.08 to 0.32%. Albumin and transferrin show equal extent of release (> 95%), in spite of the totally different $t_{1/2}$ for secretion. The release of haptoglobin and C3 also shows similarity (approx. 85% at the plateau level), although the secretion patterns are different. Plasminogen and prothrombin are the most membrane bound, as only 60 and 65% are released, respectively. Amidolytical determinations of plasminogen and prothrombin gave essentially identical results.

To get further information about the character of the membrane association of the secretory proteins, rough microsomes were treated with increasing concentrations of sodium deoxycholate and of Triton X-100. Fig. 3 shows the release of total protein by saponin compared to that of sodium deoxycholate and Triton X-100. The figure confirms that the luminal content is selectively released by 0.08% saponin, where a plateau level at approx. 30% is reached. Saponin is thus very well suited for permeabilization of rough microsomes, within a concentration range from 0.08 to 0.32%. At a protein concentration of 3–4 mg/ml and low salt medium, 0.05% sodium deoxycholate or 0.08% Triton X-100 is reported to cause a selective release of the luminal content of microsomes [30]. Fig 3 shows, however, that 40% of the total protein are released at 0.08% Triton X-100, suggesting some release of membrane proteins. Further increase in the concentration

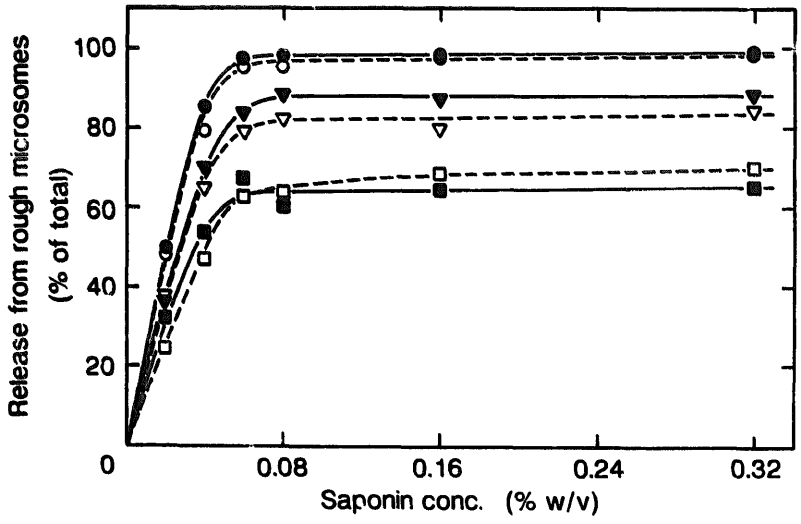

Fig. 2. Release of secretory proteins from rough microsomes. Rough microsomes prepared from five combined rat livers, were treated with increasing concentrations of saponin as described in Materials and Methods. The proteins were quantitated in the released and membrane bound material by ELISA (Materials and Methods), and the results are expressed as % released of total. Each value is the mean from two experiments differing by less than 5%. Albumin (●), transferrin (○) complement C3 (▼), haptoglobin (▽), plasminogen (■), and prothrombin (□).

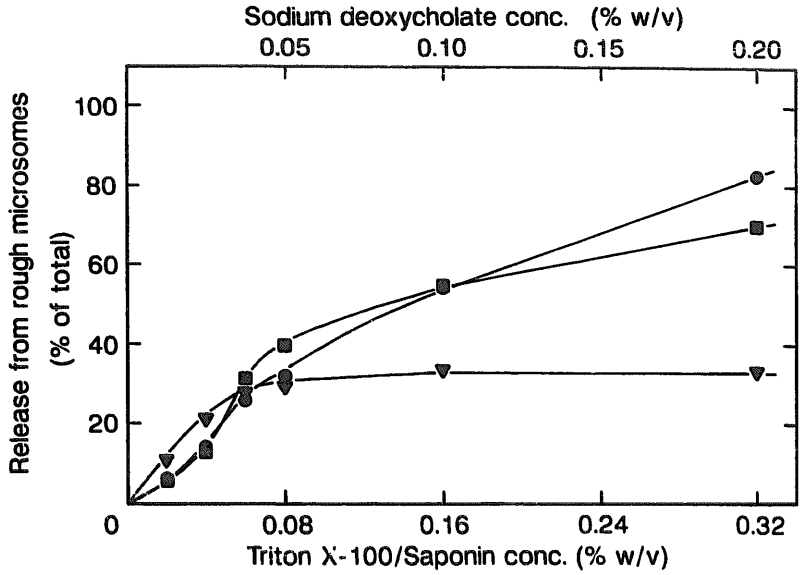

Fig. 3. Release of acid-precipitable protein from rough mirosomes after treatment with sodium deoxycholate (●), Triton X-100 (■) and saponin (▼). The preparations used were those described in Figs. 2, 4 and 5. Protein was quantitated as described in Materials and Methods, and the results expressed as % released of total.

of both of these detergents, releases increasing amounts of protein, as a result of a progressive disintegration of the membrane.

In Fig. 4 the release of specific proteins with sodium deoxycholate ranging from 0.0125 to 0.2% (w/v) is presented. At 0.05% sodium deoxycholate the release is very similar to that obtained with saponin. With further increase in the concentration of sodium deoxycholate, the release of C3, haptoglobin and prothrombin is progressively increased, indicating breakage of the membrane binding. The release of plasminogen, however, decreases somewhat at concentrations above 0.0375% sodium deoxycholate. This may be caused by detergent-dependent formation of plasminogen complexes with membrane components. The anomalous results for the release of plasminogen was confirmed by both the ELISA and the amidolytic assay.

Fig. 5 shows similar experiments using Triton X-100 (0.02–0.32%, w/v). At 0.08% Triton X-100, haptoglobin and C3 are slightly less released (80%) as compared to treatment with 0.05% sodium deoxycholate. The release of prothrombin does not increase with increasing concentration of detergent to the same extent as in Fig. 4. The difference cannot be explained solely by decreased release of total protein (Fig. 3), and may indicate some kind of ionic interactions with mem-

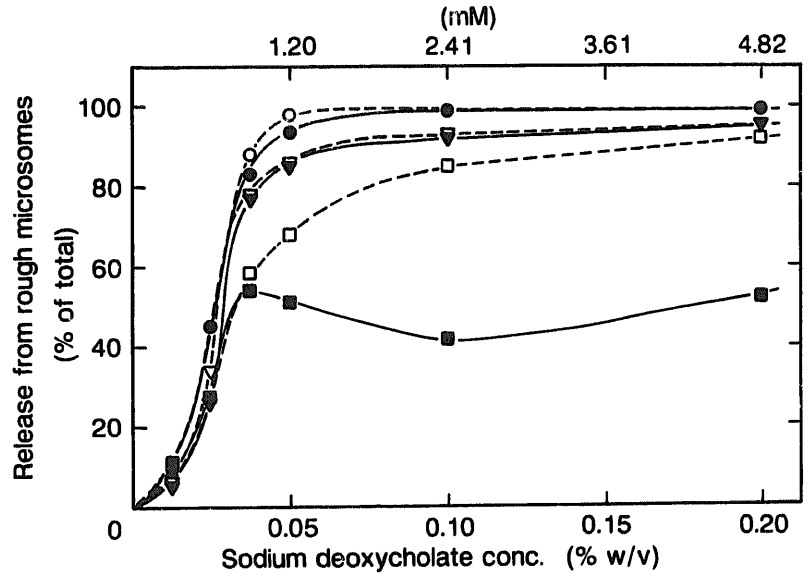

Fig. 4. Release of secretory proteins from rough microsomes with increasing concentrations of sodium deoxycholate. The proteins were quantitated in the released and membrane bound material by ELISA (Materials and Methods), and the results are expressed as % released of total. Each value is the mean from four experiments. Albumin (●), transferrin (○), complement C3 (▼), haptoglobin (▽), plasminogen (■), and prothrombin (□).

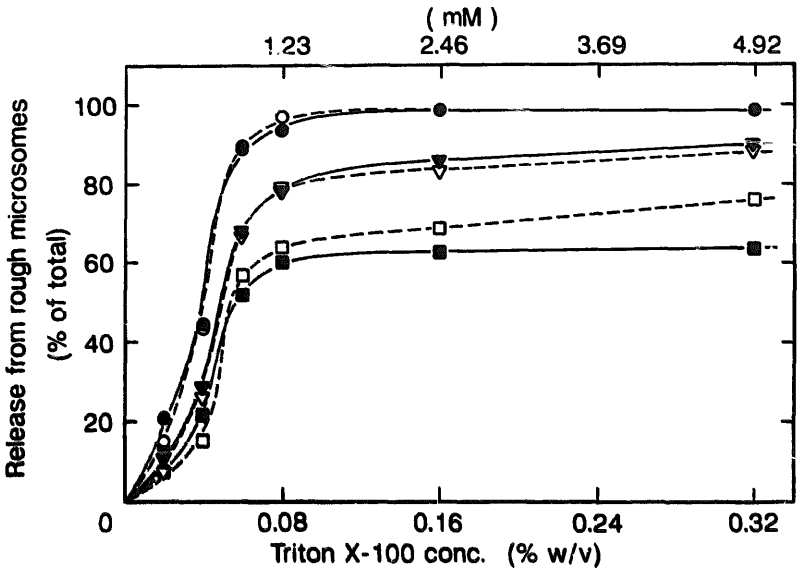

Fig. 5. Release of secretory proteins from rough microsomes with increasing concentrations of Triton X-100. The proteins were quantitated in the released and membrane bound material by ELISA (Materials and Methods), and the results are expressed as % released of total. Each value is the mean from two independent experiments differing by less than 5%. Albumin (●), transferrin (○), complement C3 (▼), haptoglobin (▽), plasminogen (■), and prothrombin (□).

brane components, which are broken by an anionic detergent. The release of plasminogen with Triton X-100 is very similar to that obtained with saponin.

The soluble and membrane fractions after treatment with 0.08% saponin, 0.05% sodium deoxycholate and 0.08% Triton X-100 were further examined by SDS-polyacrylamide gel electrophoresis (results not shown). Some minor differences were observed only in the soluble fraction. The release with sodium deoxycholate was very similar to that with saponin. After treatment with Triton X-100, a slight release of some proteins from the membrane fraction was observed, consistent with the results in Fig. 3.

Discussion

Our results show that specific proteins are released from rough microsomes to characteristic degrees irrespective of which compound used, at selected concentrations of detergent (summarized in Table I). Prothrombin and plasminogen are the most membrane bound of the secretory proteins included in our study. Approx. 40% of the total amounts of these proteins in rough microsomes, remains in the membrane fraction after the treatment. Prothrombin is during biosynthesis γ-carboxylated at several glutamic acid residues in the N-terminal part of the protein [31]. The enzyme catalyzing this carboxylation, is a resident protein in the membrane of rough endoplasmic reticulum [13,32]. Prothrombin is temporarily anchored to the enzyme by the propeptide, and possibly also by a disulphide bond [33]. Whether the fraction bound to the carboxylase may comprise 40% of the total amount of prothrombin in normal rough microsomes, is not known. During inhibition of carboxylation by warfarin treatment, 25% of the accumulated prothrombin precursor are reported to be bound to the enzyme [34]. We have observed a slight difference in release between sodium deoxycholate and Triton X-100, which may indicate that at least part of the membrane associated prothrombin is bound by ionic interactions.

Parent et al. [5] suggested that secretory proteins might be sorted in endoplasmic reticulum on the basis of structural determinants. The kringle structures of plasminogen and prothrombin [35,36] might represent such determinants in a specific recognition apparatus, leading to the observed similarities in membrane association and in rates of secretion (Table I).

Haptoglobin has a common genetic origin together with plasminogen and prothrombin [37]. Haptoglobin, however, is transported more rapidly out of the cell, with a $t_{1/2}$ of 40 min. The secretion of haptoglobin is reported to proceed more slowly than that of albumin in rat hepatocytes [38], in agreement with our results. This protein is also associated with the membranes of

TABLE I

The half-time for secretion of the six secretory proteins, and the release from rough microsomes after treatment with 0.08% saponin

The values are summarized from Fig. 1 and Fig. 2

Protein	$t_{1/2}$ (min)	Fraction released (%)
Albumin	30	98
Complement C3	55	85
Haptoglobin	40	83
Plasminogen	55	60
Prothrombin	55	60
Transferrin	75	98

rough microsomes to some extent, as approx. 15–20% remain in the membrane fraction after treatment with the detergents. Wassler et al. [17] were not able to discriminate between albumin and haptoglobin with respect to membrane association in rough endoplasmic reticulum, from experiments with permeabilized hepatocytes. In their system, however, differences in release of newly synthesized secretory proteins, may be caused both by different location in the secretory pathway and different membrane binding. Their conclusion is thus not contradictory to ours, but may solely be reflecting the different systems.

Complement C3, with a $t_{1/2}$ of secretion of about 55 min, shows approximately the same degree of membrane association as haptoglobin does. Any common structural determinants or modifying enzymes to account for the similarity in membrane association, is not known.

Our results show identical release of albumin and transferrin from rough microsomes (> 95%). Morgan and Peters [26] were also unable to detect differences in release between albumin and transferrin. Strous and Van Kerkhof [28] obtained an almost total release of both albumin and transferrin from HepG2 cells, although a higher concentration of saponin was required for transferrin. In our system, albumin and transferrin follow identical patterns of release with all three compounds tested. Furthermore, the release of all the secretory proteins in our study reaches a plateau level at the same concentration of saponin (0.08%). Pulse chase experiments have shown that the membrane association of apolipoprotein B-100 in HepG2 cells after treatment with 100 mM $NaCO_3$, decreased with increasing time after synthesis [27]. Whether this is valid for the proteins in our work, is currently under investigation.

Albumin, transferrin, plasminogen, C3 and prothrombin fit into the 'three secretory classes', suggested by Parent et al. [5], from experiments with human hepatoma cells. Haptoglobin, however, does not fit into any of these three classes. Our results thus point to a more complex system, at least in normal cells in vivo.

The current view on intracellular transport, is strongly influenced by the discovery of retention signals on stationary proteins in endoplasmic reticulum [39–42]. This has led to statements like 'secretion is the default fate for a protein containing no specific signals' [7]. Pfeffer and Rothman [43] suggested that adsorption effects along the secretory pathway, and variability in rate of protein folding and assembly, might account for the observed differences in transport rates between various secreted proteins. If this, however, was solely a result of adsorption effects, it is difficult to explain why variations primarily is observed at the level of exit from rough endoplasmic reticulum [3,4]. As to the folding and assembly, Morgan and Peters [26] concluded that the folding of transferrin was completed within few minutes, in contrast to its slow exit from rough endoplasmic reticulum.

Concerning the secretory proteins in this study, no simple explanation of the observed differences is valid. Retardation of specific proteins might be caused by different interactions with processing enzymes [44]. All of the proteins studied except albumin is glycosylated, however, and are as such modified by the same set of enzymes along the secretory pathway. Furthermore, prothrombin is secreted with the same $t_{1/2}$ as plasminogen and C3, in agreement with the observation that the carboxylation of prothrombin is not the only rate-limiting step in the transport [1].

As there is no observable correlation between membrane association and rates of secretion, it is impossible to determine how the membrane binding of proteins in this study influence on the transport rates. Variations in transport rates may moreover be caused by segregation of different secretory proteins to defined areas of the endoplasmic reticulum [5], or by interactions with soluble components in endoplasmic reticulum [11].

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