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Four secretory proteins synthesized by hepatocytes are transported from endoplasmic reticulum to Golgi complex at different rates

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Pulse-chase experiments in conjunction with subcellular fractionation and quantitative immunoprecipitation have been used to study the intracellular transport of four secretory proteins, albumin, transferrin, prealbumin and retinol-binding protein, in isolated rat hepatocytes. The proteins were found to be transported from the endoplasmic reticulum (ER) to the Golgi complex (GC) at greatly different rates ($t_{1/2}$ = 14 – 137 min), indicating that transport of secretory proteins between these organelles is effected by a selective, possibly receptor-mediated process and not through bulk phase transfers. The transport from the Golgi complex to the medium was rapid for all proteins ($t_{1/2}$ ~15 min) and possibly occurred at the same rate. Consistent with these kinetic data, the amount of a rapidly transported protein (albumin) in the GC fraction was found to be high (relative to its amount in the ER fraction) whereas the amount of a slowly transported protein (transferrin) in the GC fraction was found to be low, as determined by radioimmunoassays.

Key words: intracellular transport/secretory proteins/endoplasmic reticulum/hepatocytes

Introduction

Secretory proteins (like most membrane proteins) are synthesized by ribosomes bound to the endoplasmic reticulum and are co-translationally transferred into the lumen of the endoplasmic reticulum (ER). From the ER the newly synthesized secretory proteins are then transported, via the Golgi complex (GC), to the exterior of the cell (for reviews seen Tartakoff, 1980; Sabatini *et al.*, 1982). Very little is known about the mechanisms underlying this intracellular protein transport. Morphological studies have indicated that the transport is mediated by vesicles that bud off from one organelle and fuse with another (Jamieson and Palade, 1968; Yokota and Fahimi, 1981). The observation that two (Morgan and Peters, 1971; Strous and Lodish, 1980) or three (Ledford and Davis, 1983) different secretory proteins synthesized by the same cells are released into the medium at different rates suggests that the transport, at least in part, is effected by selective processes rather than by bulk phase movements.

We intend to use rat hepatocytes as a system to investigate the mechanisms by which secretory proteins are transported intracellularly. As an introduction to these studies we have determined the time courses for the intracellular transport of four serum proteins, albumin, transferrin, prealbumin and retinol-binding protein (RBP), in isolated rat hepatocytes. Our results show that these four proteins are transported out of the cells at greatly different rates and that the differences in the transport rates result mainly, if not solely, from differential rates of transport from the ER to the GC.

Results

Subcellular fractionation

The present investigation required the development of a simple procedure for the isolation of ER- and GC-derived membranes from a small number of cultured hepatocytes. To this end, pre-swollen cells were homogenized and cell debris; nuclei and mitochondria were removed by centrifugation. The supernatant was subjected to equilibrium centrifugation in a sucrose density gradient and the membranes in the resulting fractions were collected. The amount of protein in each fraction was determined and the activities of marker enzymes for the ER and the GC were assayed (Figure 1). The highest activities of the GC and the ER marker enzymes were found in fractions with densities of 1.13 – 1.14 and 1.18 – 1.23 g/cm^3, respectively (data from three experiments, in one of which the maximum sucrose density in the gradient was 1.24 g/cm^3).

On the basis of the data obtained from the experiments with the continuous gradient, a two-step gradient was designed (see Materials and methods), from which two fractions were collected, the lower, denser half of the gradient (density

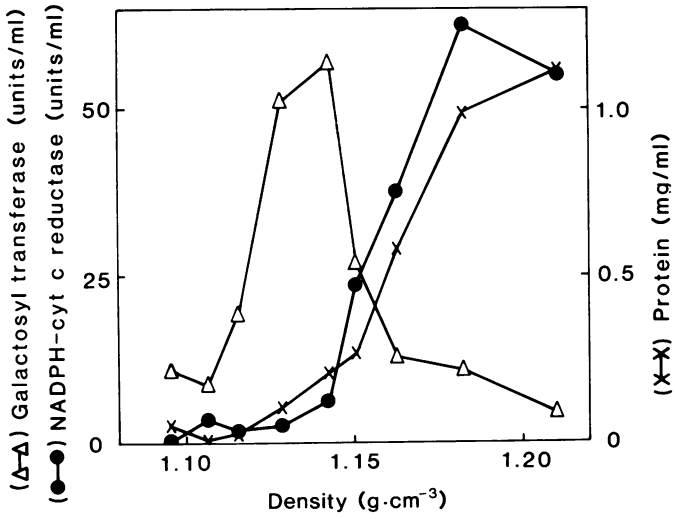

Fig. 1. Separation of ER- and GC-derived membranes from rat hepatocytes by equilibrium density centrifugation in sucrose gradient. NADPH-cyt c reductase, assayed as described (Omura and Takesue, 1970), was used as a marker for ER-derived membranes, one unit representing 1 nmol of cyt c reduced per min. Galactosyl transferase, assayed as described (Rothman and Fries, 1981), was used as a marker for GC-derived membranes, one unit representing the transfer of 1 pmol of galactose per min. Protein was determined with a modified Lowry method (Dulley and Grieve, 1975) with bovine serum albumin as standard. The densities of the fractions were determined by refraction index measurements.

Table I. Subcellular fractionation of hepatocytes

Fraction	Protein (%)	NADPH-cyt c reductase (%)	Galactosyl transferase (%)	[35S]Met-albumin 5 min pulse (%)
Homogenate	100 (3.9 mg)	100 (48 nmol/min)	100 (32 pmol/min)	100
Pellet	57 ± 4	24 ± 4	12 ± 4	36 ± 8
Supernatant	53 ± 4	71 ± 6	112 ± 7	73 ± 5
ER fraction	13 ± 1	48 ± 2	13 ± 1	51 ± 9
GC fraction	2.0 ± 0.2	6 ± 3	31 ± 7	6 ± 2

The cells were homogenized and centrifuged briefly. The supernatant was subjected to centrifugation in a two-step sucrose density gradient and two fractions were collected, the lower fraction being used as the ER fraction and the upper fraction as the GC fraction. Protein and enzyme measurements were done as described in Figure 1. Radioactively labelled albumin was detected as described in Figure 2 and was quantitated by densitometric scanning. Means ± standard errors from two to five experiments are shown.

>1.15 g/cm^3) being used as the ER fraction and the upper, lighter half as the GC fraction. Some characteristics of the membranes collected from these two fractions are given in Table I. The total recovery of the ER marker enzyme in the gradient was 54%, of which 89% was in the ER fraction. The recovery of the GC marker enzyme was 44%, of which 70% was in the GC fraction. The specific activity of NADPH-cytochrome c reductase was four times higher in the ER fraction than in the homogenate. The specific activity of galactosyl transferase was 16 times higher in the GC fraction than in the homogenate.

The recovery in the different fractions of [35S]methionine ([35S]Met)-labelled albumin from cells pulsed for 5 min was also determined (see Table I). The total recovery of the labelled albumin in the gradient was ~50%, of which 90% was in the ER fraction. However, the amount of [35S]Met-albumin in the homogenate was only half of that detected in the cells on the culture dishes (the latter amount was determined with a sample obtained from cells which had not been pre-swollen and which had been solubilized directly in the dishes, data not shown), implying that the absolute recovery of albumin in the gradient was ~25%.

Intracellular protein transport

To determine the time-course for the intracellular transport of different secretory proteins, hepatocytes in Petri dishes were pulse-labelled with [35S]Met for 10 min and sets of two dishes were incubated with an excess of unlabelled methionine for various periods of time. The incubation media were collected and ER and GC fractions were prepared from the cells with two-step gradients. Different secretory proteins were detected in the samples from the membrane fractions and in the media by immunoprecipitation followed by polyacrylamide gel electrophoresis and autoradiography. The specificities of the antisera used are shown in Figure 2. Figure 3 summarizes an experiment in which the transport of albumin, transferrin and RBP were simultaneously analyzed. Pulse-labelled albumin was found to leave the ER at a high rate initially and to rise concurrently to a high level in the GC. The transport of albumin from the GC to the medium was also fast, and at the end of the experiment virtually all of the [35S]Met-labelled albumin had been secreted. The other proteins were found to leave the ER at much lower rates and

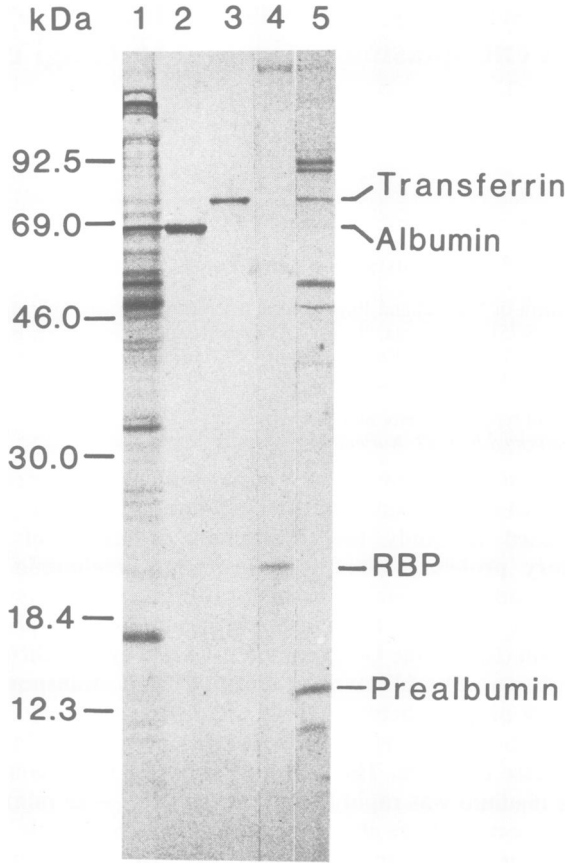

Fig. 2. Specificities of antisera. Antisera against different secretory proteins were added in excess to 10–50 μl aliquots of the ER fraction (see Table I) prepared from cells labelled for 10 min with [35S]Met. The immune complexes were analyzed by polyacrylamide gel electrophoresis followed by autoradiography. **Lanes 2–5** show the patterns obtained with antiserum against albumin, transferrin, RBP, and prealbumin, respectively. The apparent mol. wts. of albumin, transferrin, RBP and prealbumin, were 68, 75, 21 and 13.5 kd, in agreement with published data (Bergeron *et al.*, 1978; Schreiber *et al.*, 1979; Peterson *et al.*, 1973). The mol. wts. of ^{14}C-labelled markers (purchased from New England Nuclear) are given to the left. **Lane 1** shows the pattern obtained with the unfractionated cell homogenate.

their levels in the GC were low.

For quantitative analysis of the pulse-chase experiments the amounts of the different [35S]Met-labelled proteins in the gels were determined by densitometric scannings of the autoradiograms. A graphic representation of this quantitation for the experiment described in Figure 3 is shown in Figure 4. The graphs clearly show that the pulse-labelled proteins disappeared from the ER at different rates and that the levels in the GC increased only during the first 20–30 min, resulting in a high maximum level in the GC fraction for a fast moving protein and *vice versa*. Transferrin differed from RBP (and prealbumin, data not shown) in that it appeared in the GC fraction only after a clearly defined lag period. Furthermore, the amount of the pulse-labelled transferrin recovered in the ER fraction increased strongly following the pulse. These properties of transferrin indicate that this protein exists in two states in the ER which can be temporally resolved. All proteins were detected in the medium only 20–30 min after the end of the pulse, showing that the minimum time required for the transport to the cell surface was similar for all proteins. The rates at which the proteins appeared in the medium were different for all proteins and were proportional to the rates at

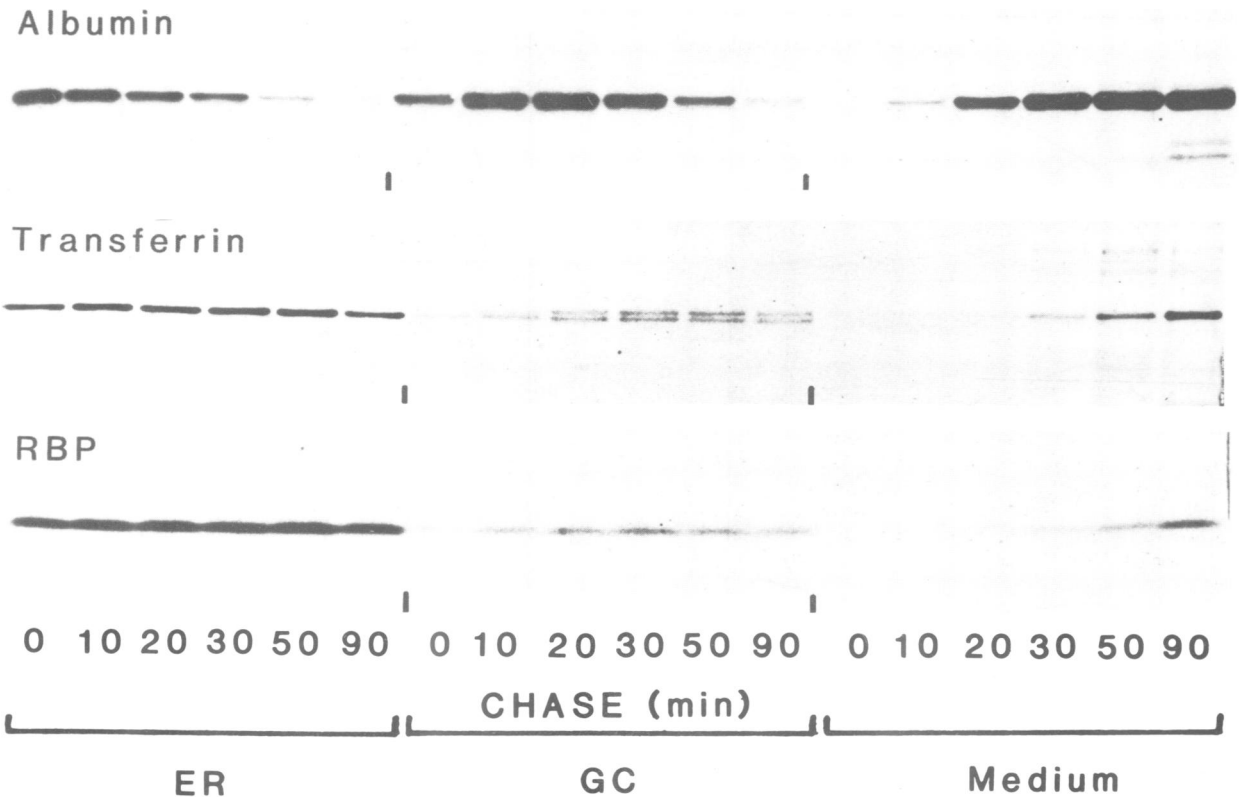

Fig. 3. Pulse-chase analysis of intracellular transport of secretory proteins. Dishes with hepatocytes were incubated for 10 min with [^{35}S]Met. After the periods of chase indicated, media from two dishes were collected and ER and GC fractions from the corresponding cells were prepared by centrifugation in two-step sucrose density gradients as described in Materials and methods. Different secretory proteins were detected by immunoprecipitation followed by polyacrylamide gel electrophoresis (see Figure 2). Only the relevant sections of the autoradiograms are shown. Note the slight decrease in electrophoretic mobility for transferrin as it passes through the GC. Similar mobility shifts have been observed for other glycoproteins and have been shown to be due to the acquisition of sialic acid residues (Knipe *et al.*, 1977; Tartakoff and Vasalli, 1979; Carlson and Stenflo, 1982). Rat plasma transferrin appears to contain 2–3 sialic acid residues (Schreiber *et al.*, 1979).

which they left the ER.

The ER- and GC-derived membranes in the two fractions obtained from the subcellular fractionation were cross-contaminated and their recoveries were different. Hence, the raw data from the pulse-chase experiments did not give a correct picture of the time-course of the intracellular transport of the secretory proteins. An attempt was made, therefore, to correct the data for these effects. To correct for the cross-contamination, we assumed that the distributions of the marker enzymes for the ER and the GC in the two fractions from the sucrose gradients (see Table I) also reflected the distributions of the secretory proteins in these organelles. This correction yielded a maximal value for the amount of radioactively labelled albumin in the GC fraction (at 10 min of chase) that was 60% higher than that of the ER fraction at the beginning of the chase period, indicating that the recovery of albumin in the GC fraction was higher than that of the ER fraction. The recoveries of the secretory proteins in the two fractions were then estimated using the assumption that the total amount of radioactively-labelled protein was constant during the chase period; if the amounts of albumin in the ER and GC fractions at all time points were multiplied by the factors 4.8 and 2.0, respectively, the sum of the resulting values and the corresponding value for the medium were approximately equal (± 10%). This calculation indicated that the recoveries of albumin in the ER and GC fractions were 21 and 50%, respectively. This value for the recovery of albumin in the ER fraction was in agreement with the value that had been determined experimentally, as discussed above. As expected, it was lower than the recovery of the (membrane-bound) marker enzyme, indicating that release of albumin had occurred during the homogenization procedure (cf., Scheele *et al.*, 1978). The calculated recovery of albumin in the GC fraction was higher than that determined for the marker enzyme for the GC. A possible explanation for this difference might be that albumin occurs throughout the GC (Yokota and Fahimi, 1981), whereas the marker enzyme is limited to a subcompartment (Roth and Berger, 1982). For the other proteins, the calculations just described yielded values that indicated that their recoveries in the ER fraction were higher (30–50%) than that estimated for albumin. Since a small but significant portion of the cells (<10%) became detached during the chase period (these cells were discarded at the end of the experiment) the estimated recoveries and transport rates, in particular those of the slowly transported proteins, were overestimated. Because the amount of radioactively-labelled protein in the GC fraction was small for the more slowly transported proteins the recoveries of these proteins in the GC fraction could be estimated only within a large range (30–100%).

Intracellular turn-over rates

To estimate the turn-over rates of the different proteins in the ER and the GC, the experimentally determined time courses (with corrected values) were simulated with a simple three-box compartment model: ER→GC→Medium, where the transfers were first order reactions. By adjustments of the two rate constants (see Materials and methods) the calculated time

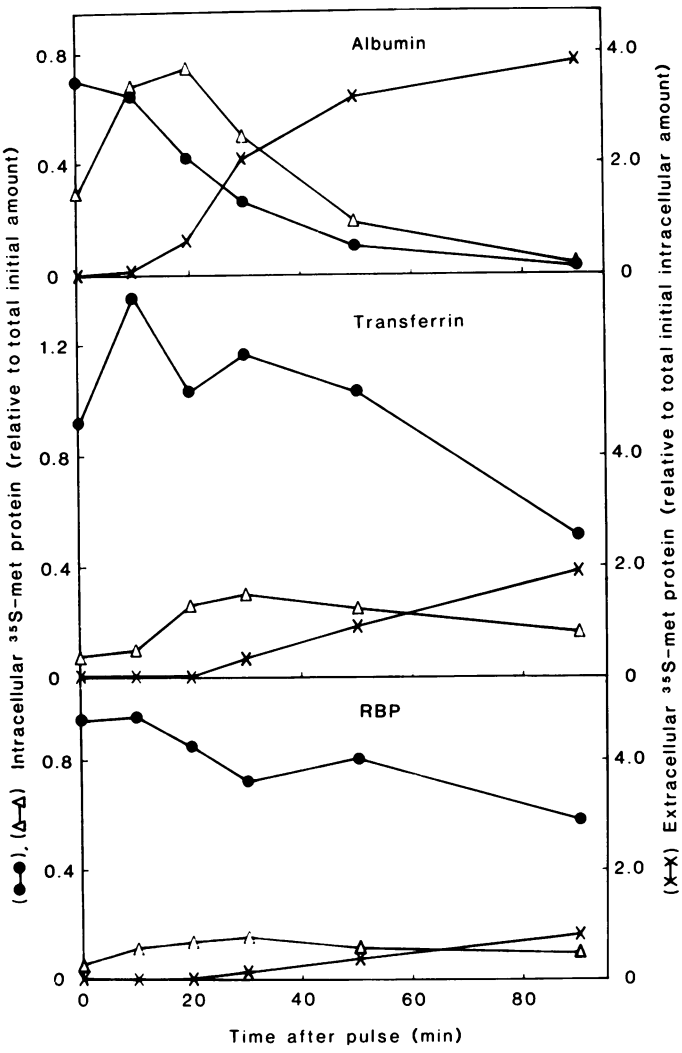

Fig. 4. Time-courses for intracellular transport by newly synthesized albumin, transferrin and RBP. The relative amounts of radioactivity in the protein bands shown in Figure 3 were determined by densitometric scanning. (●) ER fraction, (△) GC fraction and (X) medium. Because of the low recovery of the proteins in the membrane fractions the amounts of protein detected in these samples have been plotted on a scale smaller than that used for the medium.

courses were made to fit the experimental data (see Figure 5). From the rate constants (k) thus obtained the more useful half-lives ($t_{½}$) were derived from the relation $t_{½} = 0.69/k$. For pulse-labelled albumin, transferrin, prealbumin and RBP the half-lives in the ER thus estimated were 14, 73, 80, and 137 min, respectively (means of two determinations for each protein). The estimated half-life for albumin in the GC was 10–15 min. Because of the uncertainty in the assessment of the recoveries of the other proteins in the GC fraction, the half-lives of these proteins in the GC could be estimated only within a relatively large time range (5–20 min).

The experimentally determined time courses could be simulated reasonably well with the simple three-box compartment model. However, a slight but consistent difference could be seen, in particular for albumin; the experimentally determined time-course for the transport through the GC was sharper than the calculated one and the simulated time course for the release into the medium did not display a time lag as did the experimental one (see Figure 5). These results indicate that the transfer reactions in the cell occur in a more synchronous fashion than is described with the simple model used.

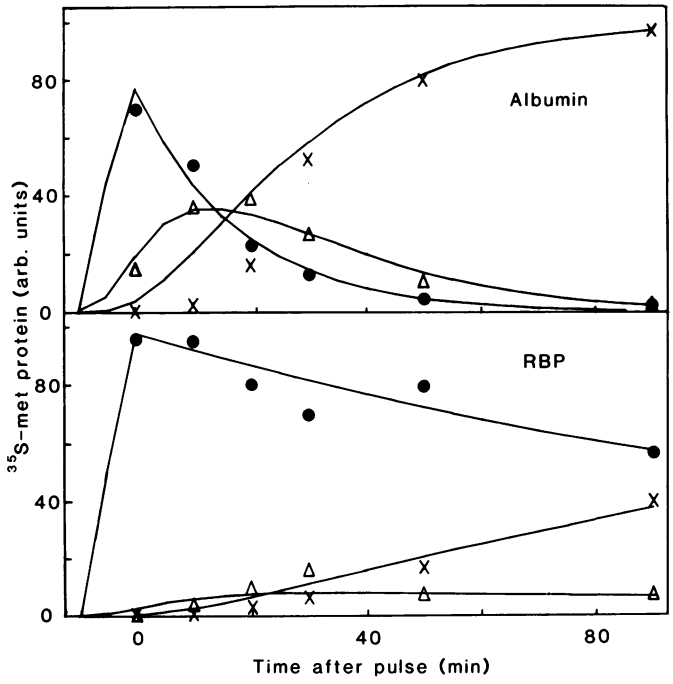

Fig. 5. Simulation of time-courses for intracellular transport of pulse-labelled albumin and RBP. The data for the ER and GC fractions (Figure 4) were corrected for cross-contamination and differential recoveries as described in the text. (●)ER, (△) GC and (X) medium. These data were then compared with computer generated time-courses describing two successive first-order reactions: ER→GC→Medium. The pulse phase was simulated with a constant flow of protein entering the ER (see Materials and methods). Through adjustments of the two rate constants of the transport reactions the simulated time courses (solid lines) were made to fit the experimental data. The $t_{½}$ values for the first and second transport reactions simulating the transport of albumin are 12 and 13 min and 110 and 15 min for RBP.

Radioimmunoassay of albumin and transferrin

The finding that the turn-over rates of the secretory proteins in the GC were high indicated that their specific radioactivities in this compartment at longer periods of chase were similar to those of the kinetically preceding ER compartment. Hence, the levels of a radioactively-labelled secretory protein in the two fractions (as shown in Figure 4) should reflect the relative amounts of the protein in the GC and the ER fractions. To test this notion, the amounts of albumin and transferrin were determined by radioimmunoassays in the fractions of sucrose density gradients in which the ER and GC membranes had been separated (Figure 6). Albumin was consistently recovered in three distinct regions: in the densest fractions, distributed roughly as the enzyme marker for the ER; in the intermediate fractions, with the highest concentration at the density where most of the activity of the GC marker was found, and at the top of the gradient, possibly in association with the low-density vesicles that mediate the transport of secretory proteins to the cell surface (Ikehara *et al.*, 1976). Consistent with the kinetic data, the amounts of albumin in the upper and lower parts of the gradient, corresponding to the GC and ER fractions in the two-step gradient, were similar, whereas most of the transferrin occurred in the part of the gradient that contained the ER membranes. In agreement with a previous study of rat liver, the amounts of albumin and transferrin in the ER were found to be similar (Morgan and Peters, 1971).

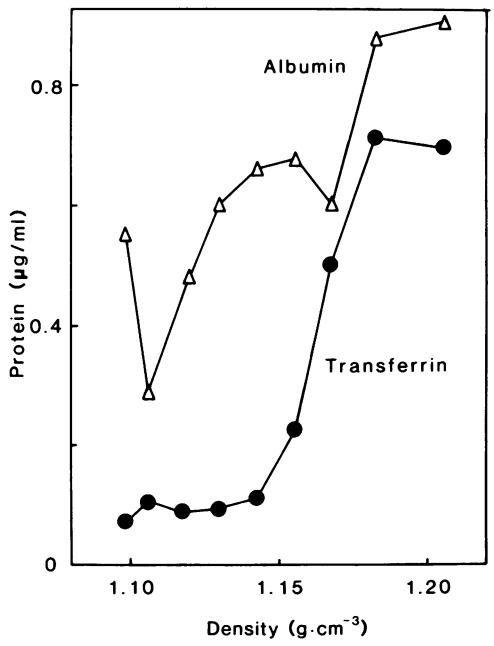

Fig. 6. Distribution of albumin and transferrin in cell extract fractionated with equilbrium density centrifugation in sucrose gradient as described in Figure 1. Quantitation was done by radioimmunoassays.

Discussion

The major finding of this study is that the four secretory proteins investigated are transported at different rates from the ER to the GC, implying that these proteins turn over at different rates in the ER. During the preparation of this manuscript a study appeared in which it was reported that five secretory proteins synthesized in a hepatoma cell line, including two of the proteins analyzed in the present study (albumin and transferrin), are also transported at different rates from the ER to the GC (Lodish *et al.*, 1983). Studies of the intracellular transport of membrane glycoproteins destined for the surface of the cell have shown that transport of this kind of proteins from the ER to the GC may occur at different rates (Strous and Lodish, 1980; Fitting and Kabat, 1982). On the basis of these observations it was proposed that the transport of newly synthesized proteins from the ER to the GC would be mediated by membrane-bound receptors that are transported to the GC: proteins that bind with high affinities to these putative receptors would be quickly and efficiently removed from the ER, whereas proteins with low affinities would be transported to the GC at low rates resulting in low turn-over rates for these proteins (Fitting and Kabat, 1982).

An alternative explanation for the fact that newly synthesized proteins destined for the outside of the cell may be transported at different rates from the ER to the GC would be that transport is unselective and is effected by bulk-phase transfers but that different proteins are retained in the ER to different degrees because of binding to the luminal face of the ER. However, the electron microscopical finding that albumin is concentrated in regions of the ER from which transport to the GC appears to occur (Yokota and Fahimi, 1981) argues against this hypothesis.

Further evidence for the notion that the transport of secretory proteins from the ER to the GC is a selective process, is provided by the observation that small changes in the primary structure of a protein, e.g., the substitution of a single amino acid residue (Wu *et al.*, 1983), may prevent secretion. Conceivably, even minor conformational changes may suffice to affect the rate of transport. Studies of the biosynthesis of RBP have revealed what may be an example of such a situation. In retinol-deficient rats, RBP accumulates in the ER of the hepatocytes (Muto *et al.*, 1972; Rask *et al.*, 1983), but upon administration of retinol to the animals (Peterson *et al.*, 1973; Smith *et al.*, 1973) or upon addition of retinol to the isolated cells (Ronne *et al.*, 1983) the accumulated RBP molecules are rapidly released. One possible explanation for these observations is that the added retinol binds to (retinol-free) RBP in the ER and that the binding induces a conformational change in the RBP molecule that leads to exposure of a recognition site for a transport receptor (Ronne *et al.*, 1983). It is possible that the culture conditions we used rendered the hepatocytes retinol-deficient and that the very low transport rate that we found for RBP represents the rate of unspecific fluid-phase transport from the ER to the medium.

Our results show that the turn-over rates in the GC of the secretory proteins studied are high. Kinetic studies of the intracellular transport of other (glycosylated) secretory proteins, where the relative amount of a protein resistant to endoglycosidase H has been used as a measure of its amount in the GC, have yielded the same result (Strous and Lodish, 1980; Lodish *et al.*, 1983). These findings imply that a secretory protein, once it has been taken up by the transport machinery in the ER, is swiftly taken through the GC and brought to the exterior of the cell. Whether this efficient transfer is mediated by receptors remains to be demonstrated.

Materials and methods

Isolation and growth of cells

Hepatocytes were isolated by collagenase perfusion (Rubin *et al.*, 1977) from male Sprague-Dawley rats weighing 150–250 g, which had been starved overnight. The viability of the cells was better than 85% as judged from the exclusion of trypan blue. 3 ml of a cell suspension containing about 5 x 10^6 cells in DMEM lacking $NaHCO_3$ with 20 mM Hepes, pH 7.4, were added to plastic Petri dishes (diameter 6 cm) which had been coated with 30 μg of human fibronectin (kindly provided by Mr. A. Ljungkvist, KABI AB, Stockholm, Sweden). By 3–4 h of culturing ~80% of the cells had attached, most of them having formed contacts with neighbouring cells.

Subcellular fractionation

All manipulations were done on ice or at +4°C. The dishes were rinsed twice with 2 ml of phosphate buffered saline (PBS) and twice with 2 ml of 50 mM sucrose. All liquid was carefully pipetted off and 400 μl of 50 mM sucrose with 1 mM phenylmethylsulphonyl fluoride (PMSF) was added to each dish. The cells from two dishes (~8 x 10^6 cells) were scraped off with a rubber stopper and were broken by 15 strokes in a Dounce glass homogenizer with a tight-fitting pestle. Sucrose (65%, w/w) was added to the homogenate to give a final concentration of 8% and after another five strokes the homogenate was centrifuged for 30 min at 1000 g_{av}. The supernatant was pipetted off and sucrose (65%) was added to give a final concentration of 50%. The sample was transferred to a centrifuge tube fitting either a Beckman SW 27 or a SW 50.1 rotor. In the former case a continuous (40–20%) sucrose gradient was layered over the sample and in the latter case 2.0 ml of 35% and 0.3 ml of an 8% sucrose solution were added. The continuous gradient was centrifuged at 85 000 g_{av} and the two-step gradient at 114 000 g_{av}, both for 15 h. Following centrifugation nine 1.5 ml fractions were collected from the SW 27 rotor tube. Two fractions were collected from the SW 50.1 rotor tube, the denser fraction containing 3.0 ml and the other fraction containing the remainder of the gradient. All fractions were diluted to 4.5 ml with ice-cold PBS and centrifuged at 190 000 g_{av} for 1 h. The pellets were solubilized in 150 μl of 20 mM Tris-HCl, pH 8.0, 20 g/l Triton, 2 mM PMSF, 1% Trasylol (Bayer, FRG) and sonicated for 2 x 5 s in an ice cooled sonicator bath. The samples were frozen in liquid nitrogen and stored at −70°C.

Pulse-chase experiments

Each Petri dish was rinsed twice with 2.5 ml PBS and 1.0 ml of DMEM without $NaHCO_3$ and methionine containing 20 mM Hepes-NaOH, pH 7.4, was added. The plates were rocked slowly and after 20–25 min of incubation the medium was replaced with 1.0 ml of the same medium containing 50–100 μCi [^{35}S]Met (New England Nuclear, 1 mCi/mmol). After another 5 or 10 min of incubation the labelling medium was removed and 1.5 ml of medium containing 4 mM methionine was added. The experiment was carried out at 37°C and all solutions were equilibrated to this temperature prior to the experiment. At the end of the chase the cell media were withdrawn and put onto ice together with the cell dishes. The media were centrifuged for 10 min at 1000 *g* and the supernatants were stored at −70°C. The cells were fractionated as described above.

Immunoprecipitation

Antiserum against albumin (isolated as described below) was prepared in rabbit. Antibody specific to this protein was isolated by affinity chromatography with albumin coupled to Sepharose 4B. Rabbit antiserum against rat transferrin was purchased from Capell Laboratories and rabbit antisera against rat prealbumin and RBP were kindly provided by Dr. L. Rask.

Prior to immunoprecipitation with specific antisera, 5 μl of normal rabbit serum were added to the samples and after 1 h of incubation at +4°C the samples were treated with protein A bearing *Staphylococcus aureus* particles. Specific antisera (1–10 μl) were added in excess to portions of the supernatants (5–75 μl) and after 4–6 h of incubation at +4°C the immune complexes were recovered with *S. aureus* particles.

Gel electrophoresis

Slab gels with 10–15% polyacrylamide gel gradients and samples were prepared as described (Dobberstein *et al.*, 1979). After electrophoresis the gels were treated with Enhance (New England Nuclear) and dried. The gels were exposed to preflashed Kodak XAR-5 or Fuji XR films at −70°C. The relative amounts of radioactivity in the protein bands were determined by densitometric scanning. The deviation from linearity within the optical density range used was <10%.

Kinetic model

The transport of pulse-labelled secretory proteins from the ER to the medium (ME) was simulated with a computer using the following equations:

$$d\mathrm{ER}/dt = \mathrm{IN} - k_{\mathrm{ER}} \times \mathrm{ER}$$

$$d\mathrm{GC}/dt = k_{\mathrm{ER}} \times \mathrm{ER} - k_{\mathrm{GC}} \times \mathrm{GC}$$

$$d\mathrm{ME}/dt = k_{\mathrm{GC}} \times \mathrm{GC}$$

where ER, GC and ME represent the amount of secretory protein in the respective compartments, IN the rate of protein entering the ER during the pulse (IN is constant for $-10 < t \leq 0$ min and 0 for $t > 0$ min) and k_{ER} and k_{GC} the rate constants for the transport from the ER and the GC compartments.

Radioimmunoassays

Albumin and transferrin were isolated from rat serum by ion exchange chromatography (Ohanian *et al.*, 1969). Albumin was further purified by adsorption chromatography on Blue-Sepharose (Travis *et al.*, 1976) and exclusion chromatography on Sephadex G-200. Transferrin was further purified by ion exchange chromatography (Ohanian *et al.*, 1969), elution through a wheat germ agglutinin gel (Vretblad and Hjort, 1977) and exclusion chromatography on Sephadex G-200. Approximately 2 μg each of albumin and transferrin were labelled with [^{125}I]NaI using the chloramine T method (Hunter and Greenwood, 1962). Standard solutions were prepared from the purified antigens and their concentrations were determined from the absorbance at 280 nm (Peters, 1962; Gordon and Louis, 1963). Quantitation of the antigens in subcellular fractions by radioimmunoassay was done essentially as described (Rask *et al.*, 1980).

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