The effects of low temperatures on intracellular transport of newly synthesized albumin and haptoglobin in rat hepatocytes

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Isolated rat hepatocytes were pulse-labelled with [³⁵S]methionine at 37 °C and subsequently incubated (chased) for different periods of time at different temperatures (37–16 °C). The time courses for the secretion of [³⁵S]methionine-labelled albumin and haptoglobin were determined by quantitative immunoprecipitation of the detergent-solubilized cells and of the chase media. Both proteins appeared in the chase medium only after a lag period, the length of which increased markedly with decreasing chase temperature: from about 10 and 20 min at 37 °C to about 60 and 120 min at 20 °C for albumin and haptoglobin respectively. The rates at which the proteins were externalized after the lag period were also strongly affected by temperature, the half-time for secretion being 20 min at 37 °C and 200 min at 20 °C for albumin; at 16 °C no secretion could be detected after incubation for 270 min. Analysis by subcellular fractionation showed that part of the lag occurred in the endoplasmic reticulum and that the rate of transfer to the Golgi complex was very temperature-dependent. The maximum amount of the two pulse-labelled proteins in Golgi fractions prepared from cells after different times of chase decreased with decreasing incubation temperatures, indicating that the transport from the Golgi complex to the cell surface was less affected by low temperatures than was the transport from the endoplasmic reticulum to the Golgi complex.

INTRODUCTION

The general pathway for the transport of secretory and plasma-membrane proteins from their site of synthesis to the exterior of the cell is well established: during their synthesis on ribosomes attached to the endoplasmic reticulum (ER), these proteins are transferred into the lumen of the ER and are then transported to the cell surface by vesicles via the Golgi complex (GC) [for reviews see Palade (1975) and Sabatini *et al.* (1982)]. Little is known, however, about the mechanisms underlying this transport. The fact that the integrity of the participating organelles is maintained despite the flow of the transported proteins (Moreé *et al.*, 1979) implies that the transport is specific, probably involving membrane-bound receptors (Fitting & Kabat, 1982; Lodish *et al.*, 1983; Fries *et al.*, 1984). In keeping with this notion, coated vesicles, which have been shown to be vehicles for receptor-mediated transport of proteins into the cell (Pearse & Bretscher, 1981), appear to mediate the transport of newly synthesized proteins to the cell surface (Rothman & Fine, 1980; Kinnon & Owen, 1983; Orci *et al.*, 1984; Griffiths *et al.*, 1985).

Biochemical dissection of the transport machinery requires procedures for the isolation of the different structures of which it is composed; such procedures are still largely unavailable. Inhibition of protein transport at specific sites may be useful in this respect, since the resulting accumulation of the secretory proteins can be used for the identification and isolation of the structures in which the proteins are arrested (Tartakoff & Vassalli, 1979; Quinn *et al.*, 1983). Temperature can be used for inhibition, since different transport steps may have different temperature-dependencies. Thus the transport from the GC to the cell surface of newly synthesized

membrane glycoproteins has been shown to be blocked at 20 °C, and their transport between the ER and the GC at 15 °C (Matlin & Simons, 1983; Saraste & Kuismanen, 1984).

In the present study we have characterized the effects of low temperature on the intracellular transport of two secretory proteins. Our data show that the transport of these proteins from the GC to the cell surface is not blocked at 20 °C and that their transport from the ER to the GC has virtually ceased below 18 °C.

MATERIALS AND METHODS

Materials

Culture media and newborn-calf serum were purchased from Labassco, Stockholm, Sweden. Human fibronectin was a gift from Å. Lundqvist, KabiVitrum, Stockholm, Sweden. Rabbit antiserum (IgG fraction) against human albumin was purchased from Pharmacia AB, Uppsala, Sweden. Affinity-purified rabbit antibodies against human haptoglobin and *Staphylococcus aureus* bacteria fixed with formaldehyde were gifts from T.-B. Karlsson and F.-P. Nilsson, respectively, of Pharmacia AB.

Cell culture

Hepatocytes were isolated by collagenase perfusion and differential centrifugation (Rubin *et al.*, 1977) from Sprague–Dawley rats weighing 175–250 g, which had been starved overnight. The viability of the cells was 80–90% as judged from the exclusion of Trypan Blue. The cells were suspended in Dulbecco's modified Eagle medium (Morton, 1970) lacking NaHCO₃, supplemented with 25 mM-glucose and 25 mM-Hepes/NaOH (pH 7.4). The suspended cells (4.0 × 10⁶ cells in 5 ml) were added

Abbreviations used: ER, endoplasmic reticulum; GC, Golgi complex.

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to 60 mm-diam. plastic Petri dishes precoated with 60 μ g of fibronectin. Newborn-calf serum, was added 30 min after plating to give a final concentration of 9% (v/v). Alternatively, 1.3×10^6 cells in 3 ml of the medium described above, supplemented with 9% newborn-calf serum, 100 units of penicillin/ml, 0.1 mg of streptomycin/ml, 10 munits of insulin/ml and 0.3 μ M-dexamethasone, were added to 35 mm-diam. dishes precoated with 30 μ g of fibronectin; this medium was changed after 3–4 h of culture.

Determination of secretion rates

Under appropriate conditions, hepatocytes in culture will continue to secrete serum proteins for several days (Auberger & LeCam, 1982). For convenience we used the cells for the determination of secretion rates only on the day after the relatively time-consuming isolation. The cells (in 35 mm dishes) were first rinsed twice with a solution containing 137 mM-NaCl, 5 mM-KCl, 1.2 mM- CaCl_2 , 0.7 mM- MgSO_4 and 10 mM-Hepes/NaOH (pH 7.4) (buffer 3; Rubin *et al.*, 1977). Then 750 μ l of Eagle's minimum essential medium (Morton, 1970) lacking NaHCO_3 and methionine, supplemented with 25 mM-Hepes/NaOH (pH 7.4), was added. After 20 or 30 min of incubation on a rocking device, 100 μ l of the same medium containing 5–10 μ Ci of [35 S]methionine (> 800 Ci/mmol; Amersham International, Amersham, Bucks., U.K.) was added. When the cells had been incubated for 10 min, the [35 S]methionine-containing medium was removed, and then 1.0 ml of the medium used for the labelling, supplemented with 2 mM-methionine, 1 mg of ovalbumin/ml and 20 μ M-cycloheximide, was added. The cells were then incubated at the appropriate temperatures for different periods of time. In experiments in which the cells were labelled for 60 min, the labelling medium contained 10 μ M-methionine.

For incubations at temperatures lower than 37 $^{\circ}\text{C}$, the dishes were put on to an aluminium slab (32 cm $\times$ 40 cm $\times$ 2 cm) (kindly provided by N. Fries) mounted on a rocking device. In each 32 cm end there was a U-shaped loop bored through the slab. Temperature gradients were obtained by pumping water from a thermostatically controlled bath (30–40 $^{\circ}\text{C}$) through one of the loops and tap water (10–12 $^{\circ}\text{C}$) through the other. Individual temperatures were obtained by pumping water

from a thermostat bath through both loops. If the temperature of the aluminium slab was 12 $^{\circ}\text{C}$, the temperature of the medium in the culture dish on the slab decreased from 37 to 13 $^{\circ}\text{C}$ in 5 min. The temperatures of the media in the dishes were checked during the experiments and were found to vary by less than 1.0 $^{\circ}\text{C}$.

At the end of the incubation the dishes were put on ice, the media removed, and the cells rinsed twice with ice-cold buffer 3. Finally 300 μ l of 20 mM-Tris/HCl (pH 8.0) containing 0.15 M-NaCl, 10 mg of Triton X-100/ml, 5 mM-EDTA, 10 μ g of aprotinin/ml and 1 mM-phenylmethanesulphonyl fluoride was added, and the cells were scraped off the dishes. Insolubilized material was removed by centrifugation for 5 min at 15000 *g*. The chase media were centrifuged for 10 min at 1700 *g*. The supernatants were frozen in liquid N_2 and stored at -70°C .

Determination of intracellular transport rates

We have previously described a procedure for preparing ER and GC membranes from hepatocytes after short-term culture (Fries *et al.*, 1984). For the determination of intracellular transport rates, this procedure was used with slight modifications. Cells cultured for 2–3 h in 60 mm dishes were first labelled with [35 S]methionine and chased as described above for cells in 35 mm dishes, except that the pulse-labelling was done in 1.0 ml of medium containing 20–40 μ Ci of [35 S]methionine and the cells were chased in 1.5 ml of medium. The dishes were then put on ice and the cells were rinsed twice with 2 ml of 250 mM-sucrose and once with 2 ml of 50 mM-sucrose. (This and subsequent manipulations were done at 0–6 $^{\circ}\text{C}$.) The remaining liquid was carefully pipetted off, and 400 μ l of 50 mM-sucrose was added to each plate. The cells from two dishes were scraped off with a rubber stopper and broken by 15 up-and-down strokes in a 7 ml Dounce glass homogenizer with a tight-fitting pestle (Kontes Glass Co., Vineland, NJ, U.S.A.). Sucrose (2.5 M) was then added to the homogenate to give a final concentration of 0.25 M, and after another five strokes of the pestle it was centrifuged for 10 min at 1700 *g*; by this centrifugation 80% of the succinate:cytochrome *c* reductase activity (King, 1967), a marker for mitochondria, was pelleted. The NADPH:cytochrome *c* reductase activity (a marker for ER membranes) of the supernatant was about 75%

Fig. 1. Analysis of secretion of pulse-labelled albumin at 37 $^{\circ}\text{C}$

Rat hepatocytes cultured for 20 h in 35 mm dishes were labelled with [35 S]methionine for 10 min and were then chased for the lengths of time indicated. The detergent-solubilized cells and the chase media were incubated with antibodies against albumin and the immunocomplexes were analysed by gel electrophoresis; the relevant section of an autoradiogram of the dried gel is shown.

of that of the homogenate, and the galactosyltransferase activity (a marker for GC membranes) was about 110%; this high value indicates that the homogenate contained inhibiting activity. Sucrose (2.5 M) was added to 500 μ l of the supernatant to give a final concentration of 1.5 M. This sample (1.6 ml) was then transferred to a centrifuge tube, fitting a Beckman SW 50.1 rotor, and 2.5 ml of a 1.18 M- and 0.5 ml of a 0.25 M-sucrose solution were added. After centrifugation for 15–16 h at 114 000 g (r_{av} 8.4 cm), two fractions were collected, the denser fraction containing 2.5 ml and the lighter fraction containing the remainder of the gradient. Phosphate-buffered saline (140 mM-NaCl, 2.7 mM-KCl, 1.5 mM-KH₂PO₄, 8.1 mM-Na₂HPO₄, pH 7.4) was added to both samples to give a final volume of 4.5 ml. The samples were then centrifuged at 149 000 g (r_{av} 8.4 cm) for 1 h. The pellets were solubilized in 200 μ l of 20 mM-Tris/HCl (pH 8.0) containing 10 g of Triton X-100/l, 5 mM-EDTA and 10 μ g of aprotinin/ml and sonicated for 10 s (Bransonic 220). The samples were frozen in liquid N₂ and stored at -70°C . The denser fraction contained 75% of the total activity of NADPH:cytochrome *c* reductase in the gradient, and the lighter fraction 82% of the activity of galactosyltransferase, essentially as reported in a previous study (Fries *et al.*, 1984). In a control experiment we established that the recovery of these enzymes in the two fractions was not affected by previous incubation of the cells at different temperatures ($< 12\%$ variation).

Immunoprecipitation

The samples (150–250 μ l final volume) were treated with antibodies (< 10 μ g) for 3–4 h at 6°C , followed by addition of 50 μ l of a suspension (10%, v/v) of fixed *Staphylococcus aureus* bacteria. After 45 min incubation, the bacteria (with bound immunocomplexes) were washed once with 300 μ l of a solution containing 10 mM-Tris/HCl (pH 7.5), 0.4 M-NaCl, 10 mg of Triton X-100/ml and 5 mM-EDTA, and once with 300 μ l of 10 mM-Tris/HCl (pH 7.4). They were then suspended in 40 μ l of 83 mM-Tris/HCl (pH 8.8) containing 300 g of sucrose/l, 30 mg of SDS/ml, 0.1 mg of Bromophenol Blue/ml, 4 mM-EDTA and 8 mM-dithiothreitol, and heated for 3 min at 95°C . After cooling, 5 μ l of 0.5 M-iodoacetamide was added to each sample and the bacteria were pelleted by centrifugation. Before analysis with haptoglobin antibodies, the samples were incubated with 10 μ g of rabbit IgG and 50 μ l of the *S. aureus* bacteria suspension for 1 h, which were then removed by centrifugation.

Gel electrophoresis

Slab gels with 10–15%-polyacrylamide gel gradients were prepared as described by Blobel & Dobberstein (1975), and the solubilized immunoprecipitates were applied. After electrophoresis, the gels were treated with 2,5-diphenyloxazole (200 g/l) in acetic acid (Skinner & Griswold, 1983), soaked in water, dried, and exposed to preflashed films (Fuji XR) at -70°C (Laskey & Mills, 1975). The relative amounts of radioactivity in the bands were determined by densitometric scanning of the films. Within the range used the absorbance of the films was proportional ($\pm 10\%$) to the radioactivity. Repeated immunoprecipitation of the samples yielded less than 10% of the radioactive protein obtained in the first precipitation.

Fig. 2. Effect of temperature on the time course for secretion of pulse-labelled albumin

Hepatocytes cultured for 20–22 h in 35 mm dishes were labelled for 10 min with [³⁵S]methionine at 37°C and then incubated at the temperatures indicated. After different times, two dishes were removed, and the amount of [³⁵S]methionine-labelled albumin in the combined cells (●) and the media (■) was analysed as described in Fig. 1. The relative amounts of radioactive albumin in the bands were determined by densitometric scanning of the autoradiograms. For clarity, the amount of albumin in the medium is shown only for 20 and 16°C . Each point is the average of the data from two experiments.

RESULTS

Effect of temperature on secretion

To obtain an overall picture of the effect of temperature on the intracellular transport of albumin and haptoglobin, we first determined the time courses for the secretion of these proteins at different temperatures. Fig. 1 shows an autoradiograph of a gel from one such experiment in which the pulse-labelled cells had been chased at 37°C and the detergent-solubilized cells and the chase media were analysed by quantitative immunoprecipitation with antibodies against albumin. These data show, in agreement with earlier studies (Oda *et al.*, 1983; Feldhoff *et al.*, 1977), that, after a lag period of 10–15 min, newly synthesized albumin is rapidly and efficiently secreted from isolated hepatocytes at 37°C . Albumin, like most other secretory proteins, is modified during intracellular transport; shortly before its externalization an *N*-terminal segment of six amino acid residues is removed (Russells & Geller, 1975; Ikehara *et al.*, 1976). The resulting increase in electrophoretic mobility is not apparent in the gel shown in Fig. 1.

Fig. 2 is a quantitative representation of part of the data from several experiments analogous to that shown in Fig. 1. In these experiments the cells were chased at different temperatures and the amounts of intra- and extra-cellular albumin were determined. The graphs obtained show that at all temperatures there was an initial phase during which there was little decrease in the amount of pulse-labelled albumin in the cells and that the length of this period increased with decreasing chase temperature. The data for the medium demonstrated that this period corresponded to a lag period in the appearance of albumin in the medium, which was about 10 min at 37°C (see Fig. 1) and 60 min at 20°C . The rate at which albumin was externalized after the lag period was also strongly temperature-dependent; the half-time ($t_{1/2}$) for externalization was about 20 min at 37°C and about 200 min at 20°C . (The amount of albumin secreted into

Fig. 3. Analysis of secretion of pulse-labelled haptoglobin at 37 and 20 °C

Hepatocytes were pulse-labelled with [35 S]methionine for 10 min at 37 °C and then chased at 37 or 20 °C. Cells and chase media were analysed with haptoglobin antibodies as described for albumin in Fig. 1. The numbers to the left indicate the positions of the different forms of haptoglobin. A precursor (band 1) is cleaved in the ER, and the so-called β -chain (band 2) and α -chain are formed. (The α -chain does not contain methionine, and was consequently not labelled.) During passage through the GC, the carbohydrates of the β -chain are modified, which leads to a decrease in its electrophoretic mobility (band 3). The arrows to the right indicate the positions of 14 C-labelled molecular-mass standards (purchased from New England Nuclear): albumin (69 kDa), ovalbumin (46 kDa) and carbonic anhydrase (30 kDa). The apparent molecular masses of the different haptoglobin forms are 46 (band 1), 34 (band 2), and 37 kDa (band 3).

the medium increased for at least 360 min at 20 °C; results not shown.) At 20 °C the intracellular amount of albumin decreased more rapidly than the amount in the medium increased, indicating that some albumin was degraded intracellularly. At 16 °C no secretion could be detected even after 270 min incubation. The inhibition at this temperature was at least partially reversible; when the cells were shifted to 37 °C after 300 min incubation at 16 °C, 60% of the pulse-labelled albumin was found in the medium after a further 60 min (results not shown).

The incorporation of [35 S]methionine from the medium into the secretory proteins was also found to be strongly temperature-dependent, being about 20 times less at 20 °C than at 37 °C (E. Fries & I. Lindström, unpublished work). All labelling was therefore done at 37 °C, and the cells were subsequently incubated at different temperatures. In a control experiment in which the cells were both labelled and chased at 20 °C, the time course obtained was almost identical with that obtained with the standard procedure.

The experimental data for haptoglobin from the pulse-chase experiments (Fig. 3) warrant some comment regarding the biosynthesis of this protein. Hanley *et al.*

(1983) have shown that haptoglobin is synthesized as a glycosylated precursor of 45 kDa (band 1 on Fig. 3), and while still in the ER this polypeptide is cleaved into one glycosylated and one unglycosylated chain, the β - (35 kDa, band 2) and the α -chain (9.5 kDa) respectively. (The α -chain does not contain methionine residues and was accordingly not labelled in our experiments.) The chains are linked intracellularly via disulphide bonds, forming tetrameric $\alpha_2\beta_2$ complexes. During intracellular transport the carbohydrates of the β -chains are processed from high-mannose to complex-type structures. The processing can be detected by gel electrophoresis as an increase in the apparent molecular mass from 35 to 38 kDa (band 3). (We have obtained slightly lower molecular masses, 34 and 37 kDa respectively.) The lower mobility of band 3 is apparently due to the presence of

Fig. 4. Effect of temperature on the time course of intracellular transport of pulse-labelled albumin

Hepatocytes cultured for 2–3 h in 60 mm dishes were labelled with [35 S]methionine for 10 min at 37 °C and then incubated at the temperatures indicated. After different periods of incubation, cells from two plates were combined, homogenized and centrifuged to yield a post-mitochondrial supernatant. An ER and a GC fraction were prepared by centrifugation in a two-step sucrose density gradient. The amount of [35 S]albumin in the ER (○) and the GC (▲) fractions and in the corresponding chase medium (□) was determined as described in Figs. 1 and 2. The results were obtained from two independent experiments. The recovery of [35 S]albumin in the gradient fractions was about 50%. Therefore the data for the medium are plotted on a compressed scale.

Fig. 5. Analysis of intracellular transport of pulse-labelled haptoglobin at 37 °C

The results were obtained as described for albumin in Fig. 4. A section of an autoradiogram of a dried gel is shown. The numbers to the left indicate the different forms of haptoglobin described in Fig. 3.

sialic acid residues on the polypeptide, because treatment with sialidase will increase its mobility to that of the 35 kDa polypeptide. The enzyme adding sialic acid residues to proteins resides in (the *trans* cisternae of) the GC (Roth *et al.*, 1985). This modification can therefore be used to monitor the passage of pulse-labelled haptoglobin through the GC.

The data in Fig. 3 clearly show that pulse-labelled haptoglobin, like albumin, appeared in the medium only after a lag period. The lag period for haptoglobin was longer (about 20 min at 37 °C) than that found for albumin, and the rate of secretion after the lag was lower ($t_{1/2} \sim 40$ min at 37 °C). As judged by the appearance of the 38 kDa form of haptoglobin (band 3), it took about 20 min for newly synthesized haptoglobin to reach the GC at 37 °C. At 20 °C only a minor fraction (8%) of the pulse-labelled haptoglobin appeared in the medium after 270 min of chase. At this temperature it took 60–90 min for haptoglobin to reach the GC and 120–150 min to reach the medium. These results showed that, with decreasing temperature, transport both from the ER to the GC (and possibly also within the GC) and from the GC to the medium was slowed down. The maximum cellular amounts of 38 kDa haptoglobin during the chase were lower at 20 than at 37 °C (2 and 10% of the total initial amount respectively).

Effect of temperature on intracellular transport

To obtain information on the effect of low temperature on different steps in the intracellular transport, we prepared two subcellular fractions from cells used in pulse-chase experiments: one fraction containing most of the ER membranes, and the other fraction most of the GC membranes. The amount of [35 S] methionine-labelled albumin and haptoglobin in these fractions, as well as in the chase medium, was then determined by quantitative immunoprecipitation. Fig. 4 shows the time courses for the intracellular transport of albumin at three different temperatures. In agreement with a previous study (Fries *et al.*, 1984), pulse-labelled albumin was found to be rapidly transported from the ER to the GC at 37 °C ($t_{1/2} \sim 15$ min) and to attain its maximum in the GC fraction by about 20 min of chase (Fig. 4, top panel). Simulation of this time course by a simple three-compartment model indicates that the turnover rates of albumin in the ER and GC are approximately equal (Fries *et al.*, 1984). At a chase temperature of 26 °C (Fig. 4), transport of albumin from the ER to the GC was

significantly slower ($t_{1/2} \sim 40$ min) than at 37 °C, and the maximum amount of pulse-labelled albumin in the GC fraction was reached by 20–30 min of chase. Beyond this time the amount of albumin in both the ER and the GC declined in parallel.

When the cells were chased at 20 °C the transport of albumin from the ER to the GC seemed to occur with a 20–30 min delay, and the subsequent transport to the GC was slow ($t_{1/2} \sim 60$ min). The maximum amount of radioactively labelled albumin, which was reached by 30–70 min of chase, was significantly lower than that observed at 26 and 37 °C. Simulation of this time course with a three-compartment model yielded a half-life of albumin in the ER of about 60 min and in the GC of about 20 min (results not shown). These results indicate that low temperature has less effect on the transport of albumin from the GC to the medium than on the transport from the ER to the GC. In cells chased at 18 °C for up to 90 min (the longest chase time used), the amount of [35 S]methionine-labelled albumin in the ER and the GC fractions was nearly constant, indicating that transport was blocked at this temperature. As discussed above, it appears that albumin was degraded intracellularly during the chase. This implies that the actual transport rates may have been lower than the rates at which the proteins were found to disappear from the subcellular fractions.

Fig. 5 shows the autoradiogram of a gel used to analyse the intracellular transport of haptoglobin at 37 °C. This shows, in agreement with the data of Hanley *et al.* (1983), that the precursor of haptoglobin (band 1) occurred almost exclusively in the ER fraction. Conversely, haptoglobin with sialic acid residues (band 3) was found only in the GC fraction, consistent with the localization of sialyltransferase in the GC. A quantitative analysis of these results (Fig. 6, top panel) showed that the amount of radiactively labelled haptoglobin in the ER fraction decreased only after about 20 min of chase ($t_{1/2} \sim 30$ min). The amount of labelled haptoglobin in the GC appeared to increase after about 10 min of chase, but was relatively low during the whole chase, indicating that the turnover rate of haptoglobin in this organelle was high.

At 26 °C the amount of haptoglobin in the ER fraction decreased only after about 40 min of chase ($t_{1/2} \sim 80$ min). The amount of haptoglobin in the GC fraction reached a plateau between 20 and 80 min of chase at a value similar to that found at 37 °C. At 20 °C the amount of haptoglobin recovered in the ER fraction remained essentially constant during the first 70 min of chase and then declined at a rate ($t_{1/2} \sim 110$ min) that was lower than

Fig. 6. Effect of temperature on intracellular transport of pulse-labelled haptoglobin

Hepatocytes were labelled for 10 min with [35 S]methionine at 37 °C and then incubated at the temperatures indicated. The amount of [35 S]haptoglobin in the ER (○) and the GC (▲) fractions and in the medium (□) were determined by immunoprecipitation and gel electrophoresis followed by densitometric scanning of the autoradiograms. The results were obtained from two independent experiments, the same as those used for Fig. 4.

that observed at 26 °C. The amount of haptoglobin in the GC fraction was low during the whole chase.

Effect of temperature on the total secretion of albumin

We have previously shown that the GC in hepatocytes contains an amount of albumin that is similar to that present in the ER (Fries *et al.*, 1984). If, as suggested above, the transport of albumin from the GC to the medium is relatively temperature-insensitive, a considerable amount of albumin (originally in the GC) should be secreted even at low temperatures. To test this supposition we labelled the cells continuously for 1 h at 37 °C, so as to label intracellular albumin homogeneously. The cells were then incubated for 180 min at different temperatures and the amount of [35 S]albumin in the cells and in the chase media was determined (Fig. 7). At chase temperatures below 30 °C a decreasing proportion of albumin was found in the medium. At 18 °C, where little transport of albumin from the ER to the GC had been detected, some 25% of the total amount of albumin was

Fig. 7. Effect of temperature on total secretion of cellular albumin

Hepatocytes cultured in 35 mm dishes for 20–22 h were labelled for 60 min at 37 °C with [35 S]methionine to yield homogeneous labelling of albumin. The dishes were then transferred to an aluminium slab with a temperature gradient. After incubation for 180 min, the amount of [35 S]methionine-labelled albumin in the detergent-solubilized cells (●) and the medium (■) was determined as described in Figs. 1 and 2.

secreted, indicating that a large part of the albumin in the GC was externalized.

DISCUSSION

Exocytosis of both secretory and membrane proteins has been found to be strongly temperature-dependent, having virtually ceased around 20 °C (Devreotes & Fambrough, 1976; Craig, 1979; Rotundo & Fambrough, 1980). More recently, it was shown that the most temperature-dependent step in the externalization of a viral membrane protein was the transport from the GC to the cell surface; at 20 °C the protein was found to accumulate in the GC (Matlin & Simons, 1983). Here we show that the two secretory proteins that we have analysed do not accumulate in the GC at this low temperature. This result was unexpected, in view of the finding that membrane and secretory proteins seem to follow the same pathway through the secretory apparatus (Strous *et al.*, 1983). A possible explanation for the different temperature-sensitivities of the two types of proteins can be deduced, however, from recent observations on the structure and function of the GC.

Analysis of the exocytosis of different types of proteins in an endocrine cell line has indicated that constitutively secreted proteins (of which albumin and haptoglobin are examples) are not actively sorted in the GC, in contrast with, e.g., membrane proteins destined for the plasma membrane (Kelly, 1985). Griffiths *et al.* (1985), studying virus-infected cells, found that on incubation at 20 °C a viral membrane protein destined for the plasma membrane would accumulate in buds on the membrane of the *trans* cisternae of the GC. The buds had a cytoplasmic coat whose structural similarity to the coated pits seen on the plasma membrane suggests that the buds are also part of a sorting device. On the basis of these observations it seems possible that the difference in tem-

perature-sensitivity seen for the exocytosis of secretory and membrane proteins is due to the membrane proteins being sorted in (the *trans* cisternae) of the GC by a process that is blocked at 20 °C.

Below 20 °C little transport of both secretory and membrane proteins from the ER to the GC seems to take place. Saraste & Kuismanen (1984), using immunohistochemical techniques on virus-infected cells, found, however, that on incubation at 15 °C a virus-encoded membrane protein accumulated in vacuoles occurring between the ER and the GC. In an experiment in which pulse-labelled hepatocytes, which had been incubated at 18 °C for 90 min, were fractionated by centrifugation in a continuous sucrose gradient, most of the radioactively labelled albumin occurred in parallel with the ER marker (E. Fries & I. Lindström, unpublished work). However, a significant portion was recovered at a density intermediate between that of the ER and the GC markers. It would be interesting to investigate whether this albumin might have resided in structures occurring between the ER and the GC in the cell.

Newly synthesized secretory proteins begin to appear in the medium only a certain time after their synthesis (Helmreich *et al.*, 1961; Morgan & Peters, 1971). The molecular basis for this delay is not known. It does not seem to represent the time required for the modification of the proteins during transport, since inhibition of the modification process, at least in some cases, does not affect the secretion kinetics (Struck *et al.*, 1978). The fact that the time lag increases markedly with decreasing temperature, as shown in the present paper for two secretory proteins and as shown previously for a membrane protein (Saraste & Kuismanen, 1984), indicates that the lag is not due to simple diffusion, since diffusion rates are proportional to the absolute temperature. Interestingly, different proteins appear in the medium with different time lags [Morgan & Peters (1971); compare results for albumin in Fig. 4 and for haptoglobin in Fig. 6]. Possibly, these differences could reflect different affinities for transport receptors (Morgan & Peters, 1971). The transport of proteins from the exterior of the cell to the lysosomes has also been found to require a relatively long time, and it has been suggested that this time may be required for the maturation of membrane compartments through which the proteins pass (Helenius *et al.*, 1983). Further studies of protein transport in cell-free systems (Balch *et al.*, 1984) will probably provide an explanation for this phenomenon.

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