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A cycloheximide-resistant pool of receptors for asialoglycoproteins and mannose 6-phosphate residues in the Golgi complex of hepatocytes

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Following *in vivo* administration of cycloheximide (20 mg/kg body weight i.p.) protein synthesis was completely inhibited (99%) in rat liver. No newly synthesized asialoglycoprotein receptor (ASGP-R) could be detected by metabolic labeling. Fluorescence immunocytochemistry of several secretory proteins and plasma membrane proteins, including the receptors for polymeric IgA (IgA-R), demonstrated a rapid loss from the Golgi complex following cycloheximide administration. On the other hand, two membrane proteins, the receptors for ASGP-R and mannose 6-phosphate (MP-R), were not altered in their cellular localization including the Golgi. Using quantitative immunoelectron microscopy with colloidal gold, we found that 2 h and 4 h after cycloheximide administration, the densities of ASGP-R and MP-R in the membranes of the Golgi complex were unaltered compared with control liver. Similarly, there was no significant effect of cycloheximide on the receptor labeling in coated vesicles and compartment of uncoupling receptors and ligands (CURL). These observations are consistent with an involvement of the Golgi and CURL pools of the receptors in intracellular trafficking, endocytosis and receptor recycling.

Key words: cycloheximide/Golgi complex/immunogold/liver/receptors

Introduction

Transportable glycoproteins are synthesized and N-glycosylated in the rough endoplasmic reticulum (ER). A portion of the mannose residues is subsequently removed by mannosidases thought to reside in Golgi cisternae (Novikoff *et al.*, 1983; Pohlmann *et al.*, 1982). Terminal carbohydrates are then incorporated by glycosyl transferases, e.g., galactosyl transferase, a resident enzyme in the membranes of the *trans*-Golgi cisternae (Roth and Berger, 1982; Strous and Berger, 1982; Strous *et al.*, 1983). On the basis of similar processing events as well as immunocytochemical localization studies, a common intracellular pathway: ER-*cis* Golgi-*trans* Golgi-cell surface has been proposed for secretory and plasma membrane glycoproteins (for review, see Palade, 1983). The time required for the entire ER to plasma membrane transit varies among protein species and cell types. In liver and hepatoma cells, the movement of albumin is relatively rapid and occurs much faster than that for glycoproteins, such as transferrin (Strous and Lodish, 1980; Sztul *et al.*, 1983; Fries *et al.*, 1984). This difference in transit times appears to arise during the ER to Golgi transport (Strous and Lodish, 1980; Lodish *et al.*, 1983; Fries *et al.*, 1984). Furthermore, membrane proteins are often slower than the bulk of secretory proteins (Sztul *et al.*, 1983). Nevertheless, most secretory and plasma membrane glycoproteins are processed and transported through their biosynthetic pathway within 2 h. Protein transport is not dependent upon ongoing protein synthesis. Inhibition of protein synthesis with puromycin or cycloheximide does not affect processing and transport kinetics of secretory (Ashley and Peters, 1969; Peters and Peters, 1972; Jamieson and Palade, 1968; Banerjee and Redman, 1977) and plasma membrane proteins (Green *et al.*, 1981; Rotundo, 1975) including receptors (Devreotes and Fambrough, 1975).

While the difference in transit time between secretory and plasma membrane proteins is in the order of tens of minutes, the intracellular life time of membrane proteins usually exceeds the transit time by many hours. For example, the major membrane glycoproteins in rat liver have half-lives of >70 h (Tauber *et al.*, 1983), similar to the 80-h half-life of the asialoglycoprotein receptor (ASGP-R) (Tanabe *et al.*, 1979). Corresponding long half-lives have been reported for other membrane receptors, presumably related to the capacity of cells to recycle membrane proteins. Thus, a single ASGP-R may recycle >250 times (Schwartz and Rup, 1983). Similarly the mannose 6-phosphate receptor (MP-R) is re-utilized about every 5 min (Sly and Fischer, 1982).

The compartments involved in the recycling of membrane proteins including receptors have not yet been identified. Using immunoelectron microscopy, we have recently defined the endocytotic compartments of ASGP-R, MP-R and the receptor for polymeric IgA (IgA-R) in rat liver (Geuze *et al.*, 1983,1984a). Following non-selective internalization within clathrin-coated vesicles, all three receptors are delivered to a compartment of uncoupling receptors and ligands (CURL). CURL was also found to segregate the non-recycling IgA-R from the ASGP-R and MP-R which are known to return to the plasma membrane. One difficulty in elucidating the recycling pathway by immunocytochemistry is the inability to distinguish newly synthesized receptors in transit from recycyling receptors. This is further emphasized by our previous immunocytochemical observation that ~20% of the ASGP-R in rat liver cells occurs in the Golgi complex (Geuze *et al.*, 1983). Given a transit time of the order of 1-2 h (Schwartz and Rup, 1983) and a half-life of 80 h, one would expect no more than a few percent of the ASGP-R within the entire biosynthetic route. The question thus arises as to the possibility that many of the receptors in the Golgi are not in transit but are recycling.

Since inhibition of protein synthesis does not interfere with transport kinetics, we have used cycloheximide to deplete rat liver cells of newly synthesized proteins. We have quantitated by immunoelectron microscopy the localization of several secretory and plasma membrane proteins including the ASGP-R, MP-R and IgA-R. We demonstrate that within 2-4 h after cycloheximide administration *in vivo* the hepatic

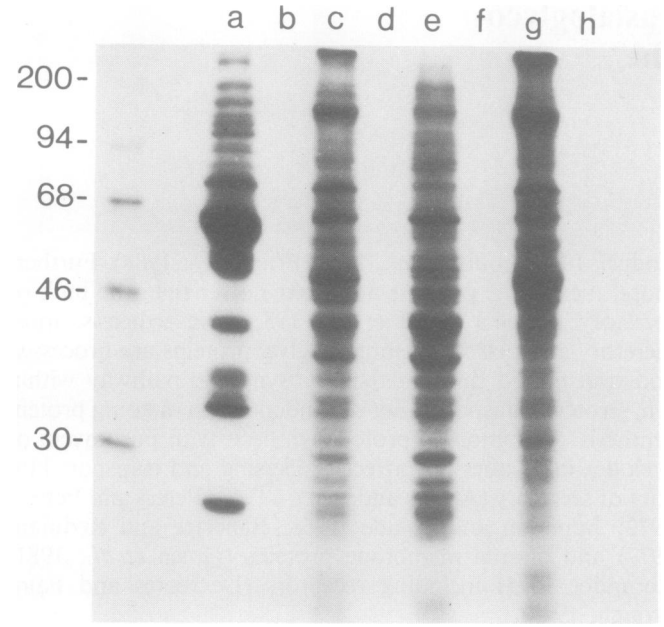


Fig. 1. Autoradiogram of a 10% SDS-PAGE gel of [^{35}S]methionine-labeled proteins in serum and liver of rats administered [^{35}S]methionine *in vivo* with or without cycloheximide pre-treatment. Aliquots of serum (1 μl), whole liver homogenate (2.6 μl), liver cytosol (5.7 μl) and Triton-extractable liver membranes (3.2 μl), from both control (**lanes a, c, e** and **g**) and cycloheximide-treated animals (**lanes b, d, f** and **h**) were analyzed by 10% SDS-PAGE and fluorography in the presence of beta-mercaptoethanol. Time of exposure was 1 day. Mol. wts. of the markers are given in kd.

parenchymal cells were devoid of proteins such as IgA-R, but that ASGP-R and MP-R were still present at essentially the control density in the Golgi cisternal membranes.

Results

Effect of cycloheximide on protein synthesis

Two hours after administration of [^{35}S]methionine to the control animal, ~30% of the radioactivity was already incorporated into liver protein, equally distributed between the cytosol and membrane fractions (Figure 1, lanes c, e and g). In addition, all of the serum proteins were labeled (Figure 1, lane a). Especially prevalent is the 67 000-dalton albumin species (Figure 1, lane a). As seen in Figure 1 the total liver cytosol (lane e) shows an enrichment of many of the labeled species seen in the whole liver homogenate (lane c) including the major band at 40 000–42 000 daltons, presumably actin. The Triton X-100 solubilized membrane fraction (Figure 1, lane g) contains numerous bands, many of which are enriched from that seen in the total liver homogenate (Figure 1, lane c). A major band at 180 000 daltons as well as bands of very high mol. wt. are markedly enriched over the whole liver homogenate. In contrast to this pattern of proteins from the control animal, identical samples obtained from the cycloheximide-treated animal are devoid of radiolabeled proteins (Figure 1, lanes b, d, f and h).

Isolation of the newly synthesized rat ASGP-R from the Triton X-100 solubilized liver membrane demonstrated a major radiolabeled species at ~45 000 daltons (Figure 2, lane b). In addition, a minor species at ~42 000 daltons presumably representing the unglycosylated form of mature 45 000-dalton species is clearly seen. Furthermore, at least three minor species of radiolabeled receptor are seen at mol. wts. between 55 000 and 65 000 daltons. These represent the two high mol. wt. low abundance species of the mature rat liver

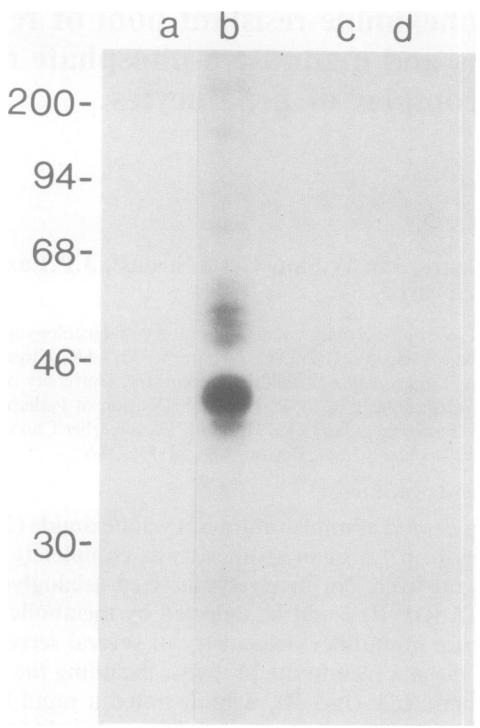


Fig. 2. Effect of cycloheximide pre-treatment on the incorporation of [^{35}S]methionine into rat liver ASGP-R *in vivo*. The Triton X-100 extract of liver membranes from both the control (**lanes a** and **b**) as well as from the cycloheximide-treated animal (**lanes c** and **d**) was each divided into two identical aliquots. One aliquot was incubated with 100 μg purified rabbit IgG (non-immune) and the other aliquot was incubated with 100 μg monospecific affinity-purified rabbit anti-rat liver ASGP-R IgG. The immune precipitates were isolated with protein A-bearing staphylococci and analysed by 10% SDS-PAGE and fluorography. **Lanes a** and **c** contained non-immune rabbit IgG while **lanes b** and **d** contained rabbit anti-rat liver ASGP-R IgG. Mol. wt. standards are noted in kd.

ASGP-R (Schwartz *et al.*, 1981) as well as the presumed biosynthetic precursor molecule(s). No radiolabeled species are seen with control antibody (Figure 2, lane a). In addition, no radiolabeled receptor was identified in the Triton X-100 extractable liver membrane from the cycloheximide-treated animals (Figure 2, lanes c and d).

Immunocytochemistry

In preliminary experiments no differences were noted between livers from animals which received 100 and 20 mg/kg body weight cycloheximide. The following data were obtained with the lower dose.

Fluorescence microscopy. Figures 3 and 4 show immunofluorescence micrographs organized into three vertical columns. Each column shows the immunoreaction of one or two proteins in liver cryosections of control (0) and cycloheximide (1, 2, 4 h) treated rats. In control cells albumin (Figure 3, left lane) gave a similar ER and Golgi (bright spots) reaction as we have described previously (Brands *et al.*, 1983). At 1 h after cycloheximide only faint fluorescent spots were found in the Golgi complexes. At 2 and 4 h, the parenchymal cells were completely negative, only sinusoidal lining cells showed some fluorescence, located presumably in endocytic vacuoles. Transferrin (Figure 3, middle column) behaved similarly, but lagged behind albumin. The cells became devoid of detectable transferrin label only after 2–4 h. 5′-Nucleotidase (Figure 3, right column, upper 2) in control cells was predominantly found in the Golgi areas and bile

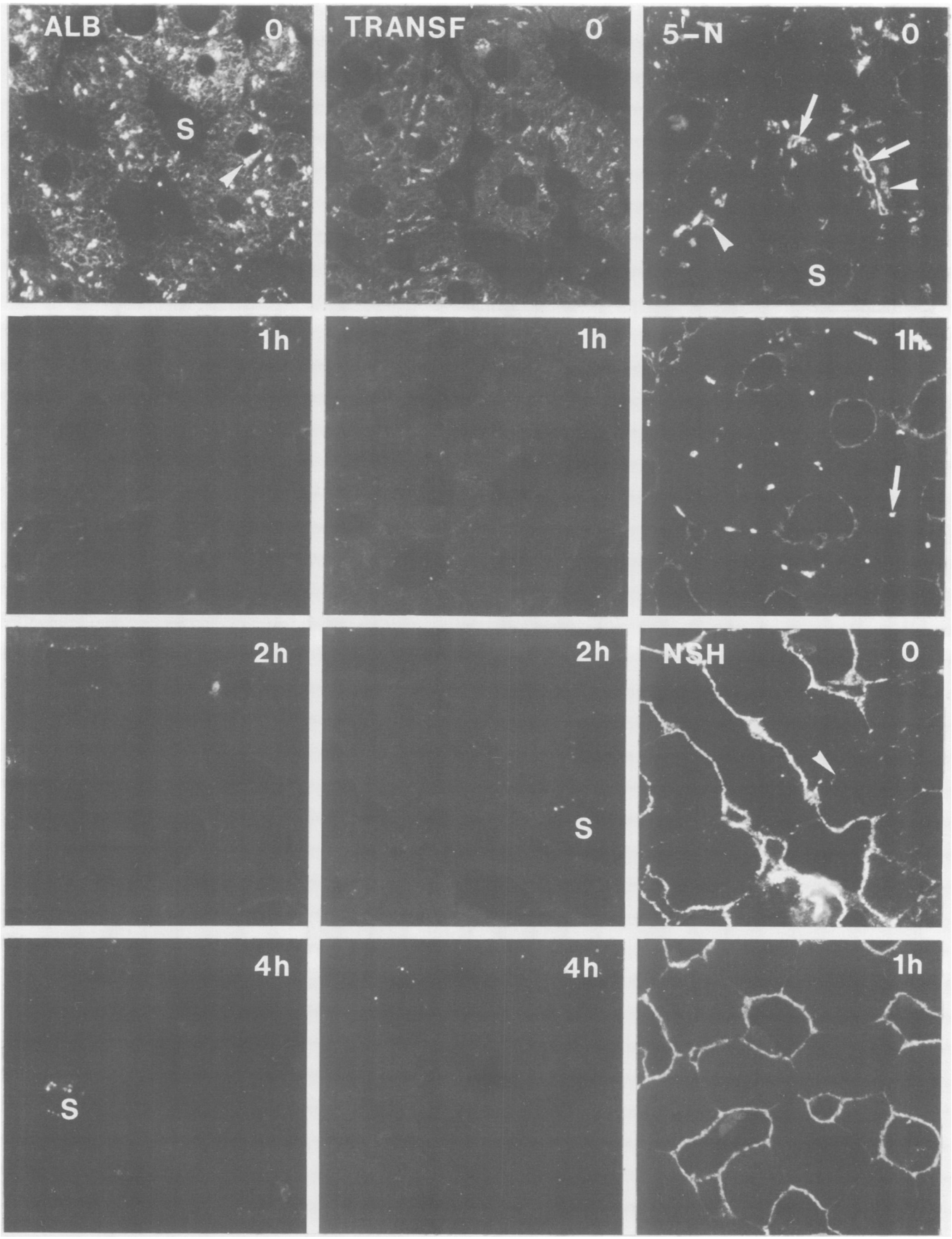


Fig. 3. Immunofluorescence micrographs of semithin cryosections of liver from control (0) and cycloheximide-treated (1, 2 and 4 h) rats. **Left column** shows albumin, the **middle column** transferrin, the **right upper two**, 5′-nucleotidase (5′-N) and the **right lower two**, nucleoside sugar hydrolase (NSH). Description is given in Results. S, sinuses; arrows, bile canaliculi; arrowhead, Golgi complexes. All magnifications 855x.

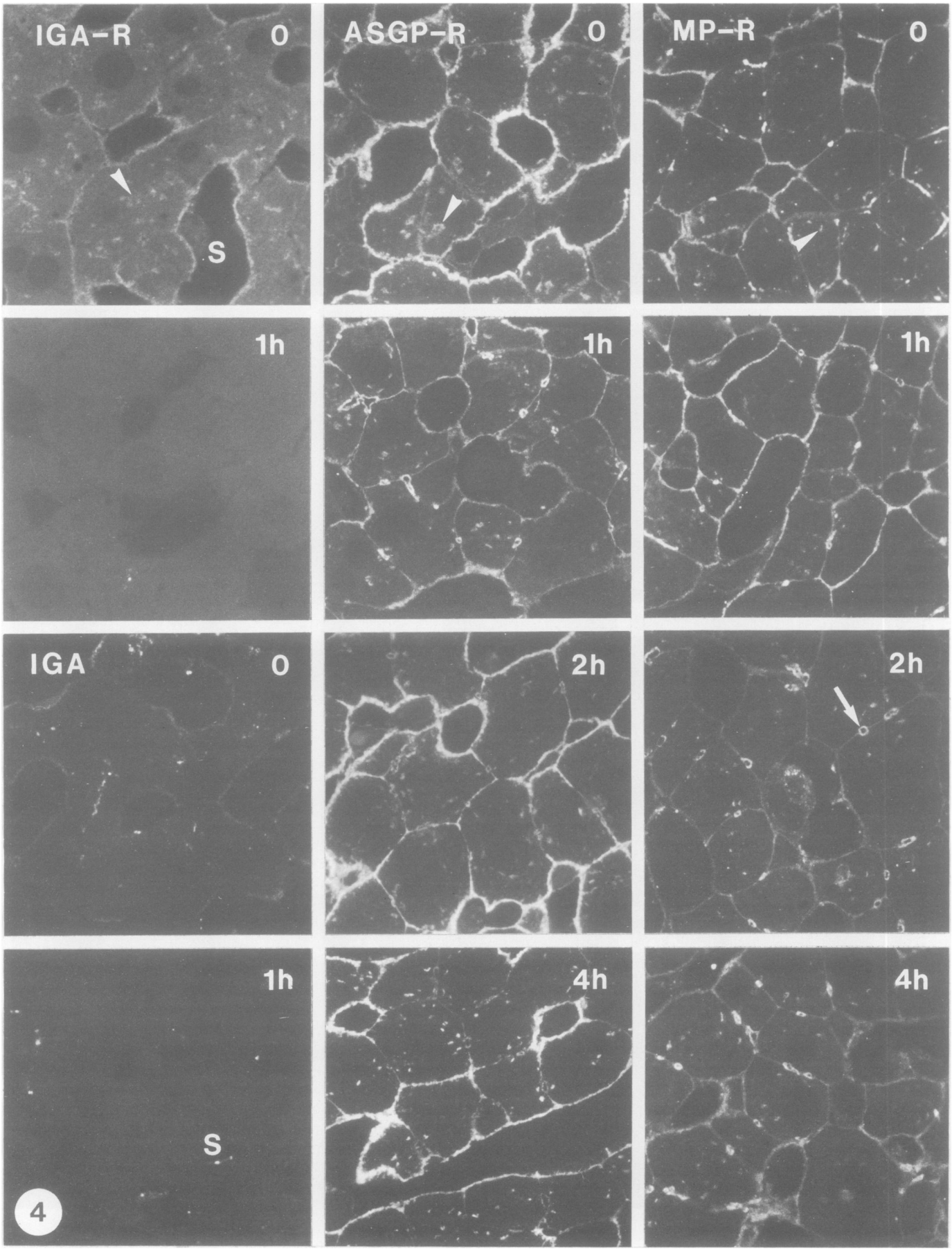


Fig. 4. See Figure 3. **Left column, upper two** micrographs, IgA-R and **lower two**, IgA; **middle** and **right columns**, ASGP-R and MP-R, respectively.

canaliculi. One hour after cycloheximide, the Golgi complexes were completely negative. Reactivity of sinusoidal and bile canalicular membranes did not change within 4 h. Nucleoside sugar hydrolase (NSH), on the other hand, reacted strongly at the sinusoidal cell surface, was absent from the bile canaliculi and was faint in the Golgi areas of control cells. After 1 h the Golgi complexes were negative. Figure 4 shows three receptors, IgA-R (left column), ASGP-R (middle), MP-R (right). In control liver, IgA-R was found in the cytoplasm, Golgi complexes, and at the sinusoidal and bile canalicular cell surfaces. After 1 h of cycloheximide, no IgA-R reactivity remained, except for weak staining in the bile cannaliculi. Consistent with this, IgA ligand was present at the sinusoidal and bile surfaces in controls, but was absent after 1 h of cycloheximide. Some fluorescence in sinusoidal lining cells persisted (lower two micrographs of left column). In contrast to all of these proteins, fluorescence of ASGP-R (middle column) and MP-R (right column) appeared essentially unchanged after cycloheximide. The only notable difference was a punctate appearance of the Golgi fluorescence (see lower micrograph, middle column), indicative of smaller Golgi complexes.

Immunoelectron microscopy. Since there was no detectable effect of cycloheximide on ASGP-R and MP-R Golgi fluorescence patterns, we used double-labeling immunoelectron microscopy to compare further 5′-N (Figures 5–8) and ASGP-R and MP-R (Figures 9–12) localization. In control cells, 5′-N was present at the luminal faces of Golgi membranes, throughout the stack of cisternae, in a *trans*-Golgi reticulum (TGR) consisting of vesicles and tubules often with lipoprotein particles (Figure 5), along the bile canalicular membranes (Figure 6), in CURL and at the sinusoidal surface (not shown). Two hours after cycloheximide injection no 5′-N reactivity remained in the Golgi, in contrast to ASGP-R which was still abundant throughout the Golgi stacks (Figures 7 and 8). The bile canaliculi of these same cells still showed 5′-N label (Figure 8), even after 4 h (not shown).

We have previously described the subcellular distributions of ASGP-R and MP-R in normal rat liver (Geuze *et al.*, 1984a,1984b). Both receptors are present in the membranes of all Golgi cisternae, in TGR, secretory vesicles, CURL, coated vesicles and along the entire plasma membrane, including the bile canaliculi. This labeling pattern was unaltered by treatment with cycloheximide (administered at two doses for up to 4 h). After 2 h both ASGP-R and MP-R occurred at the sinusoidal membrane, in coated pits and in CURL (Figure 9). The Golgi stacks remained heavily labeled (Figures 10 and 11, note reverse gold labeling). Lysosomes, as identified by their labeling for cathepsin D (not shown) were negative (Figure 10), and especially at 4 h, Golgi complexes generally appeared smaller than in controls (Figure 11), and TGR appeared less developed.

Quantitation of immunogold. To detect if any alteration (*cis* to *trans* or medial to lateral) in labeling intensity of the Golgi complexes had resulted from cycloheximide treatment, we determined the gold labeling densities over Golgi membranes in cryosections which were double-labeled with MP-R/5 nm gold, followed by ASGP-R/10 nm gold. We examined liver sections from controls and from rats treated with cycloheximide for 2 and 4 h. Perpendicularly cut Golgi stacks showing a *cis*- to *trans*-polarity were selected. The Golgi stacks, printed at high magnification, were divided into *cis*- and *trans*-halves and into lateral and medial thirds. For each Golgi complex we counted both the number of ASGP-R and MP-R gold particles present, as well as the number of times superimposed lines transsected each category of Golgi membranes. The ratio of gold frequency (%) to intersection frequency (%) was taken as a measure of membrane labeling density.

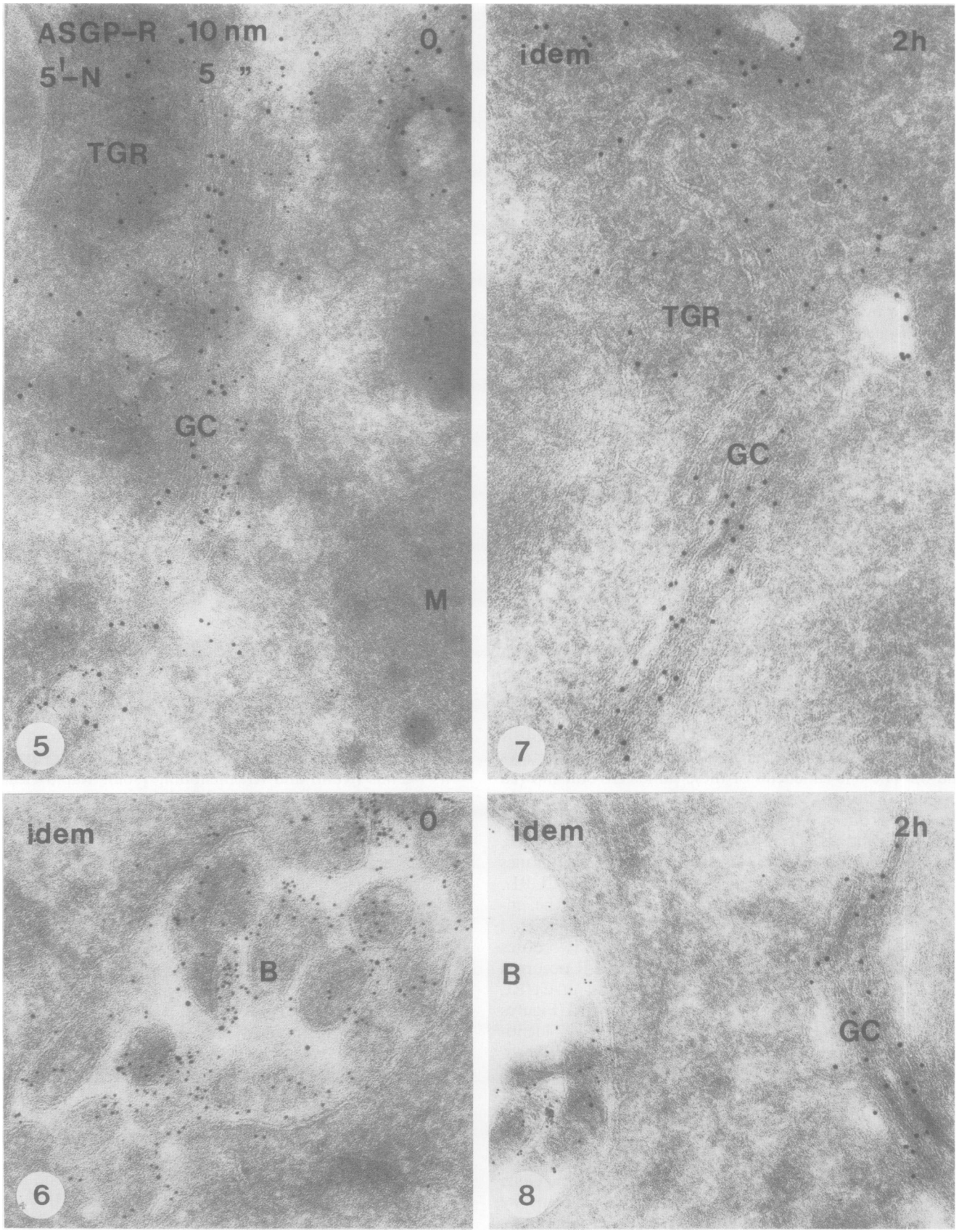
Cycloheximide had no major effect on the labeling densities of lateral and medial (data not shown), and of *cis*- and *trans*-Golgi membranes (Table I). However, the relative membrane surface area per Golgi complex decreased gradually.

Discussion

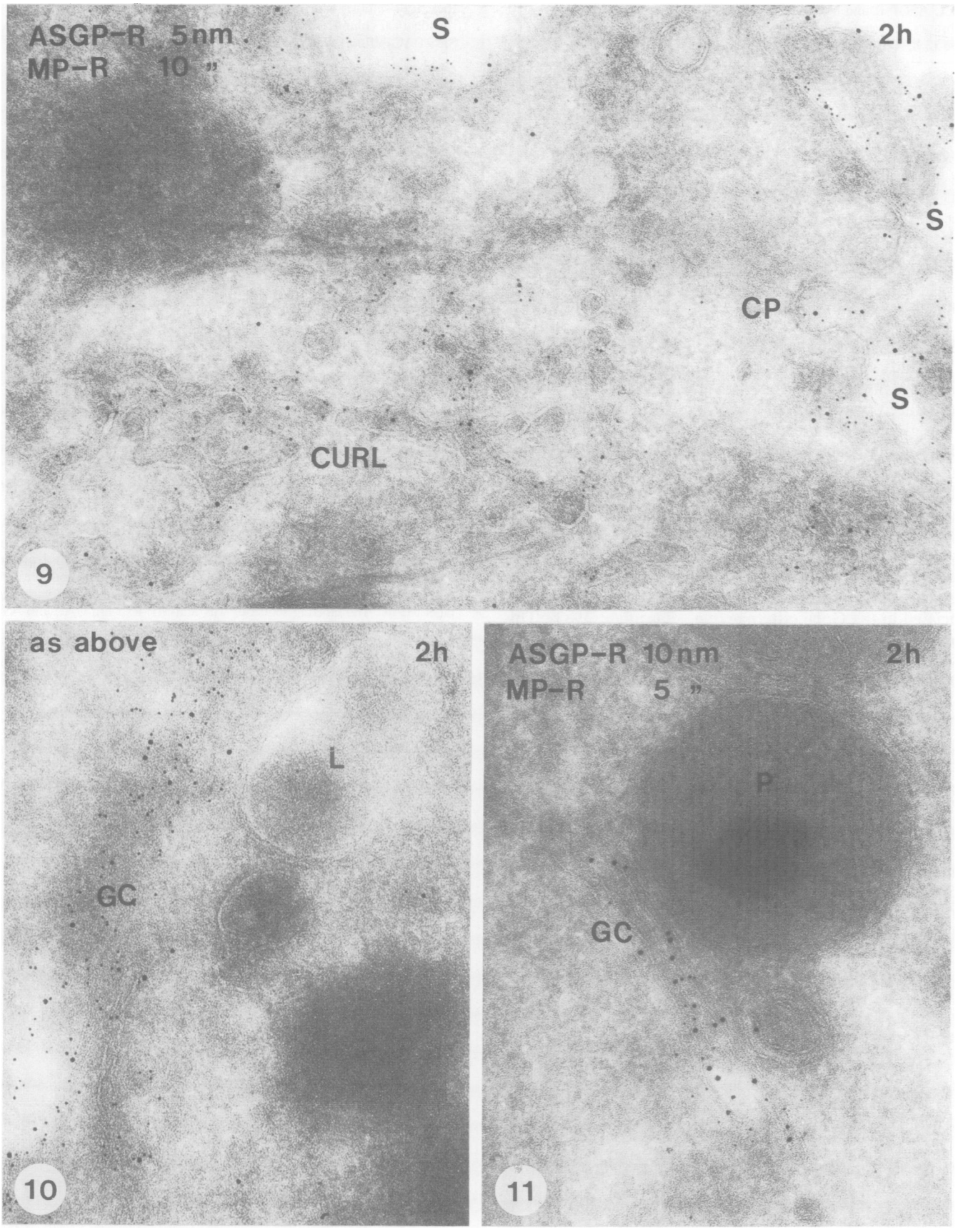
Inhibition of protein synthesis does not alter transport of previously synthesized secretory and membrane proteins. A well documented example is the spike membrane protein of Semliki Forest virus. Cycloheximide did not alter the timing of the viral glycoprotein processing in cultured cells. In addition, quantitative immunoelectron microscopy revealed that spike protein transport through the Golgi stacks proceeded normally (Green *et al.*, 1981). In the present study, cycloheximide administration abolished protein synthesis and secretion. Similarly, synthesis of membrane protein in general and ASGP-R specifically was reduced to 1% of that in controls. Thus it is reasonable to assume that within 2 h and 4 h of cycloheximide treatment, the biosynthetic pathway including the Golgi complex should be depleted of newly synthesized transportable proteins.

Indeed we found a rapid and progressive clearance of secretory and membrane proteins from intracellular compartments after cycloheximide. This was most obvious in the Golgi complex, which rapidly (within 2 h) became depleted of albumin and transferrin and IgA-R. IgA-R is known not to recycle, but is lost from the cells following transcytosis with its ligands (Kühn and Kraehenbuhl, 1982). Rapid clearance from the Golgi was also found for the two plasma membrane domain proteins 5′-N, which occurred predominantly in the bile canaliculi (Gurd and Evans, 1974), and NSH which was almost entirely restricted to the sinusoidal and lateral plasma membrane (the localization of these proteins will be detailed elsewhere). Following cycloheximide, these proteins remained unchanged in reactivity at their respective cell surface domain, indicative of a long residence time at the plasma membrane relative to the transit time.

In contrast to the membrane proteins 5′-N, NSH and IgA-R, cycloheximide had no effect on ASGP-R and MP-R Golgi labeling, both qualitatively and quantitatively. Surprisingly, cycloheximide did not even alter the average receptor density in *cis*- and *trans*-Golgi membranes. Even for MP-R, which traverses the Golgi complexes relatively slowly during biosynthesis (Sahagian and Neufeld, 1983), one would have expected a labeling density shift in a *cis*- to *trans*-Golgi direction. Both receptors also persisted in CURL tubules, coated pits and coated vesicles, indicative of ongoing endocytosis. We have previously reported that ASGP-R and MP-R in normal liver cells occur in all Golgi cisternae and in TGR (Geuze *et al.*, 1983,1984a). We have estimated by means of quantitative immunocytochemistry that ~20% of the total cellular ASGP-R is present in the Golgi complex (Geuze *et al.*, 1983). This was an unexpectedly high percentage, considering the short transit time of ASGP-R (<2 h) and the slow turnover (half-life of 80 h) (Schwartz and Rup, 1983; Tanabe *et al.*,



Figs. 5–8. Ultrathin cryosections of normal (0) liver labeled as indicated. **Fig. 5** shows a Golgi complex (GC) and *trans*-Golgi reticulum (TGR). Label of both 5′-N and ASGP-R occurs throughout the stack of cisternae and in TGR. M, mitochondrion. **Fig. 6**. Abundant labeling of 5′-N with scarce ASGP-R labeling along the microvilli of a bile canaliculus (B). **Figs. 7** and **8**. Sections of rat liver treated with cycloheximide for 2 h with labeling as in Figures 5 and 6. No 5′-N label remains in the Golgi complex (GC) in either figure, while the bile canalicular (B) membrane in the same cell remains labeled (**Fig. 8**). ASGP-R labeling in GC is unaltered. TGR, *trans*-Golgi reticulum. All figures 104 500x.



Figs. 9–11. Sections of cycloheximide-treated (2 h) livers labeled as indicated. All figures 104 500x. **Fig. 9** shows the sub-sinusoidal cytoplasm with both ASGP-R and MP-R gold at the outer surface of the plasma membrane of the sinus (S), in a coated pit (CP) and in an extensive system of CURL tubules. **Fig. 10.** Golgi complex (GC) showing MP-R and ASGP-R label throughout the stack of cisternae L, lysosome. **Fig. 11.** Small Golgi complex (GC) adjacent to a peroxisome (P). Gold labeling is reversed as compared with Figures 9 and 10.

Table I. ASGP-R and MP-R immunogold labeling densities in the membranes of liver Golgi complexes of control (0), and cycloheximide-treated (2 h, 4 h) rats

	ASGP-R			MP-R		
	0	2 h	4 h	0	2 h	4 h
cis-Golgi	0.7	0.7	0.9	0.7	1.0	1.0
trans-Golgi	1.3	1.2	1.2	1.1	0.7	0.9
Total gold	344	402	678	216	211	259
Total intersections	897	609	989	897	1156	989

Data represent the ratios of particles (%) and intersections (%) of parallel lines with the membranes of the *cis*- and *trans*-halves of the Golgi stacks. Quantitation was done with cryosections double-labeled for ASGP-R and MP-R.

1979). The cycloheximide-resistant pool of receptors detected in this study demonstrates that the vast majority of Golgi receptors is not in transit but rather represents some hitherto unrecognized population of receptors. It is tempting to relate this Golgi pool to the recycling receptors. Two observations lend support to such a relationship. (i) A considerable fraction of asialotransferrin type-3 endocytosed by rat liver ASGP-R, returns to the circulation in a sialylated form (Regoeczi *et al.*, 1982). This suggests that this ligand and perhaps the ASGP-R itself are exposed to sialic acid transferases located in or adjacent to the Golgi complex. (ii) When exogenous tracers such as cationized ferritin are bound to surface proteins, a portion of the particles appear membrane-associated in the Golgi cisternae (e.g., Farquhar, 1983). This also supports a plasma membrane to Golgi traffic for certain plasma membrane proteins.

Others have identified a large intracellular pool of ASGP-R, which are functionally equivalent to the surface ASGP-R and serve in the replacement of internalized surface receptors (Weigel and Oka, 1983; Bridges *et al.*, 1982). We have estimated the intracellular ASGP-R at 65% (Geuze *et al.*, 1983). The intracellular pool of MP-R has been reported to be even 90% in rat liver (Fischer *et al.*, 1980) and 80–85% in macrophages (Shepherd *et al.*, 1984). In addition, exposure of fibroblasts to anti-MP-R antibodies leads to a rapid degradation of >90% of the cell's MP-R, consistent with availability of the large intracellular MP-R pool to the cell surface (von Figura *et al.*, 1984). The intracellular pool of ASGP-R and MP-R in rat liver is largely comprised of receptors in CURL and the Golgi complex, thus it appears likely that both of these populations play some role in intracellular targeting of ligands as well as in endocytosis and receptor recycling.

Materials and methods

In vivo biosynthetic labeling

5 mCi [^{35}S]methionine (Amersham; 1200 mCi/mmol) was given i.v. *via* tail vein to control rats or rats which received 20 mg/kg cycloheximide i.p. 2 h previously. Two hours after [^{35}S]methionine administration, blood (serum) was sampled and the liver removed, rinsed and homogenized in 9 ml ice-cold 0.15 M NaCl, 10 mM Hepes (pH 7.3) 1 mM PMSF (buffer A) by 20 strokes with a Dounce homogenizer. The homogenate was centrifuged at 100 000 *g* for 1 h in a Beckman Ti70 rotor. The pellet was extracted twice for 20 min at 4°C in 4 ml 2% Triton X-100 in buffer A and centrifuged at 100 000 *g* for 30 min. Aliquots of the Triton X-100 soluble material were immunoprecipitated with either normal rabbit IgG (control) or affinity-purified rabbit anti-rat ASGP-R (rabbit D), and analyzed by SDS-PAGE/fluorography (Schwartz and Rup, 1983).

Aliquots of the serum, whole liver homogenate, liver cytosol (100 000 *g* supernatant) and Triton X-100 solubilized membranes were counted directly or after boiling in 10% TCA and washing in ethanol. In addition, aliquots of these four samples which contained 125 000 c.p.m. in the control animal and the identical volume from the cycloheximide-treated animal were analyzed by SDS-PAGE/fluorography.

Antibodies

Most of the antibodies used for immunocytochemistry have been described previously. We used affinity-purified rabbit antibodies against rat albumin (Brands *et al.*, 1983), transferrin (Strous *et al.*, 1983), 5'-nucleotidase (Stanley *et al.*, 1983), IgA-R, p.IgA, ASGP-R (Geuze *et al.*, 1984a) and MP-R (Geuze *et al.*, 1984b). A mouse monoclonal against rat liver nucleoside sugar hydrolase (NSH) was kindly donated by Dr W.van Dijk (Dep. Medical Chem., Free University, Amsterdam, The Netherlands) and will be described elsewhere.

Animals

Male Wistar rats (150 g for immunocytochemistry; 35 g for biosynthesis) were fasted overnight. Experimental animals received an i.p. injection of 20 and in one experiment 100 mg/kg body wt. of cycloheximide in 0.5 ml PBS. After 1, 2 and 4 h, the rats were anaesthetized for fixation.

Tissue preparation

Control and cycloheximide-treated livers were perfusion-fixed *via* the descending aorta with 4% formaldehyde in 0.1 M phosphate buffer, pH 7.4. After 5 min of perfusion, pieces of two liver lobes were cut into 1 mm slices which were further fixed in the same fixative for ~5 days prior to cryosectioning. Cryosectioning, immunolabeling, staining and embedding were carried out as described previously (Geuze *et al.*, 1983). In the case of the anti-NSH monoclonal we used a rabbit anti-mouse IgG prior to goat anti-rabbit IgG conjugated to FITC. Double-immunogold labeling was done as described (Geuze *et al.*, 1981) with two sizes of monodisperse colloidal gold particles, of 5 nm (actually 4.1nm + 10%) and 10 nm (9.6 nm + 10%), prepared according to Slot and Geuze (1981).

Quantitative immunoelectron microscopy

Immunogold labeling for ASGP-R and MP-R was quantitated using double-labeled cryosections of control and cycloheximide (2 h and 4 h) treated rats. Two liver tissue blocks from each of two rats were studied for both the control and experimental group. The most sensitive gold probe (5 nm) was used to label MP-R. The labeling sequence was anti-MP-R/5 nm gold, anti-ASGP-R/10 nm gold. From each liver 20 electron micrographs were taken from cross-sectioned Golgi complexes which clearly demonstrated the *cis*- to *trans*-Golgi polarity. The micrographs were printed at 180 000x and the Golgi stacks were divided into *cis* and *trans* halves and into medial and lateral thirds. Gold particles were then counted and attributed to the six Golgi sub-compartments. A transparent sheet with an array of 2 cm spaced parallel lines was superimposed on the prints, and the number of line intersections with the Golgi membranes in the sub-compartments was counted. The ratio of gold (%) to intersections (%) was used as a measure of receptor density in the Golgi membranes. Since Golgi stacks are anisotropic structures and were printed such that the cisternae were almost perpendicular to the lines, the line cuts provide an exaggerated estimate of the Golgi membrane surface area. Thus, these values were only used for comparison of the surfaces or ratios between the experimental groups.

Acknowledgements

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