Differential Arrest of Secretory Protein Transport in Cultured Rat Hepatocytes by Azide Treatment

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Abstract. The effect of reduced cellular ATP content on intracellular transport of two secretory proteins, albumin and haptoglobin, in isolated rat hepatocytes was studied. The cells were labeled with [^{35}S]methionine and the cellular ATP content was then rapidly reduced to different stable levels by incubation with azide at different concentrations (2.0–10 mM). The amount of the radioactively labeled secretory proteins in the cells and in the medium after 150 min of incubation was determined by immunoprecipitation followed by gel electrophoresis, fluorography, and densitometry. At progressively lower ATP levels, down to 50% of normal, the protein secretion was unaffected, whereas at even lower levels an increasing portion of the proteins remained in the cells; at 30 and 10% of normal ATP level, 25 and 75% of albumin, respectively, was arrested intracellularly.

Analysis of the carbohydrate structure of intracellularly arrested haptoglobin showed that in cells with an ATP level of ~30% of normal, the majority of haptoglobin molecules (55%) were fully or partially resistant to endoglycosidase H. This result indicates that exit from the *medial* and/or the *trans* part of the Golgi complex (GC) was inhibited under these conditions. It also shows that the protein had accumulated in the GC, since under normal conditions the fraction of the intracellular haptoglobin that is endoglycosidase H resistant is ~10%. By similar criteria it was found that at ATP levels below 10% of normal transport of haptoglobin from the endoplasmic reticulum to the *medial* GC (and possibly also to the *cis* GC) as well as from the *trans* GC to the medium were blocked.

In eukaryotic cells, secretory proteins and many membrane proteins are synthesized on ribosomes attached to the endoplasmic reticulum (ER)[1] and are cotranslationally transferred into its lumen. Here, most of the proteins acquire mannose-rich carbohydrate chains by the transfer of an oligosaccharide from a lipid donor. The proteins are then transported from the ER to the cell surface via the Golgi complex (GC), by vesicles. Little is yet known about the molecular mechanisms underlying this complex process but biochemical and genetic techniques are beginning to yield detailed information on the different compartments of the secretory pathway and of the cytosolic factors involved (for a review see Pfeffer and Rothman, 1987). Thus, it has been demonstrated that the GC can be divided into at least three functionally distinct compartments, namely the *cis*, *medial*, and *trans* GC, each consisting of one or two cisternae (for reviews see Dunphy and Rothman, 1985; Farquhar, 1985; Griffiths and Simons, 1986). Secretory and membrane proteins appear to pass through these compartments in sequence, and the compartments have been defined, at least partly, by their content of enzymes acting on the carbohydrates of proteins in transit (Kornfeld and Kornfeld, 1985; Farquhar, 1985). Hence, the *cis* GC houses an enzyme (mannosidase I) that removes two to three of the mannose residues of the proteins coming from the ER. In the *medial* GC, a transferase adds a *N*-acetylglucosamine (GlcNAc) residue to the resulting structure, a modification that can be assayed as resistance of the carbohydrate chain to endoglycosidase H (endo H). After the addition of the GlcNAc residue, another two mannose residues are removed (by mannosidase II) and a second GlcNAc residue is added, both reactions occurring in the *medial* GC. In the *trans* GC, galactose, sialic acid, and fucose residues are added.

One approach to dissecting the secretory pathway is to localize steps that depend on energy. Inhibition of respiration has been demonstrated in a number of systems to block the transport of secretory and membrane proteins from the ER to the GC and from the GC to secretory granules or to the plasma membrane (Jamieson and Palade, 1968; Fitch et al., 1968; Howell, 1972; Tartakoff and Vassalli, 1977; Kääriäinen et al., 1980; Datema and Schwarz, 1981; Godelaine et al., 1981; Novick et al., 1981; Tartakoff, 1986). Using a cell-free system Rothman and co-workers have shown that

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1. *Abbreviations used in this paper*: Endo H, endoglycosidase H; ER, endoplasmic reticulum; GC, Golgi complex; GlcNAc, *N*-acetylglucosamine.

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transport of the G protein of vesicular stomatitis virus (VSV) between the *cis* and *medial* GC requires ATP (Balch and Rothman, 1985). More recently Balch and co-workers demonstrated that the transport of this protein from the *medial* to the *trans* GC is inhibited in the absence of oxygen (Balch et al., 1986; Balch and Keller, 1986).

We are using isolated rat hepatocytes as a model system for protein secretion (Fries et al., 1984; Fries and Lindström, 1986). In the present study, we have treated these cells with graded concentrations of azide to reduce the intracellular ATP concentration to different levels, and measured the effect of this treatment on protein secretion. Azide is a water-soluble inhibitor of oxidative phosphorylation and binds reversibly to cytochrome oxidase (Hewitt and Nicholas, 1963). We have measured the secretion of albumin because this protein is synthesized at a high rate in hepatocytes and is therefore easily detected. In addition, we have studied haptoglobin, a glycosylated protein, since it can be localized intracellularly through its carbohydrate structure. The carbohydrate processing of haptoglobin has not yet been determined in detail, but all data obtained so far indicate that it is similar to that found for most other glycosylated proteins (Hanley et al., 1983).

Our results confirm the existence of at least three of the previously described ATP-dependent steps and indicate that the step requiring the highest ATP level is that of the transfer from the *medial* to the *trans* GC.

Materials and Methods

Materials

Culture media and newborn calf serum were purchased from Labassco, Stockholm, Sweden. Human fibronectin was a gift from Å. Ljungqvist, KabiVitrum, Stockholm, Sweden. Antibodies (IgG fraction) against human albumin and human haptoglobin raised in rabbits were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden and Dakopatts, Glostrup, Denmark, respectively. *Staphylococcus aureus* (Cowan I) bacteria, heat inactivated and fixed with formaldehyde, were a gift from F.-P. Nilsson, Pharmacia Fine Chemicals. Endo H was from Miles Scientific, Slough, United Kingdom and sialidase (from *Clostridium perfringens*) was from Sigma Chemical Co., St. Louis, MO.

Isolation and Growth of Rat Hepatocytes

Hepatocytes were isolated from Sprague-Dawley rats weighing 150–250 g by collagenase perfusion and differential centrifugation (Pertoft and Smedsrød, 1987). The rats were starved overnight before the perfusion, unless indicated. The viability of the cells was 80–90% as judged by exclusion of trypan blue. Isolated cells were suspended in DME (containing 25 mM glucose) lacking $NaHCO_3$ and buffered with 25 mM Hepes-NaOH (pH 7.4). The medium was supplemented with 1 mM glutamine, 9% newborn calf serum, bensyl-penicillin (100 U/ml), streptomycin (0.1 mg/ml), insulin (10 mU/ml), and dexamethasone (0.3 µM). The cells were added to 35-mm-diam plastic dishes (1.0×10^6 cells in 3 ml) that had been precoated with 20 µg of fibronectin and incubated at 37°C. After 3–4 h the culture medium was replaced with fresh medium and the cells were incubated for another 17–18 h.

Determination of ATP Content in Cells Treated with Sodium Azide

Hepatocytes cultured overnight (as described above) were 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 (pH 7.4) (buffer 3; Rubin et al., 1977). Then 900 µl was added of either buffer 3 or of MEM lacking $NaHCO_3$ and containing Hepes (25 mM, pH 7.4), glutamine (2 mM), and methionine (1 µM). After 30 min of incubation on a rocking device, 100 µl of freshly prepared sodium azide solutions in buffer 3 was added to give final azide concentrations between 0 and 10 mM. After different times, the media were removed and 1.0 ml of 5% PCA containing 2 mM EDTA was added. The cells were scraped off with a rubber stopper and centrifuged for 5 min at 15×10^3 *g*. The ATP content of the supernatants was determined by a luciferase assay as described by Borg and Bone (1985). In some experiments 5–10 µCi of [^{35}S]methionine (1,000 Ci/mmol, New England Nuclear, Boston, MA) was added to cells 5 min after the addition of the sodium azide. After 10 min of labeling, the cells were harvested as described above. The pellets were washed once with 5% PCA containing 2 mM EDTA and the radioactivity was measured.

Pulse-Chase Experiments in the Presence of Azide

Cultured cells were rinsed twice with buffer 3 and 900 µl of MEM (containing 1 µM methinine) was added. After a 30-min incubation on a rocking device, an additional 100 µl MEM containing 5–10 µCi [^{35}S]methionine was added, and the cells were incubated for either 10 or 120 min. The [^{35}S]methionine-containing medium was then exchanged for 1.0 ml of chase medium, which consisted of MEM supplemented with 2 mM methionine, 1 mg/ml ovalbumin, 20 µM cycloheximide, and different concentrations of azide. The cells were incubated for different times and then placed on ice. The media were withdrawn and the cells washed twice with ice-cold buffer 3. At this point the chase media were centrifuged for 10 min at 1.7×10^3 *g*; the supernatants were frozen in liquid nitrogen and stored at −70°C until further analyses were performed. The cells were solubilized by the addition of 300 µl of a solution containing 20 mM Tris-HCl (pH 8.0), 0.15 M NaCl, 10 mg/ml Triton X-100, 5 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 0.1 mM *N*-ethylmaleimide, and 1 µg/ml pepstatin and then scraped off the plates. Unsolubilized material was removed by centrifugation (5 min at 15×10^3 *g*) and the supernatants were frozen and stored.

Immunoprecipitation

Antibodies were added in excess to aliquots of solubilized cells or media and the volumes were adjusted to 300 µl by addition of solubilizing buffer (see above). After 3.5 h at 6°C, 50 µl of a 10% (vol/vol) suspension of *S. aureus* was added and the samples were rotated for 60 min. The bacteria were then pelleted, washed once with 300 µl of 10 mM Tris-HCl (pH 7.5), 0.4 M NaCl, 10 g/liter Triton X-100, and 5 mM EDTA, and then with 300 µl 10 mM Tris-HCl (pH 8.0). Finally, they were resuspended in 35 µl of sample buffer (83 mM Tris-HCl [pH 8.8], 100 g/liter sucrose, 30 g/liter SDS, 0.1 g/liter bromophenol blue, 4 mM EDTA, 8 mM dithiotreitol), and were heated at 95°C for 3 min. After cooling, 5 µl of 0.5 M iodoacetamide was added to each sample and the bacteria were removed by centrifugation. The supernatants were analyzed by PAGE. Samples that were to be incubated with haptoglobin antibodies were pretreated with 10 µg normal rabbit IgG and 20 µl of a 10% (vol/vol) *S. aureus* suspension at 6°C for 45 min and were then cleared by centrifugation.

Glycosidase Treatments

Endo H. Haptoglobin was immunoprecipitated as described above, and 15 µl of 50 mM Na-acetate buffer (pH 5.6) containing 3 g/liter SDS was added to the washed bacteria. The resuspended bacteria were heated for 3 min at 95°C, pelleted again, and 2.5 mU endo H in 10 µl of 50 mM Na-acetate buffer (pH 5.6) was added to 15 µl of each supernatant. After 18 h at 37°C, 25 µl of a twice-concentrated sample buffer solution (see above) was added and the samples were then prepared for electrophoresis as described above.

Sialidase. Haptoglobin was immunoprecipitated as described above and eluted from the bacteria into 15 µl of 100 mM Na-acetate (pH 5.6) containing 3 g/liter SDS. To each sample, 25 mU sialidase in 10 µl of 100 mM Na-acetate buffer (pH 5.6) was added and after 60 min at 37°C, 25 µl of a twice-concentrated sample buffer solution (see above) was added. The samples were prepared for electrophoresis as described above.

Polyacrylamide Gel Electrophoresis

Slab gels ($200 \times 200 \times 1.0$ mm) with 10–15% polyacrylamide gel gradients were prepared essentially as described by Maizel, Jr. (1971). After electrophoresis, the gels were treated with 2,5-diphenyloxazole in acetic acid (Skinner and Griswold, 1983), soaked in water, dried, and put on preflashed films (Fuji XR) (Laskey and Mills, 1975). The relative amounts of radioactivity in the bands were determined by densitometric scanning of the films.

Protein Determination

Protein was determined by the method of Hartree (1972) with BSA as the standard.

Results

Reduction of Cellular ATP Content by Azide

To be able to study the ATP dependence of the different steps in the intracellular transport of secretory proteins, we needed a procedure for reducing the cellular concentration of ATP to different levels. For this purpose we chose to use azide, a competitive inhibitor of cytochrome oxidase, rather than the more commonly used hydrophobic uncouplers, since the latter also affect membrane permeability (Hanstein, 1976). Fig. 1 *A* shows the ATP levels in cells isolated from starved rats after 15 min of incubation with azide of different concentrations. In cells treated with 2.0 mM azide, the ATP content was reduced to ∼50% of the level found in untreated cells, while treatment with 4.0 mM of the reagent resulted in a reduction to 30%, and treatment with azide at concentrations >6.0 mM resulted in a reduction to 5–10% of the level measured in the control cells. The finding that inhibition of oxidative phosphorylation in hepatocytes reduces the ATP production by >90% agrees with the results of Seglen (1974), which show that starved rats have a very low glycolytic capacity. The ATP content of untreated rat hepatocytes was found to be 13.5 ± 3.8 (n = 20) nmol per mg of cell protein, a value agreeing well with those reported earlier for cultured rat hepatocytes and for rat liver (Faupel et al., 1972; Bisell et al., 1973).

Hepatocytes isolated from fed rats differed greatly between experiments in their sensitivity to azide treatment; in some experiments they showed the same sensitivity as cells from starved rats, whereas in others they were less sensitive (Fig. 1 *B*). This variation probably reflects differences in the nutritional status of the fed rats. It is surprising that these differences could be seen, considering the long time the cells had been in culture after their isolation. To ensure uniform response to azide treatment, hepatocytes from starved rats were used in all subsequent experiments. Fig. 1 *A* also shows that the rate of protein synthesis, as measured by incorporation of [^{35}S]methionine into acid-insoluble material, was greatly affected by azide treatment. At 2.0 mM azide, the rate of protein synthesis was ∼50% of that in untreated cells and at 5 mM azide it was <5%.

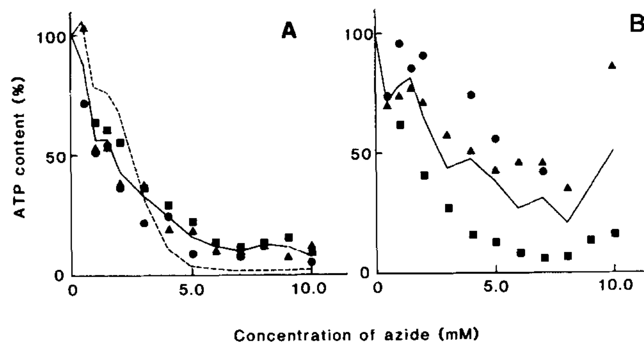

Figure 1. Effect of azide on ATP content in hepatocytes. Hepatocytes isolated from starved (*A*) and fed (*B*) rats were cultured for 20–22 h, and were then treated with different concentrations of azide. After 15 min of incubation in the presence of azide, the ATP content in the cells was determined. The different symbols (●, ■, ▲) represent results obtained from cells of individual rats. In some experiments [^{35}S]methionine was added 5 min after the start of the incubation with azide and the amount of radioactivity in acid-insoluble material was measured at the end of the incubation; *dashed line*, percent of control. The mean of three different experiments are shown.

Fig. 2 *A* shows that azide treatment brings about a stable reduction of intracellular ATP levels; essentially the same reduced levels of ATP were measured in cells incubated with azide for 150 min as in cells that had been treated with azide for only 15 min. The effect of azide treatment on the permeability of the plasma membrane was measured by uptake of trypan blue; ∼16% of the cells took up the dye after 150 min treatment with 4.0 mM azide, compared to 4% of the nontreated cells. Under the same conditions, the release of lactate dehydrogenase was <10%. However, at higher azide concentrations progressively more lactate dehydrogenase was released, ∼30 and 80% at 6.0 and 10.0 mM, respectively (data not shown). To determine if the effects of azide were reversible, cells that had been incubated with azide for 150 min were shifted to inhibitor-free medium and incubated further. Within 20 min, the ATP content in the cells doubled (Fig. 2 *B*), irrespective of the azide concentration which the cells had been treated with before the shift. Thus, in cells incubated with 4.0 mM azide for 150 min, the ATP content increased from 30 to ∼60% of the normal value when the reagent was removed. Along with the increase in the ATP content, protein synthesis was resumed as was the cells' ability to exclude trypan blue (data not shown).

Effect of Azide on Secretion of Newly Synthesized Albumin

To get an overall picture of the effect of azide on intracellular transport of a secretory protein, we determined the time course for the secretion of albumin from cells incubated with different concentrations of azide. Fig. 3 *A* shows the results from experiments in which cells pulse-labeled with [^{35}S]methionine were chased for various periods of time in the absence of azide or in the presence of different concentrations of the inhibitor. Because of its great inhibitory effect on protein synthesis, azide was added after the labeling. As reported earlier, albumin appears in the medium only after a lag period of ∼10 min in the absence of azide; by 20 min 50% is secreted and by 60 min 90% (Fries et al., 1984). A concentration of 2.0 mM azide did not affect the secretion process, while in cells treated with higher concentrations, stable levels of arrested albumin were attained after ∼120 min. Quantitatively, 25 and 75% of the labeled albumin remained in the cells after treatment with 4.0 and 6.0 mM azide, respectively. In cells incubated with 10 mM azide, the secretion of albumin was almost totally blocked. Interestingly, the time lag and half-time for the secretion of the protein that was externalized in the presence of azide were similar to those found in untreated cells.

We also determined if the azide-induced inhibition of albumin secretion could be reversed. To this end cells pulse-labeled with [^{35}S]methionine for 10 min and chased in the presence of different azide concentrations for 150 min, were further incubated in medium free of azide. The results in Fig.

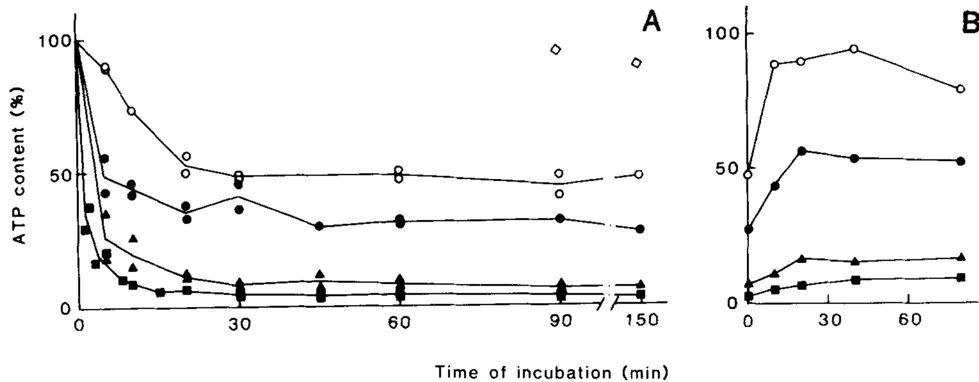

Figure 2. (*A*) Time course for the reduction of cellular ATP content upon azide treatment. Hepatocytes were incubated with different concentrations of azide (no azide, ◇; 2.0, ○; 4.0, ●; 6.0, ▲; and 10.0 mM, ■), and their ATP content was determined at the times indicated. (*B*) Reversion of the azide-induced reduction of cellular ATP content. Hepatocytes incubated with different concentrations of azide for 150 min (as described above) were incubated further in medium without azide, and the cellular ATP content was determined at the indicated times.

3 *B* show that secretion was resumed after removal of the inhibitor. The time lag for the appearance of albumin in the medium depended on the previous concentration of azide in the chase medium; for cells chased in the presence of 4.0 mM azide, the time lag was negligible, while it was ~20 or 40 min for cells that had been chased with 6.0 or 10.0 mM azide, respectively.

Effect of Azide on Secretion of Total Cellular Haptoglobin

To be able to localize the steps in the intracellular protein transport that were inhibited by the azide treatment, we extended our study to the secretion of a glycosylated protein, haptoglobin. Haptoglobin is synthesized as a proform with a molecular mass of 45 kD, which, while still in the ER, is cleaved into a β-chain (35 kD; which carries the N-linked oligosaccharide chains) and an α-chain (9.5 kD; this chain contains no methionine residues and is therefore not radioactively labeled in the experiments described) (Hanley et al., 1983). The polypeptide chains are linked intracellularly via disulfide bonds forming tetrameric $\alpha_2\beta_2$-complexes. During intracellular transport, the oligosaccharide chains are processed from high-mannose to complex-type structures, resulting in an increase of the apparent molecular mass of the β-chain to 38 kD (Hanley et al., 1983). When hepatocytes are homogeneously labeled with [^{35}S]methionine the 45, 35, and 38 kD polypeptides constitute ~40, 50, and 10%, respectively, of the total intracellular amount of labeled protein (see Fig. 4 *B*, the leftmost lane). Newly synthesized haptoglobin is secreted from isolated rat hepatocytes after a lag period of 20 min with a $t_{1/2}$ of 40 min (Fries and Lindström, 1986).

To measure haptoglobin secretion we labeled hepatocytes with [^{35}S]methionine for 120 min, which yields essentially homogeneous labeling of this protein. This procedure was chosen instead of pulse labeling for two reasons: first, haptoglobin is produced in relatively small amounts and is therefore poorly labeled during a pulse; second, and more impor-

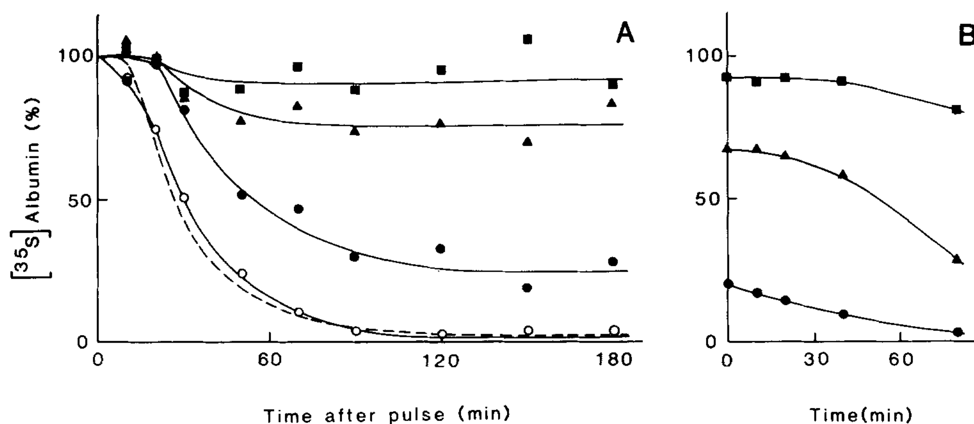

Figure 3. (*A*) Inhibition of secretion of pulse-labeled albumin by azide. Hepatocytes were labeled for 10 min with [^{35}S]methionine, and then chased in media containing different concentrations of azide (no azide, - - - -; 2.0, ○; 4.0, ●; 6.0, ▲; and 10.0 mM azide, ■). At the indicated times the media were withdrawn and the cells solubilized. Cellular albumin was detected by immunoprecipitation followed by PAGE and autoradiography, and quantitated by densitometric scanning of the autoradiogram. (*B*) Reversion of the inhibitory effect of azide on albumin secretion. Cells which had been pulse-labeled with [^{35}S]methionine and chased in the presence of different concentrations of azide for 150 min (see above) were incubated in azide-free medium. At the indicated times the media were withdrawn and the amount of radioactive albumin in the cells was quantitated.

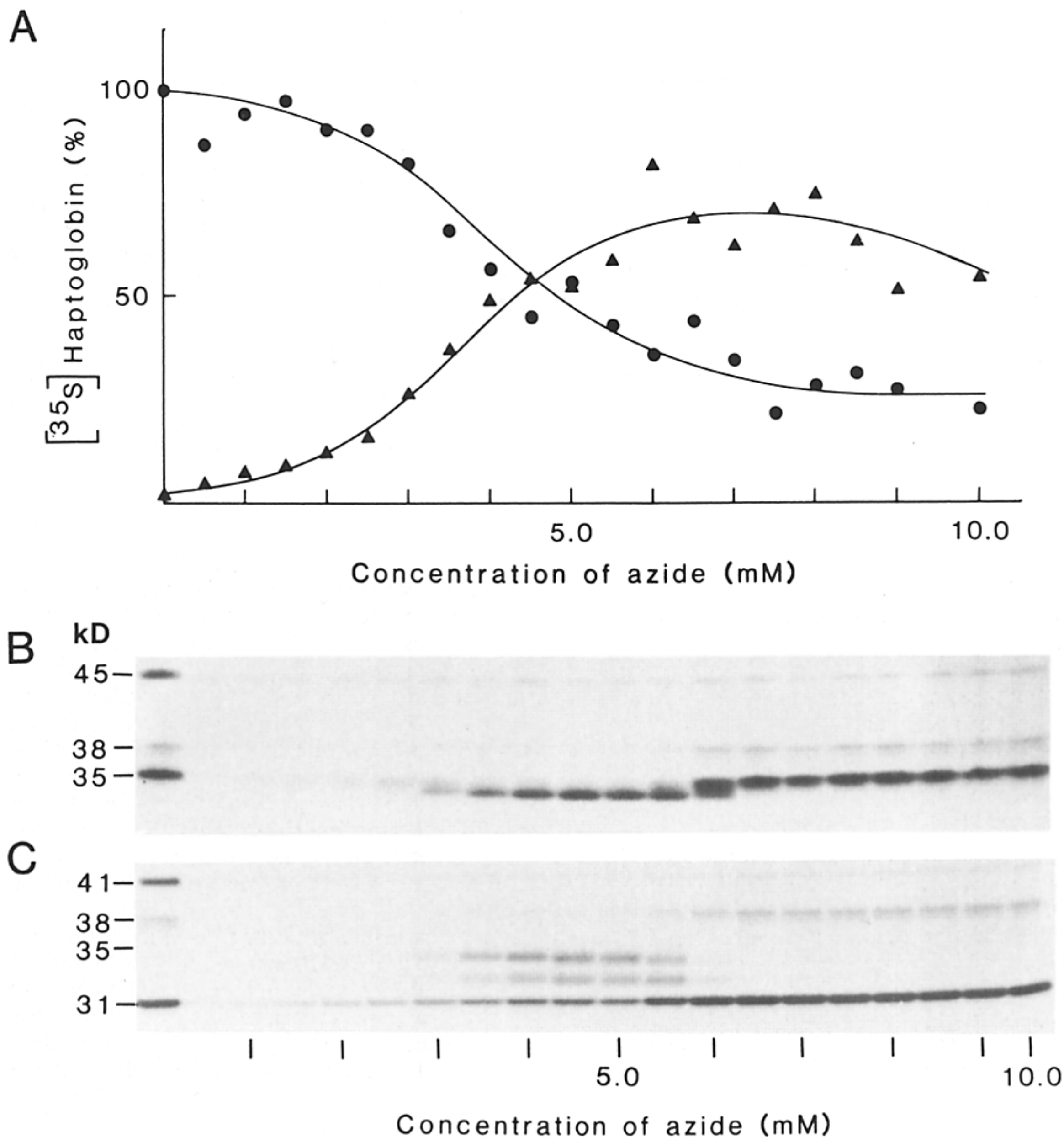

Figure 4. Inhibition of secretion and processing of haptoglobin by azide. Hepatocytes were labeled for 120 min with [^{35}S]methionine to yield homogeneous labeling of haptoglobin. The cells were then chased for 150 min in the presence of various concentrations of azide. (*A*) The relative amount of radioactive haptoglobin in the solubilized cells (▲) and the corresponding media (●) after incubation at the different azide concentrations. Quantitation was done as described in the legend of Fig. 3 *A*. (*B*) Autoradiogram of the relevant part of the electrophoresis gel used for quantifying the radioactively labeled haptoglobin in cells treated with azide of the indicated concentrations. The lane to the far left shows haptoglobin from cells harvested immediately after the labeling procedure. Molecular masses of the relevant polypeptide forms of haptoglobin are indicated. Haptoglobin is synthesized as a 45-kD polypeptide which is converted in the ER by proteolytic cleavage to a 35-kD polypeptide. In the *trans* GC, the apparent molecular mass of the 35-kD form shifts to 38-kD due to carbohydrate processing; both these forms can be seen in the samples from cells treated with high azide concentrations (6-10 mM), which block all transport steps. At lower azide concentrations (4-6 mM) an intermediate form of haptoglobin (34 kD) accumulates. (*C*) Autoradiogram of the same samples as in *B* after treatment with endo H.

tantly, after homogeneous labeling the transport from both the ER and GC can be followed simultaneously, since haptoglobin can be electrophoretically distinguished in these organelles. After labeling, the cells were chased for 150 min in the presence of different concentrations of azide. Samples of solubilized cells and media were then incubated with antibodies against haptoglobin and the immune complexes were analyzed by gel electrophoresis. The quantitative results are presented in Fig. 4 *A* and demonstrate that haptoglobin secretion was almost unaffected by azide concentrations up to 2.0 mM (i.e., at ATP levels down to ~50% of that found in untreated cells). In the presence of intermediate concentra-

tions of azide, between 3 and 5 mM, the inhibitory effect on the secretion was proportional to the concentration of the inhibitor. In cells incubated with 4.0 mM azide, ~50% of the haptoglobin remained intracellularly. Interestingly, only 25–30% of albumin was arrested at 4.0 mM azide, irrespective of whether the cells were labeled for 10 (see Fig. 3 *A*) or 60 min (data not shown). Secretion of haptoglobin was inhibited by 70–75% at concentrations above 6.0 mM azide; i.e., at ATP levels below 10% of the normal value. The secretion of albumin was inhibited to ~95% under the same conditions (see Fig. 3 *A*). It is unclear why the two proteins are differentially affected by reduced ATP levels.

One of the autoradiograms used to obtain the data in Fig. 4 *A* is shown in Fig. 4 *B*. The pattern obtained after incubation with high azide concentrations demonstrates, in agreement with Hanley et al. (1983), that conversion of prohaptoglobin (molecular mass, 45 kD) occurs in the absence of oxidative phosphorylation. The apparent molecular masses of the two major forms of haptoglobin arrested in cells incubated with azide at concentration >6.0 mM, 35 and 38 kD, were identical to two of the polypeptides found in untreated cells (Fig. 4 *B*, the leftmost lane). In agreement with Hanley et al. (1983), we found that the 38-kD polypeptide was sensitive to sialidase treatment (see below), demonstrating that this form of haptoglobin was in the *trans* GC. Thus, transport from this compartment to the medium was blocked at azide concentrations >6.0 mM. At ~6 mM azide, a new form of the β-chain could be seen, which had a molecular mass of 34 kD; at azide concentrations between 3.0 and 5.5 mM this was the dominant form.

Treatment with endo H showed, as expected, that the 38-kD β-chain was resistant and that the 35-kD chain was sensitive to the enzyme (Fig. 4 *C*). However, the 34-kD form turned out to be heterogeneous with respect to endo H sensitivity. At 6.0 mM azide, most of it was sensitive whereas at lower azide concentrations both a fully and a partially resistant form could be demonstrated. In quantitative terms, at 4.0 mM azide 30% was fully and 25% partially resistant. Haptoglobin most likely contains two N-linked oligosaccharide chains (Goldstein and Heath, 1984; Ogata et al., 1986) and it is possible that the partially endo H–resistant form resulted from only one of the chains being resistant to endo H treatment. Since endo H resistance is acquired in the *medial* GC, these experiments show that most of the arrested haptoglobin (55%) had reached this compartment. The endo H–resistant form of haptoglobin was not sensitive to sialidase treatment (Fig. 5), indicating that transport from the *medial* to the *trans* GC was inhibited. When the cells were incubated for even longer times (180 min) in the presence of 4.0 mM azide, some 75% of the arrested haptoglobin became partially or fully endo H resistant (data not shown).

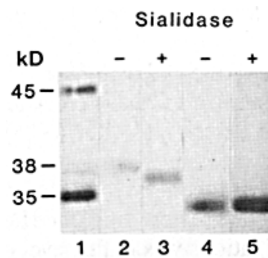

Figure 5. Sialidase treatment of immunoisolated haptoglobin. Two cultures of hepatocytes were labeled for 120 min with [^{35}S]methionine. One of them was further incubated for 150 min in the presence of 4.0 mM azide. Lane *1*, haptoglobin from the cells harvested immediately after the labeling procedure. Lanes *2* and *3*, the effect of sialidase treatment on haptoglobin from the medium of the azide-treated cells; and lanes *4* and *5*, the same effect on the corresponding cellular haptoglobin. The molecular masses of the major forms are shown to the left.

Discussion

In this communication we have used azide to reduce the intracellular ATP content of rat hepatocytes with the purpose of studying the energy-dependent steps of protein secretion. We initially established that it was possible to obtain stable, reduced levels of ATP by incubating the cells with different concentrations of azide. The azide treatment, at least at concentrations ≤4 mM, did not greatly affect the permeability of the plasma membrane as judged by the ability of the cells to exclude a vital stain and by the low degree of release of a soluble cytoplasmic protein. In addition, the effect of azide on the ATP content was reversible with a doubling of the ATP concentration occurring within 20 min after removal of the reagent.

We found that if the ATP level in rat hepatocytes was reduced to <50% of the normal level, secretory proteins were arrested intracellularly. Furthermore, we observed that the carbohydrate structure of an arrested glycoprotein (haptoglobin) was affected by the ATP level. Since the processing of the carbohydrates of secretory proteins occurs in different compartments along the secretory pathway, this finding indicated that the transport between these compartments required different threshold ATP concentrations. In cells with <10% of the normal ATP level (>6 mM azide), the secretion of haptoglobin was maximally inhibited. Under these conditions, the relative amount of sialylated haptoglobin was similar to the steady-state value in nontreated cells, indicating that transport from the *trans* GC to the cell surface was blocked. Furthermore, most of the haptoglobin in these cells (which had an apparent molecular mass of 35 kD) was endo H sensitive, suggesting that transport from the ER to the *medial* GC was also blocked. In cells with a slightly higher ATP concentration, 10–15% of normal (6 mM azide), part of the arrested haptoglobin appeared in a new form with an apparent molecular mass of 34 kD. After treatment with endo H, the electrophoretic mobilities of both these forms were the same, demonstrating that the difference was due only to the carbohydrate structure. At progressively higher ATP levels (up to 40% of the normal value), an increasing portion of the 34 kD form became endo H resistant. Interestingly, the shift in the apparent molecular mass from 35 to 34 kD occurred before the appearance of endo H resistance, indicating that it reflected a carbohydrate processing step preceding the one that confers endo H resistance. In the *cis* GC, two to three mannose residues are removed from the mannose-rich glycans arriving from the ER and the oligosaccharides formed are substrate for the glycosyltransferase in the *medial* GC that renders the glycans endo H resistant. Presumably the initial reduction in the apparent molecular mass reflected the action of the mannosidase in the *cis* GC; this has been clearly demonstrated to be the case for the G protein in cells infected with VSV (Balch et al., 1986). Thus, our results indicate that transport of haptoglobin from the ER to

the *cis* GC is blocked if the ATP level is reduced to <10% of the normal level.

The energy requirements for the intracellular transport of the G protein of VSV have been studied in detail by Balch and co-workers (Balch et al., 1986; Balch and Keller, 1986). The threshold ATP levels for the different transport steps they observed were, in general, higher than the corresponding values presented in this study. The greatest difference between the results of the two investigations was in the level of ATP needed for protein exit from the ER. Balch et al. (1986) showed that exit of newly synthesized G protein from the ER requires an ATP level >80% of the normal level, as compared with 10–15% in our system. One possible reason for this discrepancy could be that the experiments on G protein transport were performed under conditions where this protein aggregates within the ER. Dissociation of this aggregate, which is essential for the export of the protein from the organelle, requires ATP (Doms et al., 1987). Another reason could be that in our experiments the cells were first labeled with [^{35}S]methionine and then incubated with azide to reduce their ATP content. In contrast, Balch et al., who used a temperature-sensitive strain of VSV, could both label the infected cells and reduce the cellular ATP content while the transport of G protein was blocked. To investigate if the radiolabeled proteins could pass a critical step while the ATP level was being reduced, we treated hepatocytes with 2.0 mM azide for 60 min (which yielded ~50% of the normal ATP level) and pulse-labeled with [^{35}S]methionine. The secretion of albumin in the presence of 2 mM azide was then measured; the amount secreted was the same as with the standard procedure (data not shown). Finally, a third reason for the different results could be that there are differences in the energy dependence for the transport of membrane and secretory proteins.

The most striking finding in this study was that a "new" polypeptide form of haptoglobin (with a molecular mass of 34 kD) was arrested in cells with 10–40% of the normal ATP concentration (3–6 mM azide). Endo H treatment showed that part of this haptoglobin was fully endo H resistant, whereas some migrated with a mobility between that of fully resistant and sensitive forms (see Fig. 4). Rat haptoglobin probably has two N-linked glycans (Goldstein and Heath, 1984; Ogata et al., 1986) and the partially endo H–resistant form was presumably one on which only a single glycan had become resistant. The resistance to endo H is acquired through the transfer of a GlcNAc residue from UDP–GlcNAc to the protein glycan. The corresponding transferase has been localized by immunoelectron microscopy to the *medial* GC in rabbit hepatocytes (Dunphy et al., 1985). The synthesis of the carbohydrate donor requires UTP whose concentration is probably affected by the intracellular ATP level. In cells treated with azide, the transfer of GlcNAc residues will therefore occur at a lower rate. Thus, under conditions where the ATP concentration is reduced and an arrested secretory protein is found to be sensitive to endo H, one must consider the possibility that transport has occurred but transfer of the glycosyl groups has not taken place because of lack of donor. Therefore, our observation alone that haptoglobin is not converted to an endo H–resistant form in cells with ATP levels between 10 and 15% of normal, does not show that transport to the *medial* GC was blocked. However, the activity of the mannosidase located in the *cis* GC is not affected by low ATP concentrations (Tulsiani et al., 1977). With the assumption that the shift in the apparent molecular mass of haptoglobin (from 35 to 34 kD) marks the action of this enzyme, our results show that transport from the ER to the *cis* GC does occur at ATP levels between 10 and 15% of normal. Similarly, the transfer of sialic acid residues from CMP–sialic acid to protein glycans is probably also affected by the ATP level. Hence, the absence of sialic acid residues can not be taken as evidence that the arrested haptoglobin has not entered the *trans* GC. However, two pieces of evidence indicate that the majority of the haptoglobin (originally in the ER) did not enter the *trans* GC in cells with 10–40% of the normal ATP level. First, upon removal of the azide the partially endo H-resistant form became fully resistant (unpublished observation), showing that at least that fraction of the protein was still in the *medial* GC at the end of the inhibition period. Secondly, the secretion of the haptoglobin residing in the *trans* GC at the beginning of the azide treatment (the 38-kD form, see Fig. 4) occurred at an ATP (azide) concentration slightly lower (higher) than that at which endo H-resistant haptoglobin appeared (see Fig. 4). Hence, (endo H-resistant) haptoglobin, which would have entered the *trans* GC in cells with 10–40% of the normal ATP level, would have been secreted. This was not the case since the level of intracellularly arrested haptoglobin was constant during the latter part of the incubation period (shown for albumin in Fig. 3). Taken together, these results indicate that the majority of the haptoglobin that was arrested in cells with 10–40% of the normal ATP level was in the *medial* GC. Clearly, further studies are required to define more accurately the location of the arrested protein.

An accumulation of a viral membrane protein in the *medial* GC, similar to that described for a secretory protein in this paper, was found in baby hamster kidney cells which had been treated with 10 μM monensin (Griffiths et al., 1983). In the latter case, the GC was found to be vesicularized. To ascertain whether the effect of azide was related to that of monensin, we examined the azide-treated cells by EM; no morphological changes of the ER or the GC were seen. We also treated hepatocytes with 10 μM monensin for 4 h and then analyzed the intracellular transport of radiolabeled haptoglobin. Transport appeared to be blocked at a number of steps, both between the ER and the *medial* GC, between the *medial* and the *trans* GC, and from the *trans* GC to the cell surface (Persson, R., and E. Fries, unpublished results).

The secretion of albumin and haptoglobin was affected by reduced cellular ATP levels in a narrow concentration range; in cells with a 50% ATP content secretion was not impaired, while it was maximally inhibited at ATP concentrations <10% of the value in the control cells. A similar sharp ATP dependence was found for the intracellular transport of the G protein of VSV (Balch and Keller, 1986), and has also been reported for other systems of intracellular membrane traffic; e.g., for continuous endocytosis of asialo-orosmucoid (Clarke and Weigel, 1985).

Recent studies with a cell-free system, derived from cells infected with VSV, have indicated that transfer of a membrane protein from one cisterna to another is mediated by small coated vesicles, which can be seen to bud out from the

rim of the cisternae. The formation of these buds is inhibited in the absence of ATP (Orci et al., 1986). It is possible that the accumulation of a secretory protein, which we have described here, is due to differential inhibition of this vesicular traffic within the Golgi apparatus.

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