

# A soluble secretory protein is first concentrated in the endoplasmic reticulum before transfer to the Golgi apparatus

(secretion/hepatoma cells/immunoelectron microscopy)

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Contributed by S. J. Singer, March 18, 1993

**ABSTRACT** A soluble secretory protein is usually present at a much higher concentration in the Golgi apparatus than in the endoplasmic reticulum (ER) inside eukaryotic secretory cells in the steady state. We show by immunoelectron microscopic experiments with the soluble secretory protein serum albumin, inside Hep-G2 human hepatoma cells in culture, that the secretory protein is first concentrated at isolated sites within the ER before it is transferred to the cis face of the Golgi apparatus. This is contrary to expectations of the bulk-flow hypothesis of ER-to-Golgi transfer, and it suggests the involvement of concentration and transfer mechanisms within the ER that have not previously been recognized.

This paper is concerned with the segment of the secretory pathway from the endoplasmic reticulum (ER) to the Golgi apparatus in eukaryotic cells. Soluble secretory proteins are thought to be transferred out of the ER in the lumen of transition vesicles that bud off the ER, are then processed through an intermediate compartment (1), and eventually arrive at the cis face of the Golgi apparatus (2). An important factor concerning this transfer is that in the steady state the average concentration of a secretory protein is at least an order of magnitude larger in the Golgi apparatus than in the ER (3, 4). This is also consistent with the qualitative results of a great many immunofluorescence microscopic experiments (cf. ref. 5), which, at the resolution of the light microscope, have invariably shown a much more intense immunofluorescence for a soluble secretory protein over the Golgi region than in the cytoplasm of a steady-state cell.

How and where is this increase in concentration accomplished? A widely held view of the transfer process is that it occurs by simple bulk flow (6, 7). This view envisions the random capture of a soluble secretory protein, at its average low concentration in the ER, into transition vesicles that are continually budding from the ER; upon arrival at the Golgi apparatus, the secretory protein is unloaded and then the vesicular membrane is recycled back to the ER. It is the accumulated effect of such unidirectional transfers that is thought to build up the concentration of the secretory protein in the Golgi apparatus (the protein is simultaneously being removed from the Golgi apparatus into the following stages of the secretory pathway).

The main support for the bulk flow hypothesis has come from model experiments with simple peptides (7), but the interpretation of these experiments has recently been challenged (8). Furthermore, the hypothesis would require that a massive amount of membrane recycling occur between the ER and the Golgi apparatus, corresponding in a typical case to a flux of about half the mass of membrane phospholipids out of the ER every 10 min (4, 7).

One alternative possibility is that a soluble secretory protein is first concentrated at a number of sites in the ER and that transition vesicles that bud from the ER form only, or predominantly, so as to include such sites. Compared to the bulk flow process, this would clearly involve a much smaller flux of membrane mass out of the ER to accomplish the same overall rate of transfer of the soluble secretory protein from the ER to the Golgi apparatus. On the other hand, since the transfer process is thought to occur by a "default" pathway (4, 6), which implies that secretory proteins do not contain a signal that directs them to be transferred from the ER to the Golgi apparatus, such a concentration of a secretory protein in the ER, and the incorporation of the concentrate into a transition vesicle, would require that some kind(s) of concentration and recognition mechanisms operate that are not contemplated at present.

Direct experimental evidence bearing on these questions is sparse and, indeed, is not easy to obtain. In order to address this problem, we have designed immunoelectron microscopic experiments to study the process of ER to Golgi transfer of a soluble protein inside intact secretory cells. The secretory pathway followed by serum albumin (SA) within human hepatoma cells was chosen for study, because the massive synthesis and secretion of SA by these cells allow an adequate sensitivity of detection in our immunolabeling experiments. In order to select elements in the vectorial transfer of SA from the ER to the Golgi apparatus, and distinguish them from the rest of the secretory pathway, the following procedure was employed. The cultured hepatoma cells were first treated with cycloheximide for 2.5 hr to stop new protein synthesis and to empty the cells of their previously synthesized SA by secretion, which is not affected by cycloheximide (5). Upon removal of the cycloheximide, protein synthesis was resumed, and a more-or-less synchronous wave of transfer of SA from the ER to the Golgi apparatus could be detected immunocytochemically within a period of around 20 min at 37°C. At several times during this interval, cells were fixed, frozen-sectioned, and immunolabeled with polyclonal antibodies to SA and colloidal gold-labeled secondary antibodies.

These experiments have shown unequivocally that SA is first concentrated at sites in the ER before transfer to the Golgi apparatus, to a concentration comparable to that attained by SA within the Golgi apparatus at steady state. Several years ago, preliminary results were obtained by G.-A. Keller in this laboratory (9) which led us to suggest the same conclusion tentatively.

## MATERIALS AND METHODS

**Immunochemical Reagents.** Rabbit polyclonal antibodies were raised to human SA (Sigma) and were affinity-purified

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Abbreviations: ER, endoplasmic reticulum; SA, serum albumin.  
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by standard methods. As the secondary antibody reagent, 5-nm colloidal gold-conjugated goat anti-rabbit IgG (Janssen Life Sciences Products, Piscataway, NJ) was used.

**Cell Culture and Specimen Preparation.** Hep G2 human hepatoma cells were grown on 60-mm tissue culture dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum in a 5% CO<sub>2</sub> humidified atmosphere. After 24 hr in culture, some cells in the steady state were directly processed for immunolabeling as described below. Most of the cells, however, were incubated with cycloheximide at 50  $\mu$ g/ml in DMEM for 2.5 hr at 37°C. After this time, the drug was rapidly washed out by four changes of serum-free medium, and protein synthesis was resumed in fresh serum-free medium at 37°C. It was found by immunofluorescence microscopic labeling experiments that by 21 min after resumption of protein synthesis, SA reappeared throughout the Golgi apparatus, and accordingly, in order to focus on the ER-to-Golgi traffic, cells were processed at 3, 10, and 21 min after cycloheximide was removed. The cells were washed twice with sodium phosphate-buffered saline (PBS), pH 7.4, and fixed with 3% formaldehyde containing 0.1–0.5% glutaraldehyde in a 0.1 M sodium phosphate buffer, pH 7.4, for 1–2 hr at room temperature.

**Immunoelectron Microscopy.** The cells, fixed as described above, were scraped from the dishes, immersed in 2.3 M sucrose in 0.1 M sodium phosphate buffer, pH 7.4, containing

0.02% sodium azide, and then frozen and cryosectioned with a Reichert OMU-4/FC-4 cryoultramicrotome at  $-110^{\circ}\text{C}$ , essentially as described (10). Ultrathin sections 60–90 nm thick were thawed on grids and immunolabeled by incubation with the affinity-purified polyclonal rabbit anti-human SA antibodies at a concentration of 10–20  $\mu$ g/ml for 45 min and, after washing, with the colloidal gold-conjugated goat anti-rabbit IgG antibodies for 60 min. The sections were post-stained with neutral and acidic uranyl acetates and embedded in polyvinyl alcohol (11). The specimens were observed by using a JEOL 1200 EX or a Phillips EM-300 electron microscope operated at 60 or 80 kV.

## RESULTS

**Steady State.** With Hep-G2 cells in the steady state, the highest average concentration of SA inside the cell, as revealed by the density of gold labels, was in the Golgi apparatus (G, Fig. 1). Scattered gold particles were seen in elements situated between the nucleus (N) and the Golgi apparatus. In addition, there were occasional localized sites in the ER including the nuclear envelope (circles with long arrows, Fig. 1) which showed clusters of several gold labels. The numbers of labels per unit area in such a localized ER site (four to eight gold particles within a circle of 750-Å diameter)

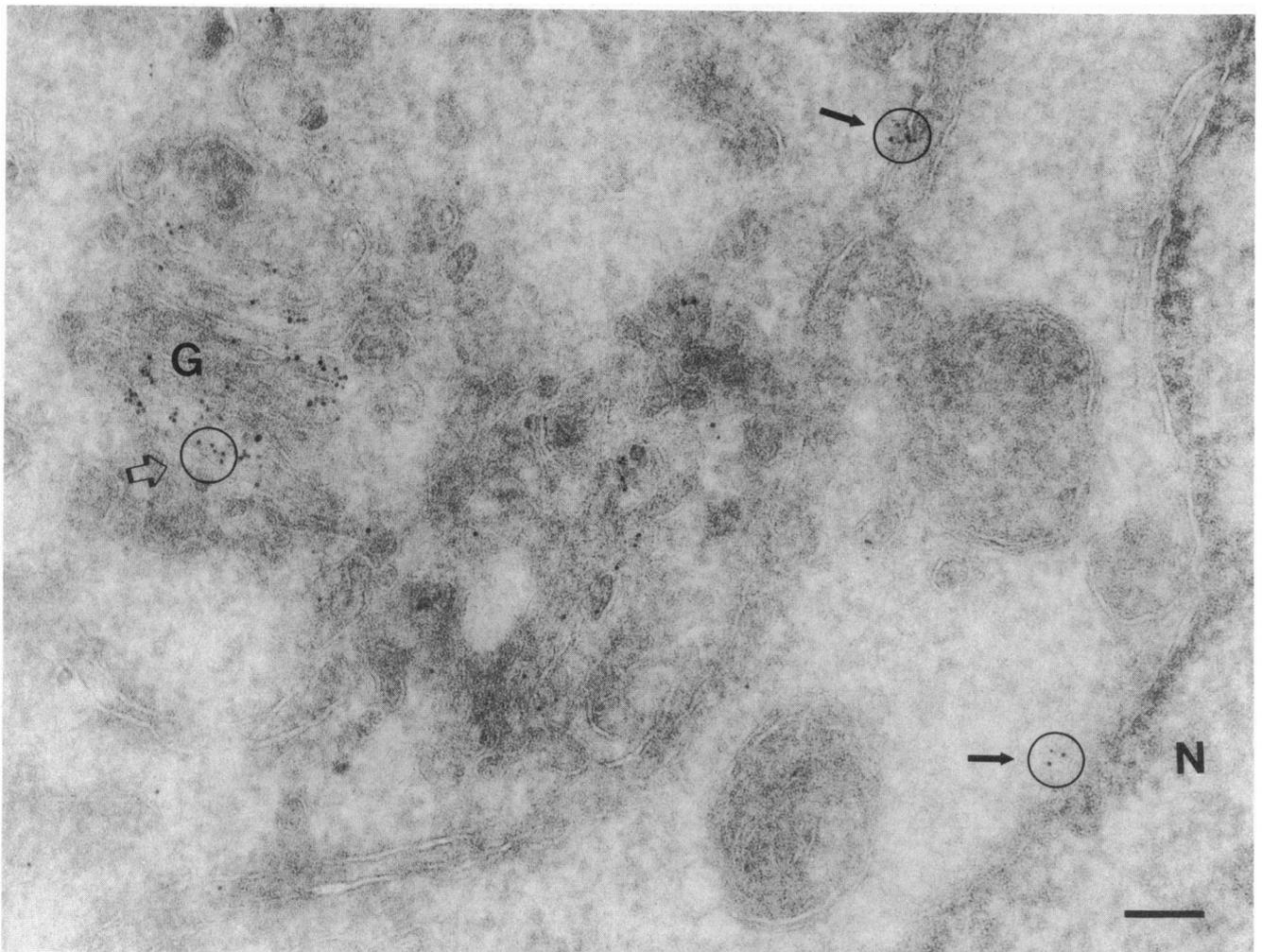


FIG. 1. Figs. 1–3 are electron micrographs of fixed ultrathin frozen sections of cultured Hep G2 cells indirectly immunolabeled for human SA by using 5-nm colloidal gold-conjugated secondary antibodies. Cells in this figure are in the steady state. Clusters of gold labels in the ER are encircled and designated with long arrows. A circle of the same diameter marks a cluster of labels in the Golgi apparatus (G), designated by a short open arrow. Note scattered individual gold particles in the region between the Golgi apparatus and the nucleus (N). (Bar = 0.1  $\mu$ m.)

were comparable to the numbers of labels per unit area within the Golgi apparatus (short open arrow and circle, Fig. 1).

**Cycloheximide-Treated Cells.** Hep G2 cells were treated with cycloheximide as described in *Materials and Methods*, and sections of some of the cells were immunolabeled for SA immediately after the cycloheximide was washed out. Typically, in fields such as those in Figs. 1–3, no or very few gold labels were found (not shown). This demonstrates that the cycloheximide treatment did indeed remove all previously synthesized SA from the cells and that nonspecific labeling was negligible.

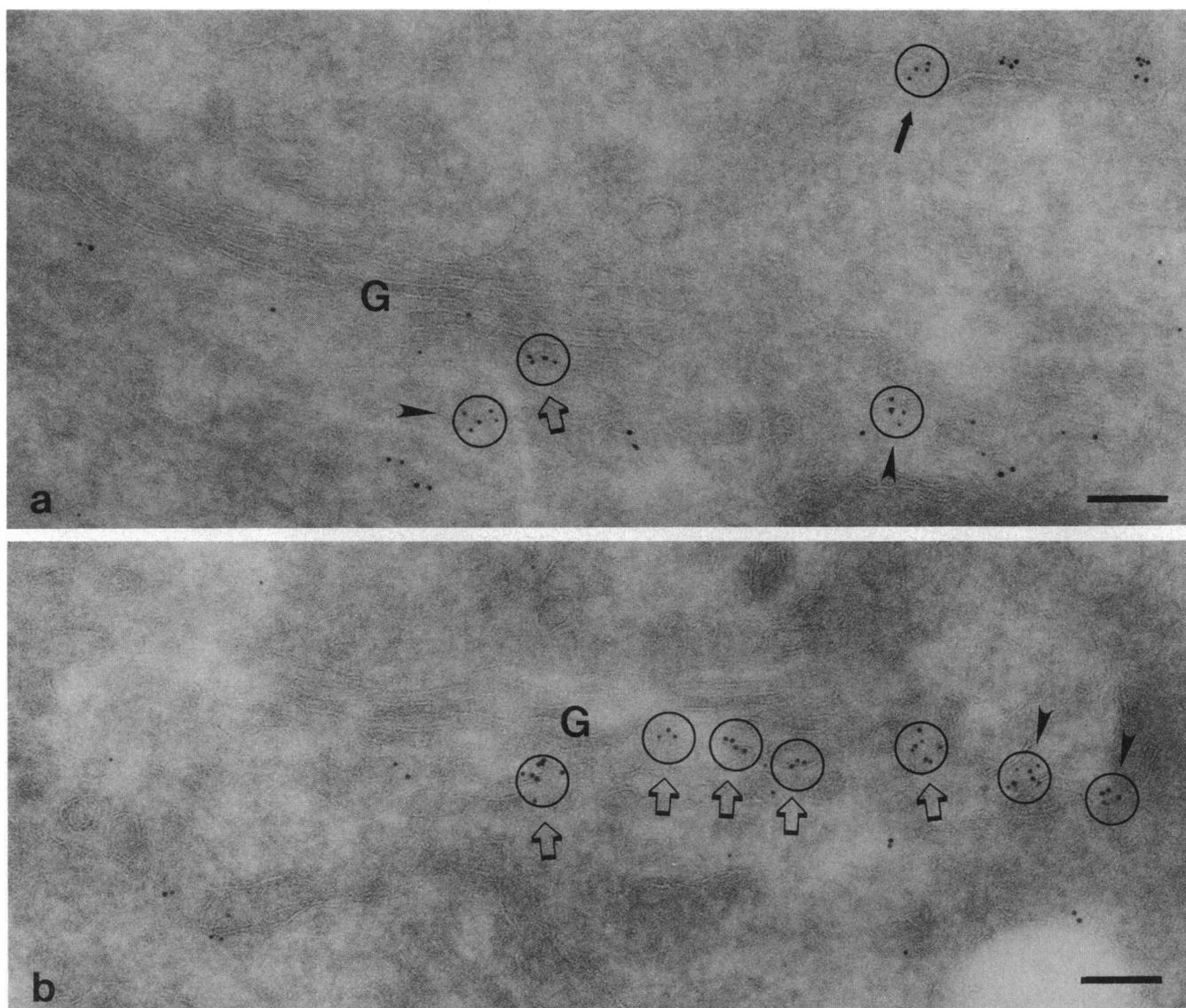
By 10 min after the washout of the cycloheximide and the resumption of SA synthesis, significant immunogold labeling for SA was seen (Fig. 2). The Golgi apparatus was still mostly free of labels, but clusters of gold particles (as demarcated by circles and short open arrows) appeared by this time to be entering the Golgi apparatus, presumably therefore at the cis face. In addition, other clusters of labels were observed (circles with arrowheads) which appeared not to be associated with the Golgi apparatus, and which must therefore have

been either in the ER or in transition between the ER and the Golgi apparatus.

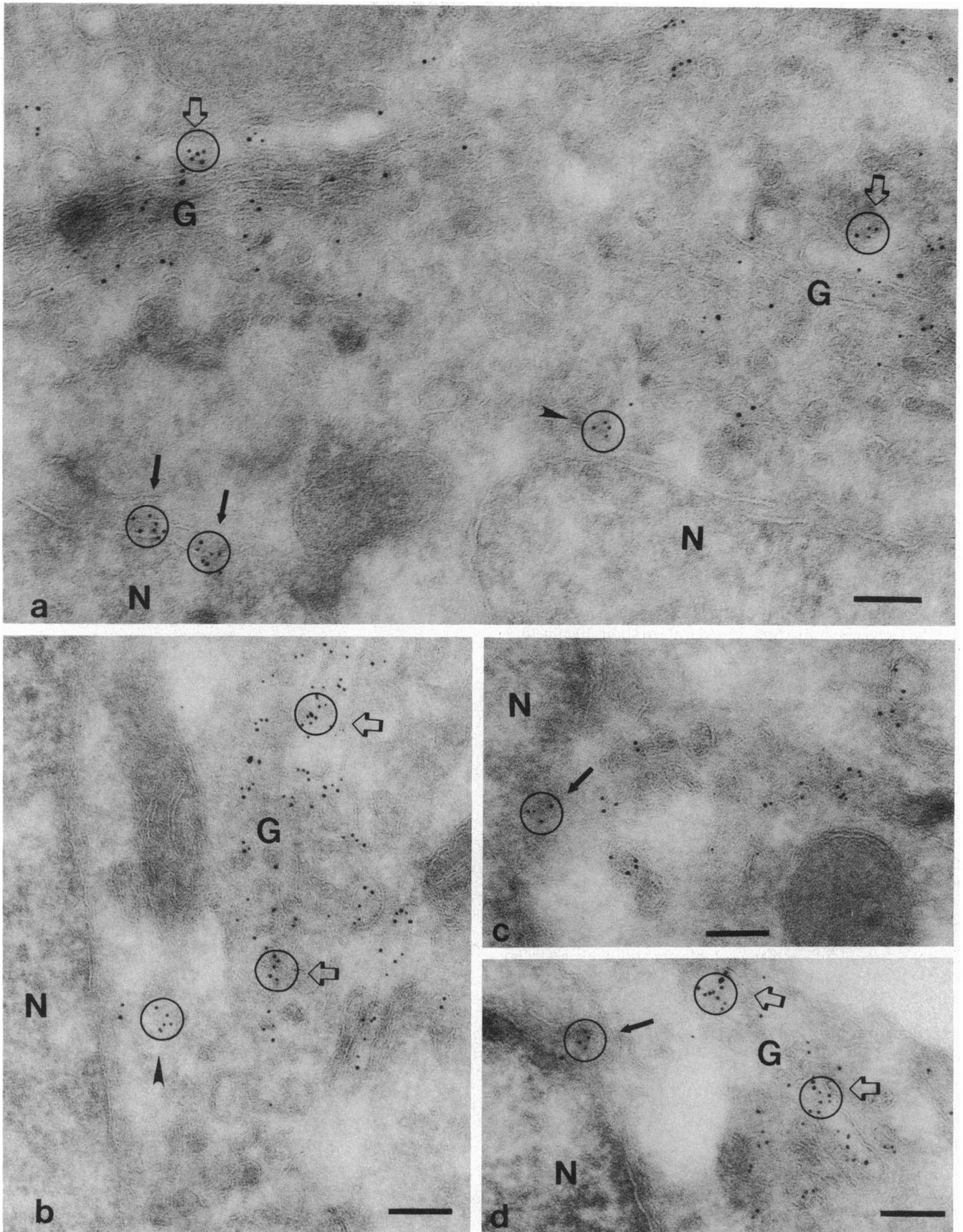
By 21 min after washout of the cycloheximide, the overall labeling for SA had increased substantially (Fig. 3). SA was now present throughout the Golgi stacks (G in Fig. 3 *a, b*, and *d*) but not yet in secretory elements beyond the Golgi apparatus (not shown). Of particular interest for our purposes was the appearance of isolated clusters of gold labels in the ER at the nuclear envelope (circles with long arrows in Fig. 3 *a, c*, and *d*). The numbers of labels per unit area in such isolated ER clusters are comparable to the maximum numbers of labels per unit area in the Golgi apparatus (see circles with short open arrows in Fig. 3 *a, b*, and *d*).

## DISCUSSION

The average concentration of a soluble secretory protein in the Golgi apparatus of a steady-state cell is known to be generally at least one order of magnitude larger than its average concentration in the ER (3, 4). This conclusion is



**FIG. 2.** Cells 10 min after washout of cycloheximide and resumption of protein synthesis. (*a*) A cluster of gold labels in the ER is encircled and designated with a long arrow. Other clusters in a pre-Golgi compartment, perhaps a transition vesicle, are encircled and designated with an arrowhead. Another cluster at the cis face of a Golgi apparatus (G) is encircled and designated with a short open arrow. (*b*) Several clusters of gold labels at the cis face of a Golgi stack are visible (circles with short open arrows), but the remainder of the Golgi apparatus is still unlabeled at this early time. Other clusters in a pre-Golgi compartment are encircled and designated with arrowheads. (Bars = 0.1  $\mu\text{m}$ .)



**FIG. 3.** Four examples of cells 21 min after resumption of protein synthesis. In each panel, representative clusters of gold labels are encircled; they are designated with a long arrow for the ER on the nuclear envelope, with an arrowhead for a presumptive transition vesicle, and with a short open arrow for the Golgi apparatus (G). By this time, the Golgi apparatus is labeled throughout. N, nucleus. (Bars = 0.1  $\mu\text{m}$ .)

confirmed by the high-resolution immunoelectron microscopic experiments herein described. Visual inspection shows that the *average* density of immunogold labeling for SA throughout the Golgi stacks (Fig. 1; Fig. 3 *a, b, and d*) when the Golgi apparatus was filled with secretory protein was much greater than the *average* density of labeling over the ER (e.g., of the nuclear envelope); in fact, in the latter case, most areas of the ER showed no labeling at all. However, and this is the key finding of the present paper, in isolated areas of the ER clusters of four to eight immunogold labels were often found, both in cells in the steady state (Fig. 1, circles with long arrows) and in cells shortly after SA synthesis was reinitiated (Fig. 3 *a, c, and d*, circles with long arrows). The numbers of gold labels per unit area in such ER clusters were similar, on average, to the maximum numbers of labels per unit area within the filled Golgi apparatus. To help visualize this similarity of labeling densities circles of 750 Å were drawn around the labels in Figs. 1–3.

We conclude from this result that there are isolated sites in the ER where the concentration of SA attains a value close to the average of that found throughout the Golgi apparatus. The absolute value of this concentration is difficult to derive from the density of gold labels for a number of reasons, including the unknown degree to which SA is solubilized and lost from the section in the course of the glutaraldehyde fixation and preparation of the section, and the degree of accessibility of the polyclonal primary and secondary antibodies to their respective antigenic epitopes in the section. This uncertainty about absolute concentrations does not, however, affect the conclusion that the *relative* concentrations of SA in the isolated clusters in the ER and throughout the Golgi apparatus are closely similar.

Another important finding is that in the initial entry of SA into the Golgi apparatus (which takes place about 10 min after washout of the cycloheximide and reinitiation of SA synthesis) clusters of four to eight immunogold labels (Fig. 2) were observed at the cis face which were closely similar to those seen in the ER (Figs. 1 and 3). This strongly suggests that it is the SA in the isolated clusters in the ER which is then transferred to the cis face of the Golgi apparatus.

Our results are therefore consistent with the conclusion that the SA is first concentrated at isolated sites in the ER before transfer out of the ER and into the Golgi apparatus. There appear to be no further concentration steps operating for this constitutively secreted protein either in the intermediate compartment or within the Golgi apparatus beyond that occurring in the ER, judging from the closely similar densities of gold labels for SA in the ER clusters and throughout the Golgi apparatus when it is filled.

Apart from our earlier preliminary findings (9), we know of no other demonstration that a soluble constitutively secreted protein is concentrated in the ER *before* transfer to the Golgi apparatus in the physiologically normal secretory pathway. In exocrine pancreatic cells in the steady state, granules occasionally appear inside the ER (12) that contain soluble secretory protein in a concentrated form (13), but these

granules have generally been interpreted (14) as the consequence of a backing-up of the rest of the secretory pathway rather than as a normal concentration event occurring in the ER. In our studies, in which the cells were first emptied of their soluble secretory protein, the concentration of SA that we observe in the ER cannot be attributed to a backing-up of the secretory pathway.

As indicated in the Introduction, a transfer mechanism from the ER to the Golgi apparatus that involves a prior concentration of a soluble secretory protein in the ER, and the inclusion of the concentrate into transfer vesicles, makes much less demand on membrane recycling between the ER and Golgi apparatus than does the bulk transfer mechanism. However, it also raises many questions. How is the concentration achieved? Does it involve SA in its native or unfolded conformations? If unfolded, are chaperone proteins directly involved (cf. ref. 15)? Are other soluble secretory proteins included in the SA concentrate? [Of possible relevance here is the fact that different secretory proteins in the same cell can be transferred from the ER to the Golgi apparatus at significantly different rates (cf. ref. 16).] How is the concentrate recognized so as to be included in a vesicle budding from the ER? Must such concentrates be included for a bud to form? What is the relationship between the vesicular transfer of soluble secretory proteins and membrane integral proteins from the ER to the Golgi apparatus (17)? These and other questions now call for investigation.

We thank Mrs. Margie Adams for excellent technical help and Dr. K. T. Tokuyasu and Dr. Anne Dutton for advice and discussion. These studies were supported by National Institutes of Health Grant GM-15971 to S.J.S.

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