

## RECOVERY OF SURFACE MEMBRANE IN ANTERIOR PITUITARY CELLS

### Variations in Traffic Detected with Anionic and Cationic Ferritin

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#### ABSTRACT

Cells dissociated from rat anterior pituitaries were incubated with native or cationized ferritin (CF) to trace the fate of surface membrane. Native ferritin, which did not bind to the cell surface, was taken up in small amounts by bulk-phase endocytosis and was found increasingly (over 1–2 h) concentrated in lysosomes. CF at 100-fold less concentrations bound rapidly to the cell membrane, was taken up by endocytosis in far greater amounts, and within 15–60 min was found increasingly within multiple stacked Golgi cisternae, around forming secretion granules, and within elements of GERL, as well as within lysosomes. The findings demonstrate that the fate of the tracer—and presumably also that of the surface membrane—varies with the same molecule differing only in net charge: vesicles carrying anionic ferritin (net negative charge) fuse only with elements of the lysosomal system whereas those carrying CF (net positive charge) can fuse not only with elements of the lysosomal system, but also with elements along the secretory pathway (Golgi cisternae and condensing granules) as well.

**KEY WORDS** membrane recycling · secretion · anterior pituitary · cationic ferritin

Using dextran as a tracer, Herzog and I (12) have recently obtained evidence that after stimulation of exocytosis in exocrine cells of the rat lacrimal and parotid glands, luminal membrane is retrieved and reaches multiple cell compartments, i.e., the stacked Golgi cisternae, condensing vacuoles, and lysosomes. The new finding was the demonstration that the tracer can reach condensing vacuoles and many or most of the cisternae in a given Golgi stack. Earlier observations (cf. reference 12) had demonstrated that exogenous macromolecular tracers can reach lysosomes in secretory cells. Moreover, it had been shown in a few cases by us and by others (7, 10, 19) that a tracer taken up by endocytosis can reach the Golgi complex, but in these prior studies uptake was limited to a single cisterna on the trans-side of the Golgi stack.

In this communication, native anionic ferritin (pI = 4.6) and cationized ferritin (CF) (pI = ~8.5) have been used to trace the fate of surface membrane in dissociated anterior pituitary cells.

The findings obtained indicate that membrane is removed from the cell surface in vesicles and that the intracellular pathway taken by these vesicles is influenced by the net charge of the tracer.

#### MATERIALS AND METHODS

Pituitaries were obtained from young (150–200 g) male or female rats of the Sprague-Dawley strain. Some females were given six daily injections of 17  $\beta$ -estradiol (6) before sacrifice to stimulate secretion of prolactin by mammothrophs. Pituitaries were dissociated, and the resultant cell suspension was cultured overnight as described previously (13). Cells were then washed (by centrifugation at 70 g for 9 min through 4% bovine serum albumin) and resuspended in incubation medium. Experiments were carried out as follows: 2–4 ml of cell suspension ( $1-2 \times 10^6$  cells/ml) was incubated in Medium 109 (Grand Island Biological Co., Grand Island, N. Y.) containing CF (Miles Laboratories, Kankakee, Ill.) at concentrations of 0.05, 0.1, or 0.5 mg/ml or native anionic ferritin (cadmium-free; Calbiochem, San Diego, Calif.), 1 or 5 mg/ml. Incubation was carried out at 25° or 37°C. Cell samples were removed at intervals from 2 min to 3 h, fixed in Karnovsky's fixative, and processed as described elsewhere (6, 13). Sections were

stained briefly in lead or bismuth and examined in a Philips 301 electron microscope.

## RESULTS

Pituitary cell suspensions, like the gland *in situ*, contain five secretory cell types plus nonsecretory (endothelial and follicular) cells (7). This report emphasizes findings on the mammothroph or prolactin cell because more information is available on this cell type than the others (5-7). The mammothroph has variable numbers of 600-900 nm granules which are assembled in a stepwise fashion over a 3-h period (Fig. 1).

Approximately 95% of the cells cultured overnight excluded trypan blue. Control cells or those incubated in native ferritin, showed only a small decrease (to 85-95%) in viability over a 3-h incubation period. Cells incubated in the highest concentration of CF showed a decline in viability to ~70% at 1 h and ~60% at 2 h. However, at lower concentrations of CF, the number of surviving cells was greater (75% at 2 h). The observations recorded apply only to intact, undamaged cells.

When CF was added to a suspension of pituitary cells it immediately bound to the cell surfaces, and the cells agglutinated into clumps of 3-10. Binding of CF to the cell surface could be detected at the gross level because the pellets of cells incubated in CF were reddish-brown in color whereas those of controls were white. At the electron microscope level abundant CF molecules were seen adhering to the entire cell surface of mammothrophs at all concentrations used and at all time intervals (Figs. 2 and 3). Along the free surfaces of the cells, the ferritin was aggregated into clusters that varied in size from a few particles to large masses (Figs. 2 and 3). This clustering was evident as early as 2 min after exposure to the tracer. Along the cell



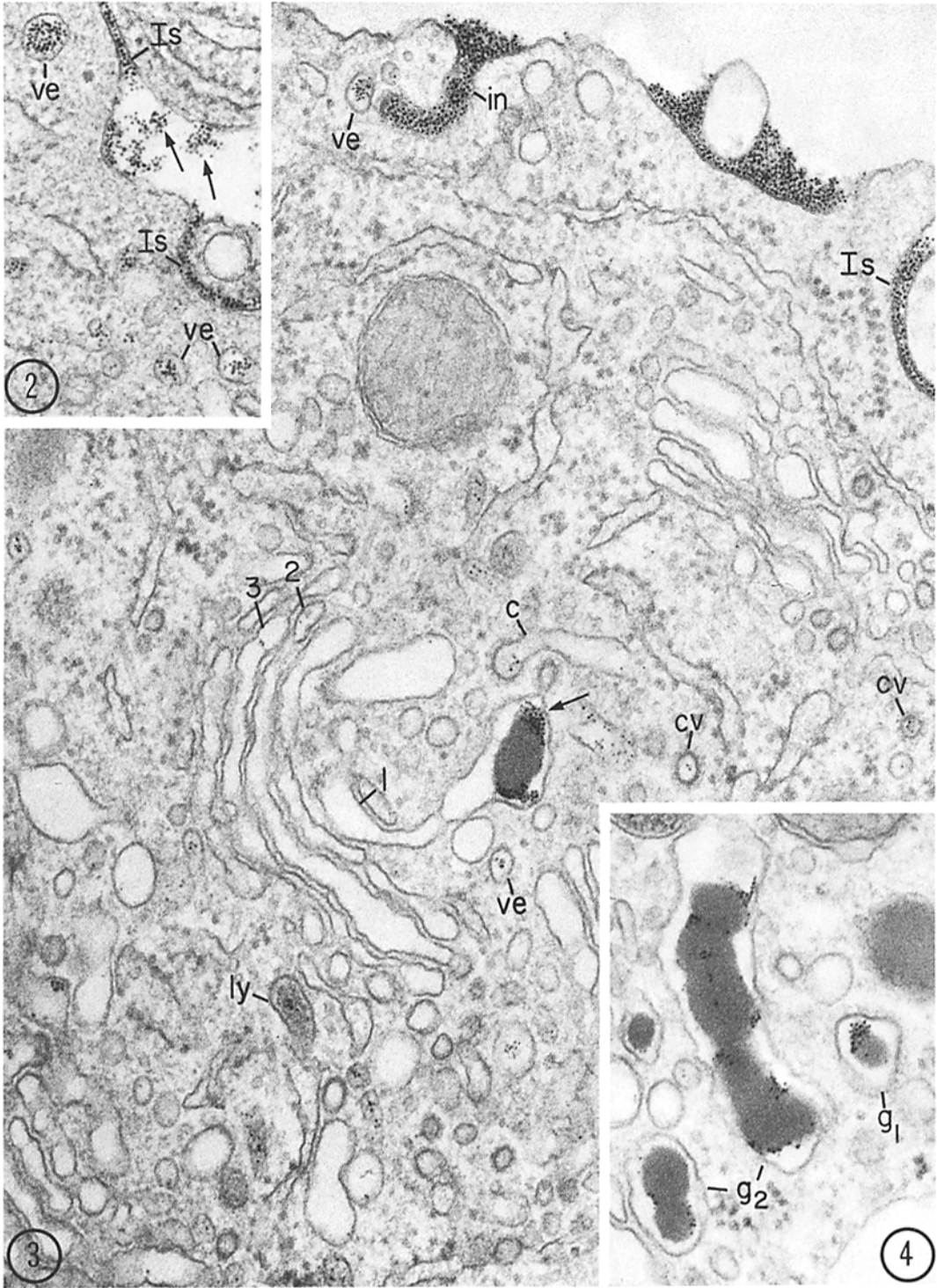
FIGURE 1 Diagram to show types of granules found in mammothrophs. Prolactin is initially concentrated in small (type I) granules within the transmost Golgi cisterna; these pinch off and aggregate into polymorphous forms (type II) which round up, and assume simpler contours (type III) before achieving their mature rounded or ovoid shape (type IV). (From reference 6.)

surfaces which faced other cells the ferritin was distributed regularly and uniformly - sometimes in a single, monomolecular layer (Fig. 2) except at points where there was local distention of the intercellular spaces. In specimens resuspended in CF-free medium before fixation, the amount of ferritin adhering to the free cell surfaces was reduced but not eliminated entirely, whereas that in the intercellular spaces appeared untouched.

A considerable amount of CF was also taken up into mammothrophs. Its location varied as a function of time: At 2-5 min it was located predominately within invaginations of the cell membranes, some of which were "coated," or within vesicles of variable size (600-900 Å) located near the cell surface, some of which were coated and others smooth (Fig. 2).<sup>1</sup> By 15 min and at all periods thereafter (up to 3 h), CF was found within the stacked Golgi cisternae (Figs. 3 and 6), within forming granules (Figs. 3, 4, and 7-11), within rigid lamellae corresponding to elements of GERL (17) (Fig. 3), and within lysosomes (Figs. 3 and 11) of some mammothrophs. The amount found in these locations increased with time up to

<sup>1</sup> At 0.5 mg/ml some CF-loaded membrane was also internalized in larger segments or patches.

FIGURES 2-4 Mammothrophs from estrogen-treated females incubated with CF (0.1 mg/ml). Fig. 2 shows that initially (after 15 min of incubation) CF is seen binding to the cell membrane or within numerous vesicles (*ve*) located in the cytoplasm near the cell membrane. CF molecules are aggregated on the free cell surface (arrows), but form a regular layer (one or two molecules deep) in the intercellular spaces (*Is*). Inside the vesicles the CF is also aggregated. Fig. 3 shows that later (after 60 min), CF aggregates are still seen along the free cell surface (upper right), within membrane invaginations (*in*), and within smooth (*ve*) or coated (*cv*) vesicles. Some molecules are also found within the stacked Golgi cisternae and around a forming secretion granule, as well as in a lysosome (*ly*). Here, CF molecules are present in three (*1-3*) of the stacked Golgi cisternae. It is concentrated at the periphery of a granule (arrow) condensing within the transmost cisterna. Several molecules are also present within the coated tip of another smooth cisterna (*c*) which may correspond to GERL (17). Fig. 4 (60 min) shows CF within immature type I (*g<sub>1</sub>*) and type II (*g<sub>2</sub>*) prolactin granules, sticking to the periphery of the dense content. Figs. 2 and 3,  $\times 70,000$ ; Fig. 4,  $\times 75,000$ .



60 min. The distribution of CF in different granule types varied with time: At 15–30 min CF was seen primarily in type I granules either condensing within Golgi cisternae or located near the trans Golgi face. By 1 h, in addition to its presence in type I granules (Figs. 3, 4, and 6), it was regularly found in type II (Figs. 4, 7, and 10) and type III granules as well. After 2–3 h it was also seen in type IV granules. Within all these granule types CF was typically located between their membrane and dense content, adhering to the periphery of the dense content (Figs. 3, 4, and 9). Within the Golgi complex small numbers of CF molecules could frequently be found in multiple cisternae in a given stack (Figs. 3 and 6). The largest concentrations were seen in the transmost cisternae with condensing secretion granules (Figs. 3, 8, and 9). Sometimes continuity was seen between the membrane of a CF-loaded vesicle and a Golgi cisterna (Fig. 6) or forming granule (Fig. 7), suggesting that the CF gains access to these compartments by fusion with endocytic vesicles. Some of the CF within vesicles and Golgi cisternae was located in close proximity to the membrane apparently adhering to it (Figs. 5 and 6); however, in other cases, the CF formed part of the vesicle content (Fig. 3). The size and number of lysosomes and the amount of ferritin found within them also increased progressively over this time period. CF was never seen within the rough ER regardless of concentration or time interval.

CF was seen in larger amounts in Golgi cisternae and granules of mammothrophs which were rapidly synthesizing and discharging prolactin as determined by the presence of many forming secretion granules and few stored, mature secretion granules. Thus, uptake of ferritin into compartments along the secretory pathway was greater in mammothrophs from estrogen-treated females, which are known to be producing and secreting high levels of prolactin (6), than in mammothrophs from normal cycling females.

The fact that CF distribution is limited to the earliest forming (type I) granules at 30 min, appears also in type II and III granules after 1 h, and is not seen in more mature (type IV) granules until after longer intervals (2–3 h), indicates that CF is added to the granule contents primarily at the earliest steps in granule concentration, and appears only in more mature forms after maturation and assembly of these early labeled forms. It can be concluded (a) that incoming CF-laden vesicles fuse with the Golgi cisternae, rather than

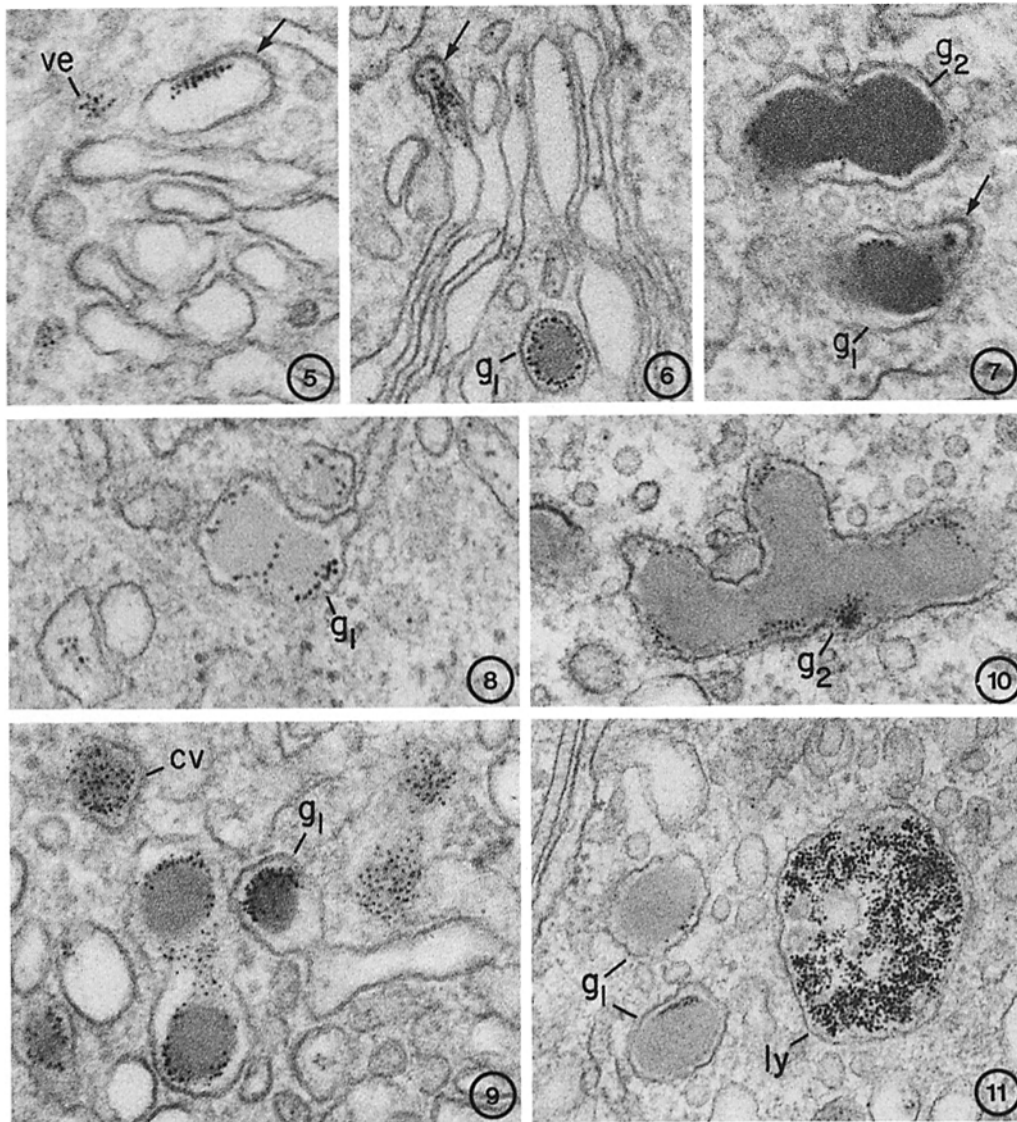
with more mature granules, and (b) that these vesicles fuse with all the Golgi cisternae, but fusion with the transmost cisternae from which the granules arise is more frequent.

Anionic ferritin did not bind to pituitary cells: pellets of cells incubated in native ferritin appeared white like the controls, and relatively little ferritin was seen along cell surfaces in electron micrographs. Some particles were seen adhering to the cell periphery in specimens incubated at high concentrations (5 mg/ml). If preparations were resuspended in ferritin-free medium before fixation, particles were rarely seen along the cell membrane, indicating that ferritin molecules were not firmly bound to the cell surface. Small amounts of native ferritin were also seen within mammothrophs where it was confined to endocytic vesicles and to lysosomes (Figs. 12 and 13). It was not seen within forming granules, the stacked Golgi cisternae, or elements resembling GERL. The amount of ferritin taken up was greater in cells incubated at the higher concentration of ferritin (5 mg/ml). For a given concentration, the amount found in these intracellular compartments increased with time, being smaller at 10 min and gradually increasing up to 3 h; however, even at 5 mg/ml it never reached the amounts seen with the lowest CF concentration (0.05 mg/ml).

Very similar observations to those recorded for mammothrophs were made on all other secretory cell types—somatotrophs (Fig. 14), thyrotrophs, gonadotrophs, and corticotrophs.

## DISCUSSION

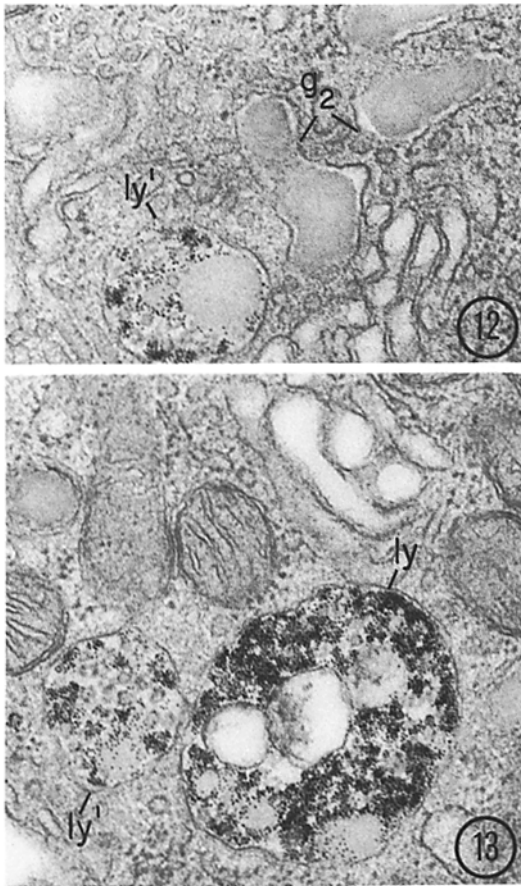
CF—but not native ferritin—binds to the surfaces of dissociated anterior pituitary cells and hence can be used as a tracer to investigate the fate of membrane retrieved from the cell surface in these secretory cells. When CF was present in the incubation medium at low concentrations (down to 0.05 mg/ml), it bound immediately (within 1–3 min) to the cell membrane and was rapidly taken up into pituitary cells by endocytosis. As in the case of dextran uptake into parotid and lacrimal gland cells studied previously (12), CF was subsequently found in several cell compartments: in multiple stacked Golgi cisternae, in condensing granules, in elements corresponding in description to GERL (17), and in lysosomes. Presumably, CF has access to all these compartments as a result of their fusion with vesicles derived from the cell surface. This work provides another example in addition to that reported previously (12) of a



FIGURES 5-11 Small fields from mammothrophs incubated for varying periods in CF to show intracellular sites of tracer uptake. Figs. 5-7 show that elements which contain the tracer often appear coated on part of their surfaces. Fig. 5, at 60 min, depicts a row of CF molecules, lined up in regular array, attached to the membrane of a partially coated Golgi cisterna (arrow). Figs. 6 and 7 are images suggesting fusion between CF-loaded coated vesicles (arrows) and a Golgi cisterna or condensing granule after 60 min and 15 min respectively. Figs. 8 and 9 illustrate CF at the periphery of forming granules ( $g_1$ ) condensing within the transmost Golgi cisternae after 60 min. Fig. 10 shows uptake into a type II granule ( $g_2$ ) after 60 min. Fig. 11 shows CF within two type I granules ( $g_1$ ) and a lysosome ( $ly$ ) after 3 h. Note that in all cases CF sticks to the dense granule content rather than to its membrane. Figs. 5, 8, and 9,  $\times 100,000$ ; Figs. 6 and 7,  $\times 87,000$ ; Fig. 10,  $\times 76,000$ ; Fig. 11,  $\times 93,000$ .

secretory cell in which surface membrane reaches—directly or indirectly—multiple stacked Golgi cisternae. By contrast, native, anionic ferritin present in the medium in quantities up to 100

times that of CF, was taken up in much smaller amounts by pituitary cells, and its distribution was limited to endocytic vesicles and lysosomes. Since native ferritin does not bind to the cell surface, it



FIGURES 12 and 13 Mammothrophs incubated 60 min with native ferritin (5 mg/ml) showing that the distribution of the tracer is limited to lysosomes. Molecules are present within a large dense body (*ly*) and two multivesicular bodies (*ly'*), one of them (Fig. 12) with evidence of crinophagy. None is present within forming granules (*g<sub>2</sub>*) or Golgi cisternae.  $\times 51,000$ .

can be considered a content rather than a membrane marker. CF begins as a membrane marker and in some cases retains its original relation to membranes, but in others it apparently detaches and clumps inside of vesicles or binds to the content of secretion granules. Detachment from the membrane could be due to competition for binding with other acidic groups, e.g., sulfated proteoglycans, which are known to be present in prolactin (9), and growth hormone (25) granules.

The new finding of interest in this work is that the route of incoming vesicle traffic varies for the same tracer molecule depending on its surface charge. It can be concluded that charge interaction influences the fate of the incoming membrane.

since incoming vesicles loaded with negatively charged ferritin fuse only with the vacuolar or lysosomal system, whereas incoming vesicles loaded with the same protein positively charged fuse with compartments of the secretory pathway, i.e., Golgi cisternae and condensing granules as well. At present, it cannot be determined whether the two differently charged forms of the molecule are incorporated in different pockets of surface membrane or whether both are taken up randomly, and the presence of the tracer reroutes the vesicle upon internalization.<sup>2</sup> The fact that CF is found more frequently in Golgi cisternae and condensing granules of cells actively synthesizing and discharging prolactin granules, supports the assumption that the vesicles marked by CF represent membrane retrieved from the plasmalemma to compensate for that added during exocytosis.

CF was introduced by Danon et al (4) as a technique for determining the distribution of negatively charged sites on the cell surface—primarily in fixed cells. It has been widely used for this purpose (2, 11, 23, 24). As early as 1961, Nachmias and Marshall (16) prepared methylated ferritin and used it as a probe, to study effects of charge on protein binding and pinocytosis in living cells (amoebae). More recently, CF has been used to study the distribution and fate of negatively charged sites in a variety of living cells (3, 11, 23, 24). In all cases when binding was carried out on unfixed cells, the CF was not evenly distributed; patches or caps of anionic sites were formed which were shown in several studies (23, 24) to be subsequently internalized. In the only case in which the fate of the internalized CF was followed (macrophages), it was traced to lysosomes (24) and was not found in Golgi cisternae. The fate of internalized CF has not been previously studied in a typical secretory cell. On the other hand, the fate of native ferritin has been investigated in a variety of cells including several secretory cells (8, 14) and was always found to be restricted in its distribution to the lysosomal system as reported here.

<sup>2</sup> While the existence of two different recovery routes seems likely, the possibility of a single route with the lysosome as a first station and the Golgi as a second station cannot be ruled out at present. This would require that the incoming vesicle lose its content at the first station and carry the membrane marker to the next station where the interaction of the marker with the local content (e.g., proteoglycans) would be stronger than with the membrane.

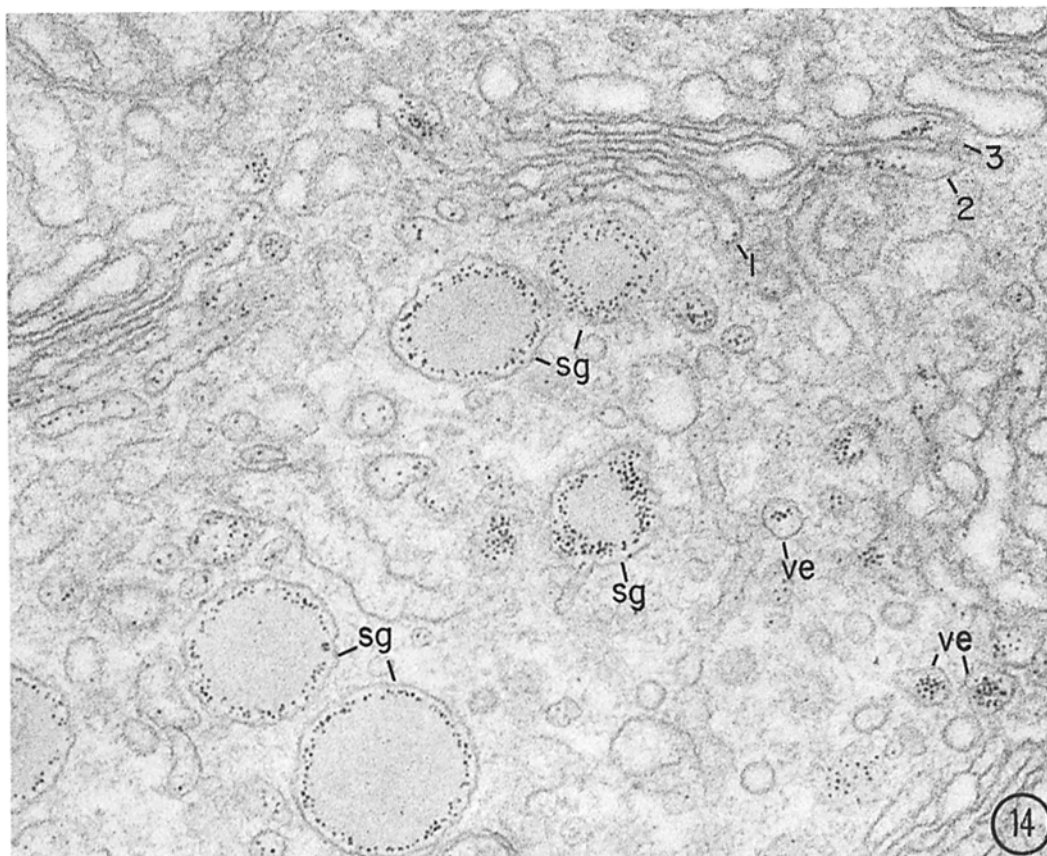


FIGURE 14 Somatotroph from a male rat incubated 60 min in CF (0.5 mg/ml). CF molecules are present within several stacked Golgi cisternae (1-3), multiple smooth vesicles (ve) in the Golgi region, and within numerous growth hormone secretion granules (sg) of varying size. Note that the CF molecules are located exclusively at the periphery of the dense granule contents.  $\times 85,000$ .

Polycations have been shown to exert a variety of effects on different cells. They act as polyvalent ligands which bind to sialic acid residues and other positively charged groups on the cell surface and induce capping in lymphocytes (15), pinocytosis in tumor cells in culture (21), disturbances in epithelial cell architecture (20, 22), stimulation of hormone effects on endocrine cells (27), and altered lipoprotein uptake (3) in fibroblasts. The precise nature of these effects and interactions are unknown. In the case of pituitary cells it is not known whether the effects of net charge on the route of the tracer is due to binding to specific sites or to aggregation of surface proteins.

We have recently reported (12) that the incoming vesicle traffic in secretory cells is complicated, involves several cell compartments including the stacked Golgi cisternae and condensing vacuoles, and is not restricted to lysosomes (1, 8, 14, 18,

26) or specific Golgi (7, 19) or GERL (10) cisternae as previously found. It is not known whether the differences in the disposition of tracers in our work as well as that of others is related more to the cell types investigated, the physiologic state of the cell, or the nature of the tracer. Our previous observations suggest that the physiologic state of the cell is one factor which influences vesicle traffic. The present work clearly demonstrates that the nature of the tracer is also important in determining its eventual fate by demonstrating that a difference in net charge influences the route of internalized ferritin. It is of further interest to note that so far anionic tracers, i.e., those with a net negative charge, have been restricted in their intracellular traffic to the lysosomal system, and that the only tracers which have been shown so far to gain access to elements along the secretory route in a few cells are those

with a neutral or net positive charge (e.g., dextrans [12], horseradish peroxidase [7, 19], and CF).

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