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Internalization of ferritin-concanavalin A by the lactating mammary cell in vivo

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Summary. Ferritin-concanavalin A (Fer-Con A) was used to label the apical plasma membrane of the lactating cell to determine whether membrane internalization takes place. Rat glands were infused in vivo via the teat with 0.2 mg of Fer-Con A in 0.2 ml tris buffer (pH 7.0) containing 0.1% trypan blue, the latter acting as a marker of the infusate. Tissues were obtained from separate animals 5, 10 and 60 min postinfusion. Fer-Con A was seen in alveolar lumina bound to the outer surfaces of apical plasma membrane, microvilli and milk fat globules. It was observed within lactating cells on the inner membrane surfaces of endocytotic vesicles, Golgi cisternae, and secretory vesicles containing casein micelles, and in multivesicular bodies and lysosomes. Internalization of the ferritin-lectin conjugate into casein-containing secretory vesicles was detectable in the 5-min postinfusion tissue. Lysosomes were the only structures in control tissue that contained particles bearing some resemblance to Fer-Con A. The data provide evidence that apical plasma membrane is internalized and distributed to a number of intracellular compartments.

Key words: Mammary gland – Ferritin-concanavalin A – Concanavalin A – Endocytosis – Membrane reuse – Rat

Membranes are involved in the process of milk secretion from the lactating cell. Milk-fat globules are expelled from the cell by envelopment in the apical plasma membrane. In milk of cow, goat and human these globules represent about 4% of the total volume. The other 96%, the so-called skim milk or non-fat phase, is secreted by exocytosis when the vesicle membrane fuses with the plasma membrane. If there were no compensation for the addition of membrane by exocytosis to the cell surface, cells would grow larger in surface area. In the case of the lactating cell, envelopment of fat globules during secretion results in retrieval of a small amount of plasma membrane. However, there is a much greater amount of membrane involved in exocytosis of the skim milk phase because its volume is greater than that of the fat by about 24:1; the secretory vesicles are on average smaller than milk fat globules (approximately 0.5 μm and 3 μm respectively), and they thus have a greater surface (membrane) area per unit volume. For pertinent literature on these two mechanisms of milk secretion see Saacke and Heald (1974); Patton and Keenan (1975); Franke et al. (1978); Keenan et al. (1978); Sasaki et al. (1978) and Wooding (1977).

For some time, we have sought ultrastructural evidence for the reabsorption of the membrane of emptied secretory vesicles. No large collapsed vesicles have ever been seen in the vicinity of the plasma membrane in our specimens. Rather, as noted by Franke et al. (1976), the membrane of the emptied vesicle becomes extended and continuous with the apical plasma membrane. However, we have observed regions of secretory vesicles that resembled coated pits (Buchheim and Welsch 1973). Coated vesicles and their precursors, coated pits, represent a pathway of plasma membrane internalization (Roth and Porter 1964; Silverstein et al. 1977; Pearse and Bretscher 1981). This matter is complicated by the variety of vesicle-type and coated membrane surface in the lactating cell (Franke et al. 1976). We report here experiments utilizing ferritin-concanavalin A (Fer-Con A), a lectin conjugate that binds to mannosyl and glucosyl moieties in cell surface components (Nicolson and Singer 1974; Nicolson 1974) as a means of labeling the apical plasma membrane of the lactating cell. Our findings show that this membrane is internalized in endocytotic vesicles, the membranes of which appear to fuse with those of other intracellular compartments. A preliminary report of this work has been given in abstract form (Welsch et al. 1982).

Materials and methods

Ferritin-concanavalin A infusion. Twentyeight Wistar rats (310–350 g body-weight) in the fifth to twentieth day of lactation were used. After the animals were anesthetized with diethyl ether, their mammary glands were infused with Fer-Con A (Polysciences Inc., Warrington PA), 1 mg/ml in tris buffer (pH 7.0) with 0.1% trypan blue. Variation of the quantity of lectin derivative established that an infusion of 0.2 mg of the lectin in 0.2 ml was a satisfactory dose. Some glands of animals so treated were also infused with trypan blue (0.1%) in saline (containing no Fer-Con A). The materials were infused by inserting a No. 32 needle connected to a loaded 1-ml syringe into the teat canal via the nipple. The treated animals were revived, retained (with-

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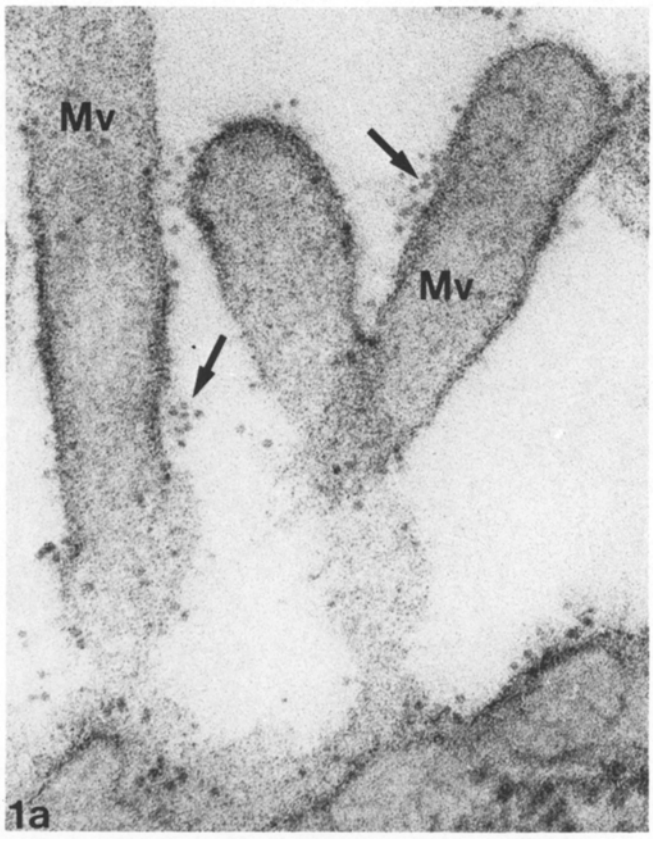


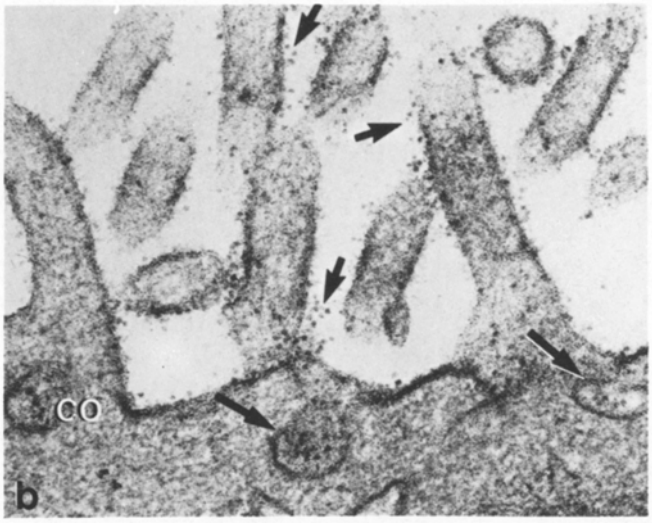
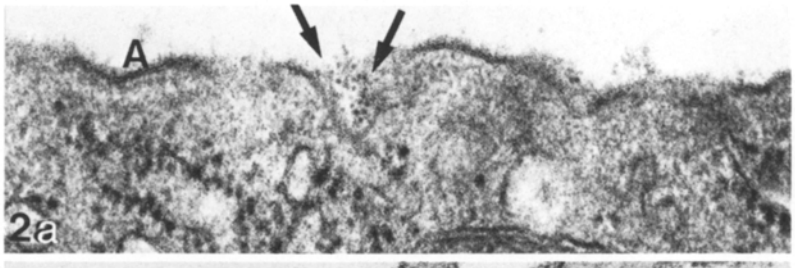
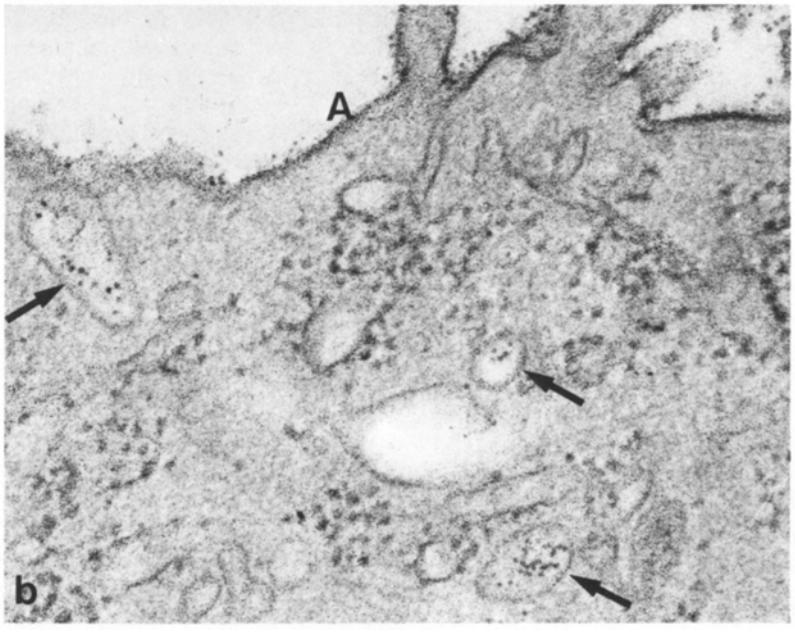
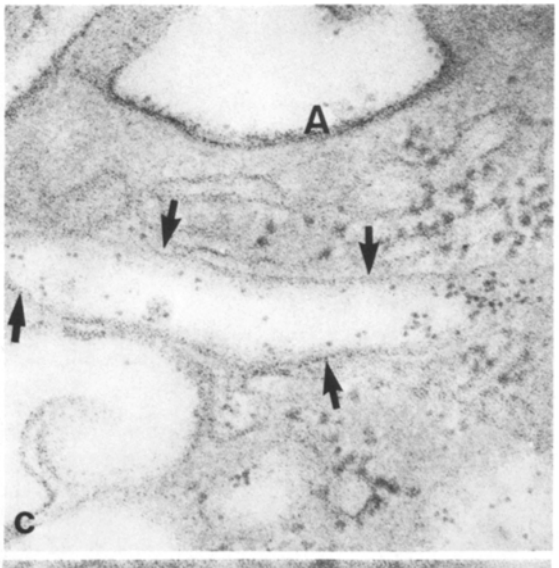


Fig. 1 a, b. Distribution of the Fer-Con A complex (*arrows*) at the apical plasma membrane of rat lactating epithelial cells. Animal sacrificed 5 min after infusion of the lectin. **a** At microvilli (*Mv*), **b** at microvilli and in apical pits (tangentially cut) and vesicle, *co* presumed coated pit or vesicle. Bismuth stain. (a) ×125000, (b) ×93400







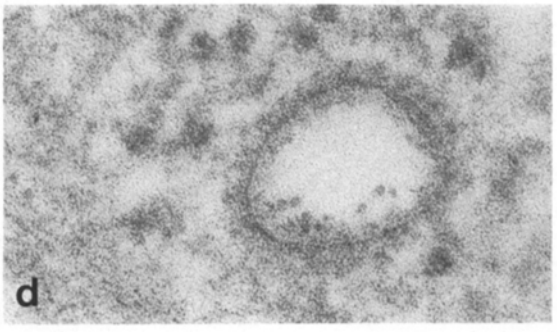


Fig. 2 a–d. Lectin-ferritin complex in apical pits or vesicles. Animals sacrificed 10 min after infusion of the lectin. *A* apical membrane. **a** Apical invagination with Fer-Con A particles (*arrows*), lead stain; **b** variously sized and shaped apical vesicles (*arrows*), bismuth stain; **c** elongated apical cisternae with the Fer-Con A complex (*arrows*), possibly part of the Golgi system, bismuth stain; **d** coated vesicle with Fer-Con A, lead stain. (a), (b), (c) ×83400, (d) ×180000

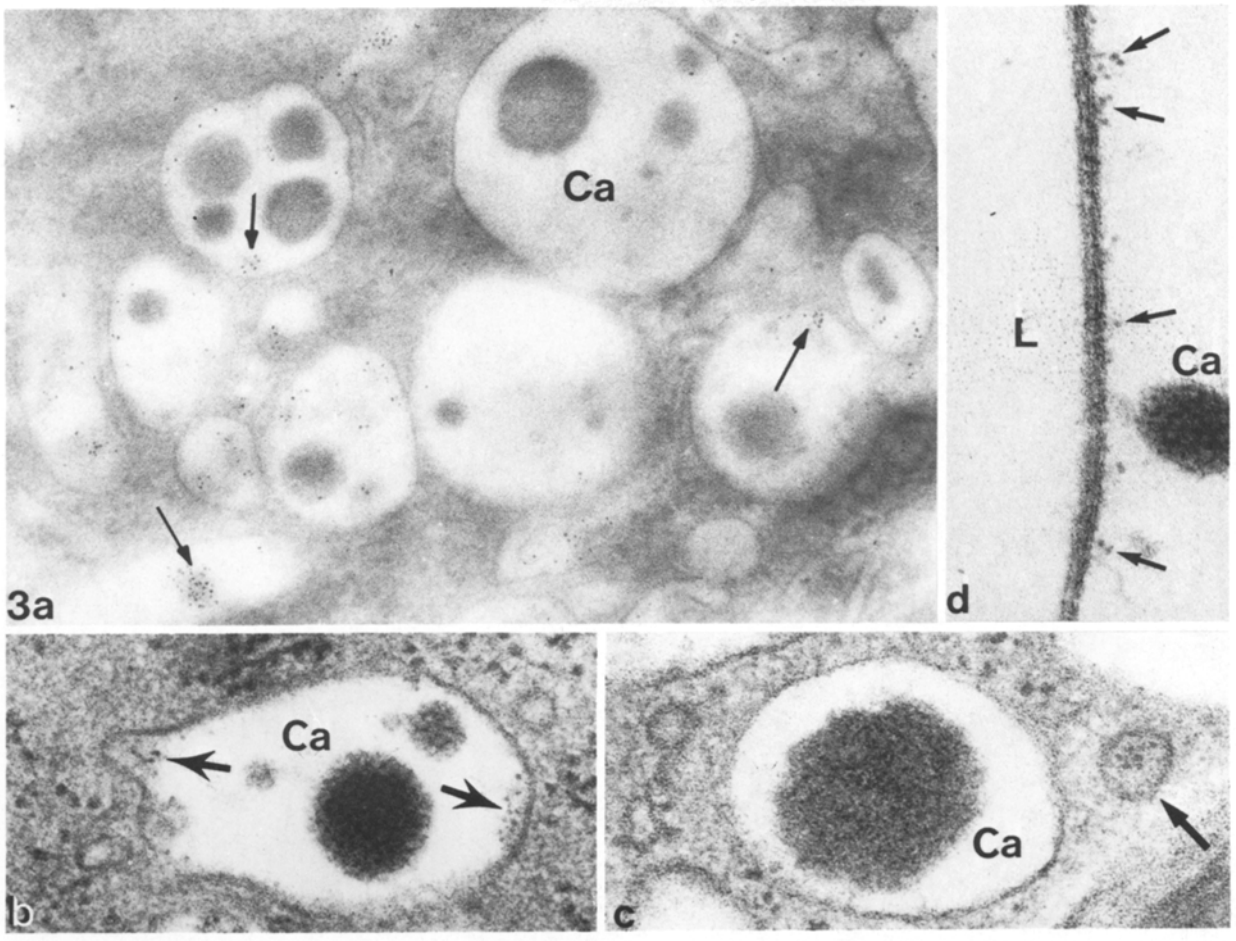


Fig. 3a–d. **a** Lactating cell of animal sacrificed 60 min after infusion of the lectin; unstained section, the majority of casein vesicles (*Ca*) still near the Golgi apparatus. **b–d** Tissue from animals sacrificed 10 min after infusion of the lectin. **b** Casein vesicle (*Ca*) with Fer-Con A particles (*arrows*), at the left side the image suggests the fusion of an endocytotic vesicle (containing Fer-Con A) with a casein vesicle, lead stain; **c** small endocytotic vesicle with the lectin conjugate near immature casein vesicle (*Ca*), lead stain; **d** Fer-Con A complex (*arrows*) at plasma membrane covering lipid droplet (*L*) bulging into alveolar lumen, *Ca* casein micelle, lead stain. (a) ×71000, (b) ×93400, (c), (d) ×121000

out young) for varying times, reanesthetized with ether and dissected to remove infused mammary glands. Animals were sacrificed at 5, 10 and 60 min postinfusion. A successful infusion can be readily identified if the blue dye has permeated the entire gland.

Tissue preparation. Freshly sectioned mammary gland was minced with a razor blade into small pieces of about 2 mm largest dimension. These pieces were fixed in cold 3.5% glutaraldehyde, buffered with phosphate (pH 7.4) for 2 h. Post-fixation of tissue in 2% osmium tetroxide was evaluated at 10, 30, 60 and 120 min. It was found that the 10- and 30-min treatments were adequate to give a good outline and contrast of tissue ultrastructure and yet afford contrast for the more opaque ferritin granules. After dehydration in ethanol and propylene oxide the tissue was embedded in Araldite. Thin sections were prepared, left unstained and viewed in a Philips 300 electron microscope. For guidance on the appearance of Fer-Con A in the electron microscope, the lectin derivative was also fixed, embedded, sectioned and examined. In addition to the osmium treatment for detection of the Fer-Con A, two staining procedures were employed. One of these, the basic lead citrate technique of Reynolds (1963) improves definition of the tissue beyond that of osmium postfixation without obliterating the presence of Fer-Con A. The other, a bismuth staining technique (Ainsworth and Karnovsky 1972), enhances the size and electron opacity of ferritin.

Control experiments with ferritin. Plain uncharged ferritin (Serva, Heidelberg) was injected into rat mammary glands at a concentration of 1 mg/ml. Tissues were studied after 10 and 30 min. The processing of the tissues was the same as above.

In addition, the binding and uptake of cationized ferritin proved useful for comparison. Details of these experiments are not given here.

Results

In unstained sections (osmium treatment only), Fer-Con A appeared as a relatively opaque particle about 5–10 nm in diameter (see Fig. 1). All three procedures (no stain, lead stain or bismuth stain) gave the same results with respect to the localization of Fer-Con A at the surface of and within epithelial cells of the lactating mammary gland. The bismuth stain facilitates detection of Fer-Con A since the

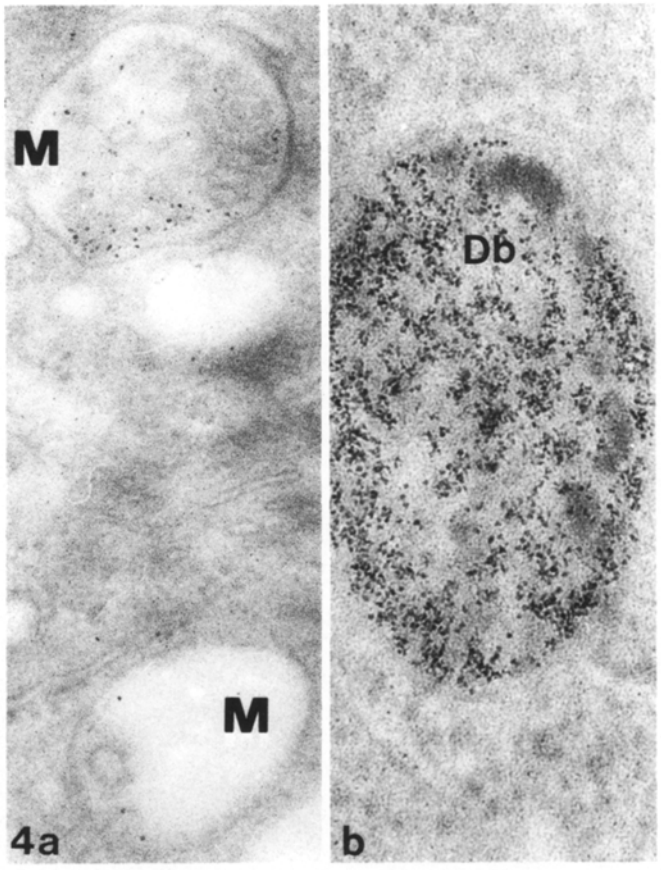


Fig. 4a, b. Fer-lectin conjugate in the lysosomal system, unstained sections. **a** In multivesicular bodies (*M*), tissue from animal sacrificed 10 min after infusion of the lectin; **b** in dense body (*Db*), tissue from animal sacrificed 60 min after infusion of the lectin. (a) ×93400, (b) ×121000

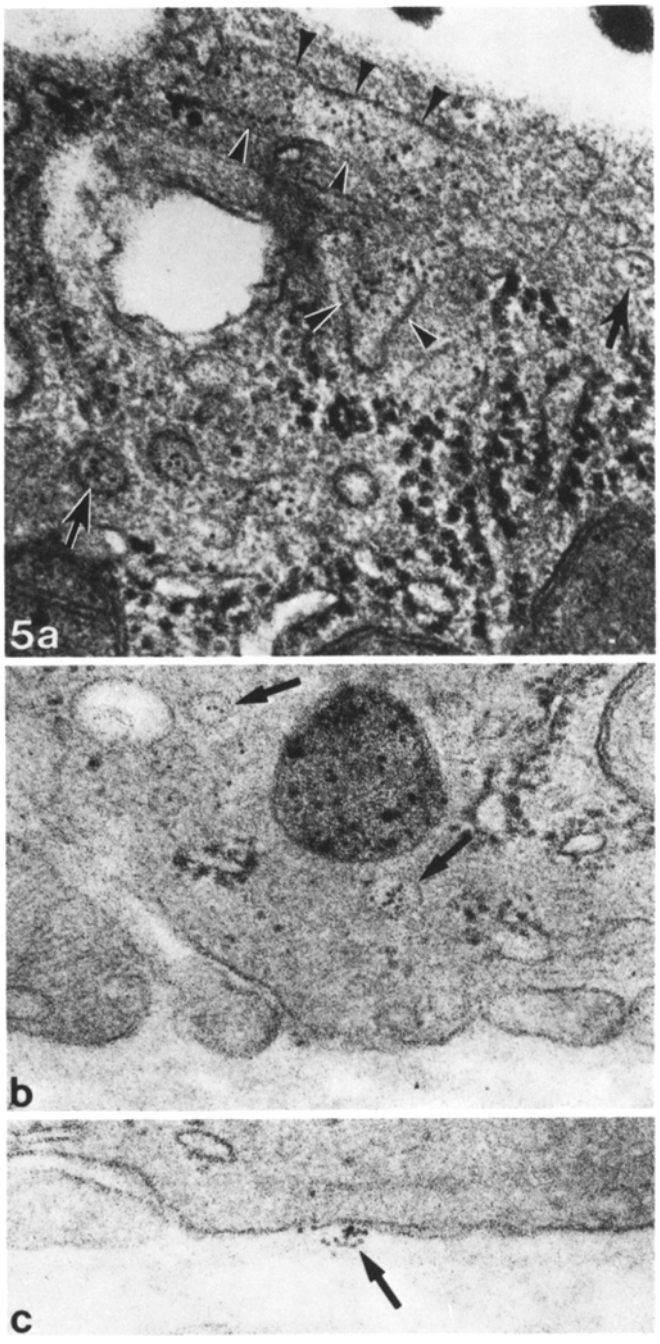


Fig. 5a–c. a Fer-Con A particles in vesicles (*arrows*) and cisternae (*arrowheads*) between rough ER and Golgi field, lead stain; **b**, **c** lectin conjugate within basally located vesicles (**b** *arrows*) and just outside the basal plasma membrane (**c** *arrows*), bismuth stain; all preparations from tissue of animals sacrificed 10 min after infusion of the lectin. (a) ×93400, (b), (c) ×77160

enlarged ferritin particles contrast well against the background (see e.g., Fig. 1).

On infusion into the rat mammary gland, the lectin conjugate was located primarily on the outer apical surface, within various vesicular structures and in lysosomal structures of the lactating cells. Figs. 1–3 show the characteristic distribution of Fer-Con A: 1) over microvilli, on smooth surfaces, and in pits of the apical plasma membrane, and 2) within small smooth and coated endocytotic vesicles of 50–150 nm in diameter, and within "receptosomes", close to the cell surface and deeper in the cell. Such vesicles were also observed near secretory vesicles containing casein micelles (Fig. 3), in the surroundings of the Golgi apparatus, and between the rough endoplasmic reticulum and Golgi apparatus (Fig. 5). In addition we found label in apically-located elongated or sausage-shaped cisternae.

The lectin conjugate was also sighted frequently in lysosomes (dense bodies and multivesicular bodies, Fig. 4). Control tissue, i.e., infused with trypan blue in saline, showed no particles similar to Fer-Con A, except that a few lysosomes (dense bodies) were seen to contain dark granular material that was somewhat smaller and more irregular in shape than the ferritin-lectin complex.

Fer-Con A was seen in developing and mature secretory vesicles (containing well-developed casein micelles) with the lectin conjugate adhering to the inner membrane surface (Fig. 3). Electron-opaque ferritin particles could be seen in relation to some pitted or asymmetrical surfaces of secretory vesicles (Fig. 3b). In regions containing Golgi, Fer-Con A was located on the inner membrane surface of cisterns, mainly in those areas of the cis-face where inflated cisterns transform into immature secretory vesicles (Fig. 3a). Short smooth cisterns intermediate between rough ER and the typical cisterns of the forming face of the Golgi apparatus occasionally contained Fer-Con A particles (Fig. 5a).

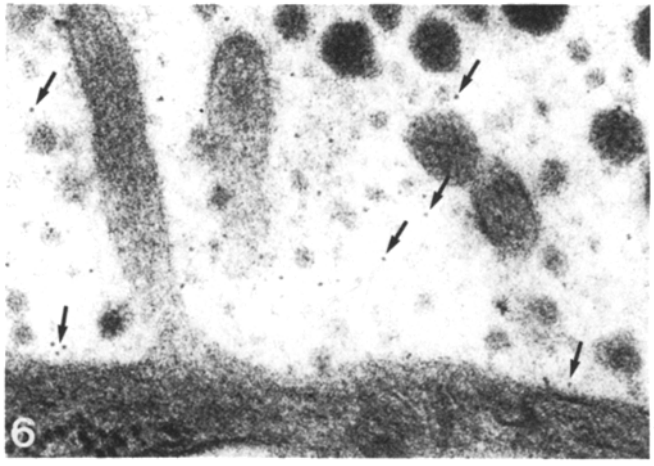


Fig. 6. Part of mammary gland alveolus infused with plain uncharged ferritin. Note paucity of ferritin granules (*arrows*) adhering to the apical membrane, although other granules float free in the lumen, lead stain; tissue obtained from animal sacrificed 10 min after infusion of the ferritin. × 81 000

Binding of Fer-Con A to the surface of milk fat globules was noted in the alveolar lumina by the three detection methods.

The length of time that Fer-Con A was allowed to stay in the mammary gland influenced its penetration and distribution in the lactating cells. After 5 min, the lectin derivative occurred predominantly over the cell surface, in closely associated small (50–150 nm) vesicles, and on milk fat globules in alveolar lumina. A secretory vesicle containing casein micelles was sometimes seen to be decorated with Fer-Con A on the membrane inner surface in this tissue. No labeling of lysosomes (dense bodies) was observed after 5 min. A careful search of these samples did not reveal the presence of multivesicular bodies. Extensive internalization of the lectin derivative had occurred by 10 min; at 60 min it was found in all locations at which it had previously been observed. The principal difference between the 10- and 60-min treatments was the greater concentration of label in lysosomes at 60 min. The detection of Fer-Con A in mature (casein micelle-containing) secretory vesicles after 5 min suggests relatively rapid movement of internalized membrane.

Fer-Con A was not seen on or within nuclei, endoplasmic reticulum, mitochondria or intracellular fat droplets of lactating cells. It was seen, however, within vesicles and multivesicular bodies in the basal region of these cells and even outside the basal plasma membrane (Fig. 5b, d). No evidence for disrupted alveoli was found in the tissue areas studied.

No systematic observations were made regarding the Con A derivative and other cell types present in the mammary gland.

The control experiments demonstrated that unconjugated ferritin is internalized but shows little or no tendency to bind to membranes (Fig. 6).

Discussion

Our experiments provide evidence that Fer-Con A, when infused into the rat mammary gland, binds to the apical plasma membrane of lactating cells and becomes internalized by endocytosis. The pathway observed appears to involve coated pits, coated vesicles, endosomes or receptosomes (as characterized by Pastan and Willingham 1981) seen in the lactating cell (e.g., Franke et al. 1976) and other cell systems (for reviews see Silverstein et al. 1977; Pearse and Bretscher 1981; Pastan and Willingham 1981). The fact that these structures are readily evident and characteristic in lactating tissue shows that Fer-Con A is not essential to their induction. The distribution and degree of mobility of Con A receptors in the apical plasma membrane of the lactating cell have not yet been demonstrated but it appears that they are incorporated into coated pits and vesicles.

It is reasonable to assume that Fer-Con A is a satisfactory membrane marker under our experimental conditions (see Nicolson and Singer 1974; Beaudoin et al. 1978; Zweig and Singer 1979; Petty and Ware 1981). Con A binding to milk fat globule membrane has already been observed in vitro by Sasaki and Keenan (1979). This membrane is originally plasma membrane and is acquired by the globule on secretion from the cell; it is sufficiently strong to withstand isolation of the globule from milk, removal of membrane from the globule by the detergent, Triton X-100, and sedimentation of the membrane at 50000 × *g* for 1 h (Patton and Hubert 1983). Specific binding of Fer-Con A (i.e. mannoside-inhibited) to milk fat globules has been shown by Sasaki and Keenan (1978). Our control experiments with uncharged ferritin have demonstrated that very little of this substance becomes associated with the apical plasma membrane. It seems reasonable, therefore, to assume that the strong labeling of membranes in the Fer-Con A experiments corresponds to lectin binding and uptake.

According to our findings, the membrane internalized in small vesicles proceeds to two principal destinations within the cell, as shown by Fer-Con A. Part of it goes to Golgi membranes and secretory vesicles and part to the lysosomal system (multivesicular bodies and lysosomes). Exocytosis of the secretory vesicles bearing Fer-Con A would complete a membrane cycle to the initial labeling point, the apical plasma membrane. Evidence of plasma membrane reuse in the intact animal has been obtained by Heuser and Reese (1973) and Herzog and Farquhar (1977). Our research provides the first direct evidence for internalization of plasma membrane by the lactating cell. Further research will be required to confirm reuse of this membrane. In lactating cells, the speed of the internalization appears to be rapid and roughly corresponds to that of the uptake of dextran particles as observed by Herzog and Farquhar (1977) in the parotid gland.

The fact that Fer-Con A can be seen in lysosomal structures (prelysosomal compartment (Farquhar 1983), multivesicular and dense bodies) does not define the intracellular directions of vesicle traffic or the role of lysosomes. Lysosomes in the lactating cell have received very little study. Evidence has been obtained, using other cells, that lysosomes assist in plasma membrane recycling rather than exclusively in its degradation (Tulkens et al. 1977; Muller et al. 1980). As pointed out by Tulkens et al. (1977), complete hydrolytic degradation of all plasma membrane being internalized by cells would create a large task for lysosomes, especially in the case of the lactating cell with its extensive membrane flow. It is of interest to note that elongated or sausage-shaped smooth cisterns containing the lectin conjugate have been seen in the apical cytoplasm; these resemble structures in other exocrine glands (Oliver and Hand 1981)

and have been termed the prelysosomal compartment by Farquhar (1983). We cannot exclude that the formation of these cisterns is induced by the uptake of the lectin conjugate. Further studies are required to determine whether they occur in normal tissues.

Apart from the two principal pathways taken by the internalized Fer-Con A (Golgi-secretory vesicles and lysosomal system), a third route for the lectin complex may exist, i.e., all the way through the cell with release (exocytosis) at the basal membrane. Evidence for this pathway has been found in the 10- and 60-min experiments. Such a pathway may account for the movement of some milk constituents into the circulation via interstitial fluid and lymph. It has been reported (Leary and Larson 1982) that small vesicles travelling in the opposite direction transport immunoglobulins across the lactating cell into milk.

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