Endosomes and Golgi Vesicles in Adsorptive and Fluid Phase Endocytosis

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ABSTRACT We studied with morphometric methods the endocytosis by pheochromocytoma cells of a conjugate of wheat germ agglutinin with ferritin (WGA-Ft) and of horseradish peroxidase (HRP).

Quantitative studies indicated that WGA-Ft was cleared slowly from cell surfaces and that it was not recycled to the surface.

Cells labeled with WGA-Ft for 15 min at room temperature were washed and incubated in medium containing HRP for 15 or 30 min at 37°C. The greatest proportion of labeled vesicles and tubules contained only WGA-Ft (83.4% at 15 min and 85.3% at 30 min). A very small fraction of labeled vesicles and tubules contained only HRP (0.2% at 15 min and 0.9% at 30 min). Vesicles and tubules at the Golgi apparatus were labeled almost exclusively with WGA-Ft (97% at 15 min and 30 min); the rest had both labels. Most labeled lysosomes contained both labels (80.1% at 15 min and 80.8% at 30 min). Of the remainder more contained WGA-Ft alone (20% at 15 min and 10.9% at 30 min), then HRP alone (none at 15 min and 8.2% at 30 min). In contrast to the various and varying patterns of labeling with WGA-Ft and HRP of the other organelles studied, the vast majority of endosomes contained both markers (94.1% at 15 min and 100% at 30 min); the rest contained WGA-Ft only. These results demonstrate that endosomes are recipients of both fluid phase and adsorptive endocytosis markers; these findings are consistent with the hypothesis that endosomes mediate the sorting out and subsequent intracellular traffic of membrane bound and fluid phase markers.

Cisterns of the Golgi apparatus did not contain WGA-Ft; in sharp contrast, when WGA-HRP was used, the cisterns of the Golgi apparatus consistently contained HRP.

According to current concepts, adsorptive endocytosis is a process by which cells selectively concentrate and internalize ligands that bind to plasma membrane receptors "... without ingesting a corresponding large volume of solution" (1). Molecules without affinities to plasma membrane moieties enter cells by fluid phase or bulk endocytosis. Fluid phase endocytosis is considered to be a "constitutive" process, i.e., it is not modulated by extracellular ligands or by other known natural inducers (2).

Certain pathologically active ligands such as lectins and toxins bind specifically to a large number of cell surface receptors and undergo adsorptive endocytosis (3–8). Also, a heterogeneous class of physiologically active ligands, such as hormones, growth factors, low density lipoprotein particles (LDL),[1] asialoglycoproteins, α^2-macroglobulin, etc., bind to a relatively small number of cell surface receptors and are internalized by a process referred to as receptor-mediated endocytosis (9–13). Generally, in receptor-mediated endocytosis, the receptor–ligand complexes accumulate in coated pits of the plasma membrane; whereas, in the adsorptive endocytosis of cholera and tetanus toxin, noncoated membrane invaginations are involved in the initial binding and endocytosis (8, 9–12). Another difference between receptor-mediated and adsorptive endocytosis is the reported number of plasma membrane receptors to a given ligand. In the receptor-mediated uptake of LDL, there are 20,000 receptors to LDL per fibroblast, while in the adsorptive endocytosis of

[1] *Abbreviations used in this paper*: DAB, diaminobenzidine tetrahydrochloride; GERL, Golgi endoplasmic reticulum lysosome; HRP, horseradish peroxidase; IgG-Ft, immunoglobulin G-ferritin; LDL, low density lipoprotein; NGF, nerve growth factor; WGA-Ft, wheat germ agglutinin-ferritin.

toxic ricin we calculated 8×10^7 receptors per neuroblastoma cell (7, 9, 10).

Most of the morphologic and biochemical studies have been focused on the receptor-mediated endocytosis of various ligands, while adsorptive endocytosis has received less attention, although a number of extremely active molecules such as toxins and lectins bind specifically to a large number of cell surface receptors and undergo adsorptive endocytosis.

Studies of receptor-mediated endocytosis have implicated several intracellular compartments in the processing of the ligand and the retrieval of the receptor (9–15). Receptosomes, endosomes, compartment of uncoupling of receptor from ligand, and the Golgi apparatus and GERL (Golgi endoplasmic reticulum lysosome) have been recognized as potentially important steps in the endocytic pathway(s) of ligands (9, 11, 13–16).

Another question of considerable interest is whether fluid phase and adsorptive endocytosis are mediated through the same or different endocytic vesicular compartments. To answer this question, Rodewald and Abrahamson (17) used simultaneously a tracer of fluid phase endocytosis, horseradish peroxidase (HRP), and an immunoglobulin G-ferritin (IgG-Ft) conjugate that binds to intestinal cell IgG receptors of suckling mice. Using this double-labeling method to study fluid phase and adsorptive endocytosis, they concluded that the two tracer molecules entered cells together in the same vesicles. However, later on, the two tracers were "sorted" within the cell; while HRP was confined to the apical cytoplasm in small vacuoles and lysosomes, the IgG-Ft conjugate was transported in small vesicles to the basal aspect of the intestinal cell and subsequently released in the interstitial space (17). In the present study we used a similar double-labeling method to study the adsorptive endocytosis of a monomeric conjugate of wheat germ agglutinin with ferritin (WGA-Ft) and the fluid phase endocytosis of HRP in cultured rat pheochromocytoma cells (PC-12 line). The PC-12 line, established by Greene and Tischler, has been extensively used as a model for neurons. After stimulation by nerve growth factor (NGF) the cells undergo morphologic and biochemical changes similar to those seen in sympathetic neurons exposed to NGF (19–21).

This study was done to (*a*) examine the localization of the two markers in the initial endocytic vesicles, endosomes (receptosomes), Golgi apparatus, and lysosomes; (*b*) to assess quantitatively the distribution of WGA-Ft in the various endocytic compartments during early and late phases of endocytosis; and (*c*) to determine whether the marker molecule modifies the endocytosis or detection of the ligand by comparing the endocytosis of WGA-Ft with that of WGA-HRP.

MATERIALS AND METHODS

Materials: Wheat germ agglutinin (WGA) (lot 103378) was bought from Calbiochem-Behring Corp. (La Jolla, CA); ferritin (Ft) (6 × crystal lot 18) was from Miles Laboratories, Inc. (Elkhart, IN); horseradish peroxidase (HRP), type VI, was from Sigma Chemical Co. (St. Louis, MO), and Aclar 33c, 5-mm, plastic strips was from Allied Chemical Corp., (Morristown, NJ). An *N*-acetylglucosamine affinity column was purchased from Bio-Rad Laboratories (Richmond, CA). Chitobiose (*N,N'*-diacetylchitobiose hexaacetate) was purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture: The PC-12 rat pheochromocytoma cell line, obtained from Lloyd Greene (Department of Pharmacology, New York University), was grown on Aclar plastic strips according to previously described methods (18); PC-12 cells were exposed to NGF for 7–10 d before use. Mouse 2.5 S NGF, prepared by the method of Bocchini and Angeletti (21), was given to us by R. H. Angeletti (Department of Pathology and Laboratory Medicine, University of Pennsylvania).

Conjugation of WGA with HRP: The conjugate was made according to the method of Avrameas and Ternynck (22) using glutaraldehyde as the cross-linking agent. A detailed description of the conjugation procedure has been published (23). The molar ratio of horseradish peroxidase to wheat germ agglutinin in the conjugate is 1:1:1.

Preparation of Monomeric Lectin-Ferritin Conjugate: This was done following the basic methods of Avrameas and Ternynck and of Pfenninger and Jamieson, with glutaraldehyde as the cross-linking agent (22, 24). Wheat germ agglutinin was iodinated at a specific activity of 8×10^5 cpm/μg with the chloramine T method (25). Iodinated wheat germ agglutinin and an equimolar amount of ferritin in 0.01 M potassium phosphate buffer, pH 7.5, containing 0.15 M sodium chloride (PBS) were combined in the presence of a 200 molar excess of glutaraldehyde. The solution was allowed to react at room temperature for 2 h with gentle stirring. The reaction was quenched by the addition of a 1 M lysine solution to give a final concentration of 0.1 M of lysine. This mixture was dialyzed against PBS at 4°C for 48 h and then applied to an *N*-acetylglucosamine affinity column previously equilibrated with the same buffer. Unconjugated ferritin was not adsorbed, while the wheat germ-ferritin conjugate and free lectin were eluted with 0.5 M *N*-acetyl-glucosamine. Monitoring of ferritin was done spectrophotometrically at 310 nm and the radioactivity of the lectin was measured in a Searle gamma counter. The eluted material was concentrated through a P-10 membrane, dialyzed against several changes of PBS, and then separated chromatographically on a Biogel A5m (Bio-Rad Laboratories) column developed with PBS. The elution profile consisted of three peaks: a small peak consisting of high molecular weight WGA-Ft aggregates appearing at the void volume, a second peak corresponding to the elution position of conjugated ferritin and finally one peak of free wheat germ agglutinin as judged by the absence of absorbency at 310 nm. The appropriate tubes were combined and concentrated. The ratio of ferritin to lectin in the conjugate is 1:1. All experiments were done with the same preparation of WGA-Ft, and with the same concentrations of wheat germ agglutinin (20 μg/ml) and ferritin (320 μg/ml).

Fixation and Processing of Cells for Electron Microscopy: These methods were described in detail in previous papers from this laboratory (3–6). To fix cells we used 2.5% glutaraldehyde, 1% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.3 containing 0.002% $CaCl_2$. Cytochemical stain for HRP was done using diaminobenzidine tetrahydrochloride (DAB) as substrate according to the method of Graham and Karnovsky (26). Quantitative studies were done with unstained sections mounted on uncoated copper grids; qualitative studies were done with sections of cells stained with 4% uranyl acetate in 50% ethanol. A JEOL-100CX electron microscope was used.

Experimental Protocols: Cells were washed for 10–15 min at room temperature with Eagle's minimal essential medium buffered with 0.01 M HEPES (*N'*-2-hydroxyethyl-piperazine-*N*-2-ethanesulfonic acid) at pH 7.6 (buffer). Subsequently cells were incubated with WGA-Ft at 4°C or room temperature for various periods of time. Endocytosis of HRP and WGA-Ft was studied in cells incubated in full culture medium at 37°C for various periods of time.

Double Labeling: After incubations with WGA-Ft cells were washed in buffer and then incubated at 37°C in full culture medium containing 5 mg/ml HRP for 15 and 30 min. In cells incubated for 2 h at 37°C, HRP was present in the culture medium only for the initial 15 min. Incubation times and temperatures are given in Results. After all incubations at 37°C, cells were washed at room temperature with the buffer as above, fixed at room temperature for 20–30 min, washed in buffer and, in experiments in which HRP was used, cells were incubated with DAB for 10 min at room temperature before osmication and embedding in Araldite.

Morphometry and Statistical Analysis of Data: For counts of ferritin particles at the cell surface, electron micrographs were printed at a final magnification of 47,000. The length of a cell's plasma membrane was measured with a cartographer's wheel. Only segments of plasma membrane not contiguous with other cells, processes, or debris were used. Tangential sections of plasma membrane were not used. For counts of cytoplasmic compartments containing ferritin, HRP, or both labels, electron micrographs were printed at a final magnification of 40,000; a compartment containing two ferritin particles was registered as positive. For counts of ferritin within compartments magnifications of 54,000–65,000 were used. To make an unambiguous distinction between HRP and unlabeled compartments, electron micrographs of sections not stained with uranyl or lead were employed. Because of the relatively low contrast in these electron photomicrographs, the number of unlabeled vesicular and tubular compartments was not counted; unlabeled endosomes and lysosomes were counted. To avoid counting the same cell twice, all pictures were

taken from a single section. The following compartments or organelles were identified: vesicles (0.1 μm in maximal diameter), tubules (0.1 μm in maximal diameter), lysosomes (>0.1 μm in maximal diameter), endosomes (vacuoles with lucent center, >0.1 μm in maximal diameter), vesicles and tubules within 0.3 μm of the *trans* face of the Golgi apparatus, and cisternae of the Golgi apparatus. The total cytoplasmic area and the areas of the various compartments were calculated by the point-counting method from printed electron micrographs perforated by a grid with points 1 cm apart. The area of the Golgi apparatus and associated vesicles and tubules was outlined on each picture before the actual counting of the various compartments. The number of labeled compartments per 100 μm^2 was computed as follows: vesicles and tubules per 100 μm^2 cytoplasm (not including the area occupied by the Golgi apparatus); lysosomes and endosomes per 100 μm^2 of total cytoplasmic area (there were virtually no lysosomes or endosomes within the area of the Golgi apparatus); vesicles and tubules in the Golgi area per 100 μm^2 of Golgi area. Means were weighted by the cytoplasmic area counted. For calculations of standard deviations and standard error of measurement (SEM), one cell was treated as one sample. Means for plasma membrane lengths were weighted by the length of plasma membrane measured in each cell. Means for the percentages of labeled compartments were weighted by the number of the appropriate labeled compartment per cell.

RESULTS

Qualitative Studies

SURFACE DISTRIBUTION OF WGA-FT: After cells were incubated with WGA-Ft for 15 min at room temperature, ferritin particles were evenly distributed at the cell surface (Fig. 1). The electron-dense iron cores of ferritin, measuring 5-nm diam, were seen either at the plasma membrane or up to 30 nm from the plasma membrane. Frequently ferritin particles were in rows 1–3 deep on the plasma membrane; this arrangement of ferritin particles in rows is probably due to the thickness of the section (50–70 nm) and the superimposition of ferritin particles from several levels. After incubation of cells at 37°C, surface WGA-Ft particles were fewer and usually were clustered (Fig. 2). At all time points WGA-Ft was found on smooth segments of the plasma membrane, smooth invaginations or caveoli, and coated pits (Fig. 3).

ENDOSOMES: (Figs. 4–6). Endosomes were defined as round, oval or irregular, membrane-bound organelles with empty appearing centers. The average diameter of endosomes was 0.4 μm. Almost all endosomes of cells incubated with WGA-Ft and HRP contained both labels, (see below, quantitative data). Frequently, vesicles or tubules with only WGA-Ft or with WGA-Ft and HRP were connected with the wall of the endosome (Figs. 4 and 5). Several endosomes containing both labels showed the "straight, fuzzy, lamellar" edge at the defining membrane as originally described in receptosomes by Willingham et al. (12) (Fig. 6). We found no evidence of fusion of coated pits or coated vesicles with the wall of endosomes.

VESICLES AND TUBULES: Numerous smooth walled vesicles and branching tubules containing ferritin, HRP or both were found near the plasma membrane, as well as dispersed throughout the cytoplasm (Fig. 7). The diameters of vesicles and tubules ranged between 0.05 and 0.1 μm. Often, vesicles appeared to bud off or connect with a tubule. On several occasions a coated vesicle (or pit) appeared to be continuous with a smooth walled vesicle or tubule (Fig. 8). Rat pheochromocytoma cells exposed to NGF show neurite-like processes; the cytoplasm and processes of these cells contain a large number of secretory granules, ~0.1-μm diam, with characteristic electron-dense cores that are separated from the limiting membrane by a narrow lucent band (19). We did not find WGA-Ft or HRP in secretory granules.

LYSOSOMES: For quantitative purposes we made no attempt to separate the relatively few multivesicular bodies from lysosomes. In Fig. 9 we illustrate two membrane-bound organelles one of which contains only ferritin and membranous or vesicular profiles while the other contains both peroxidase and ferritin. The diameter of lysosomes ranged from 0.5–2.0 μm.

THE GOLGI APPARATUS: Cells contained numerous groups of characteristic flat, parallel, smooth cisternae displaying typical *cis* and *trans* polarity. Quite often ferritin was found in vesicles and tubules in the immediate vicinity of the Golgi apparatus, especially at its *trans* aspect. However, we were not able to find ferritin particles within any cisterna of the Golgi apparatus (Fig. 10). By comparison, cultures of PC-12 cells incubated with WGA-HRP under identical conditions of lectin concentration, temperature, and time showed HRP stain not only in vesicles but also in one or more cisternae of the Golgi apparatus (Fig. 11).

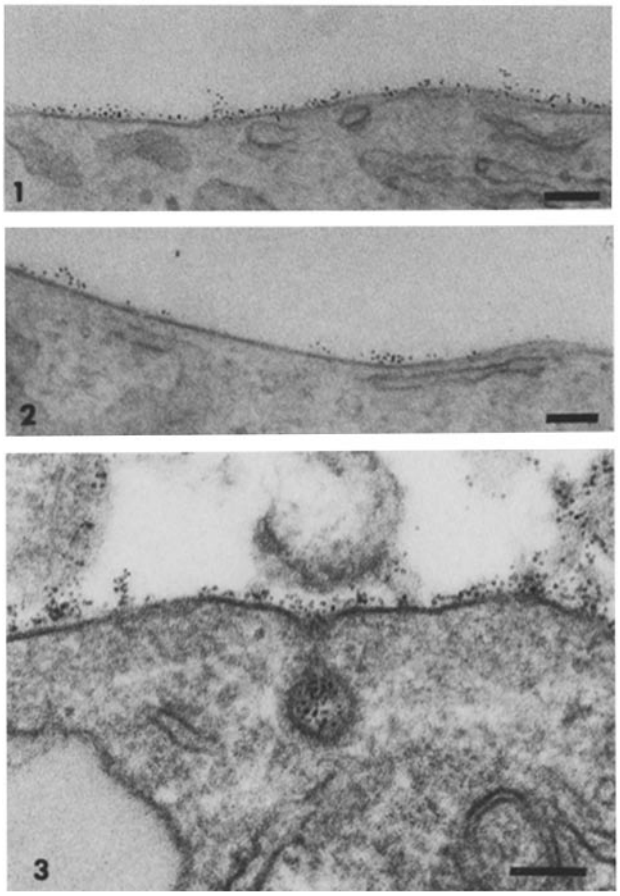
Quantitative Studies

SPECIFICITY OF THE CONJUGATE OF WHEAT GERM AGGLUTININ WITH FERRITIN: After the initial wash in buffer, cells were incubated for 1 h at 4°C with WGA-Ft in buffer, or with WGA-Ft and a 100-fold excess of free wheat germ agglutinin. Cells were then washed in 4°C buffer and processed for electron microscopy. In cells incubated with WGA-Ft, 63.6 + 4.8 ferritin particles per micrometer of surface were counted (based on 1,072 ferritin particles counted from sections of 10 cells). In cells incubated with WGA-Ft and free wheat germ agglutinin, 2.2 + 0.5 ferritin particles per micron of surface were counted (based on 58 ferritin particles counted from sections of 16 cells); thus, excess of free wheat germ agglutinin inhibited the binding of WGA-Ft (96.5% inhibition). To rule out any nonspecific binding of native ferritin to cell surfaces, cells were treated in an identical fashion except the incubation medium contained, instead of WGA-Ft, free native ferritin at a concentration of 320 μg/ml. In this experiment 3 + 0.2 ferritin particles were counted per micrometer of cell surface (based on 187 ferritin particles counted from sections of 11 cells).

Having determined that the conjugation of wheat germ agglutinin with ferritin did not alter detectably the affinity of wheat germ agglutinin for its plasma membrane receptor(s) and that native ferritin did not bind significantly to cell surfaces, we investigated whether HRP binds to cell surfaces nonspecifically and whether it has an affinity for wheat germ agglutinin. This experiment was done in two ways, first by using a wheat germ agglutinin Sepharose column, and second by a morphological approach.

LACK OF BINDING OF HRP TO WGA-SEPHAROSE: HRP, 1 mg/ml, was applied to a Sepharose-WGA 6 MB column. The column did not retain HRP which was fully recovered.

MORPHOLOGIC DEMONSTRATION OF LACK OF BINDING OF HRP TO WGA-FT: Cells grown on Aclar strips were washed with buffer and incubated for 1 h at room temperature with 20 μg/ml WGA-Ft and 5 mg/ml HRP (Sigma VI, Sigma Chemical Co.) in the same buffer. Cells were washed at room temperature, fixed in the standard fixative, washed again in buffer, stained for HRP with DAB and processed for electron microscopy. Sections not stained with lead or uranyl salts were examined for surface stain of HRP. There was no surface stain for HRP in 25 sections of different cells examined.

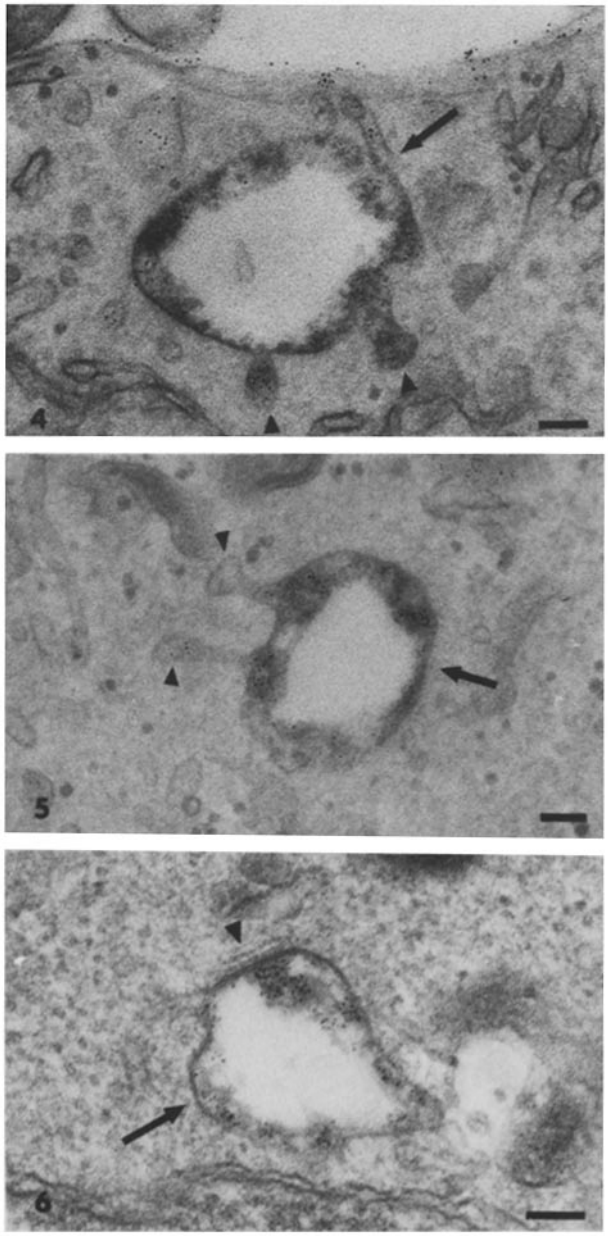


FIGURES 1–3 Fig. 1: PC12 cells incubated for 15 min at room temperature with WGA-Ft before fixation. Section not stained with lead or uranyl (unstained section); all magnification lines in this and subsequent electron micrographs represent 0.1 μm. × 70,000. Fig. 2: Cells incubated with WGA-Ft 15 min at room temperature, washed and incubated in full culture medium containing 5 mg/ml HRP at 37°C for 15 min before fixation and incubation in DAB. Unstained section. Note small clusters of ferritin and segments of plasma membrane that are not covered by ferritin. × 70,000. Fig. 3: Cells incubated with WGA-Ft as in Fig. 2. Ferritin particles within a coated pit; stained with uranyl. × 100,000.

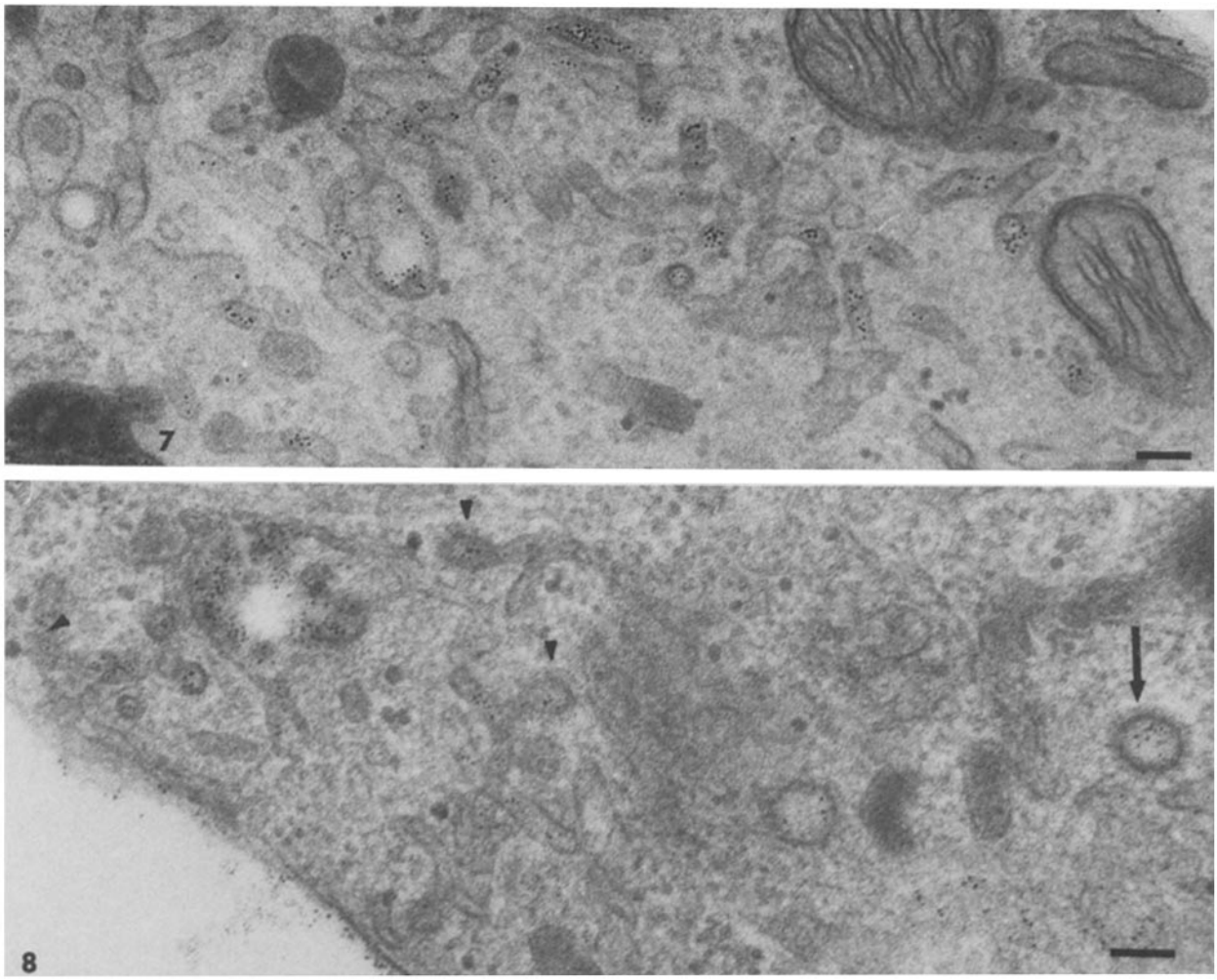
CLEARANCE OF WGA-FT FROM THE CELL SURFACE: Cells were incubated with WGA-Ft for 15 min at room temperature, washed, and fixed. Sister cultures were incubated with WGA-Ft for 15 min at room temperature, washed, and incubated in medium for 15, 30, and 2 h at 37°C before fixation. Morphometric analysis of plasma membrane ferritin shows a 66% clearance of the initially bound ferritin on cells incubated for 30 min at 37°C; a lower but probably significant number of surface ferritin particles was detected on cells incubated for 2 h at 37°C (Fig. 12).

EFFECT OF CHITOBIOSE ON SURFACE BOUND WGA-FT: Cells were incubated with WGA-Ft in buffer for 15 min at room temperature, washed with buffer, and incubated in culture medium for 15 min at 37°C; the cells were then washed with buffer for 5 min at room temperature and fixed for electron microscopy. The number of ferritin particles per micron of plasma membrane was 40.0 ± 2.8 (based on 2,866 particles counted from sections of 28 cells). Sister cultures were treated in an identical fashion except that in the second wash just before fixation, the buffer contained 0.2 M chitobiose. The chitobiose wash resulted in the removal of 86% of surface ferritin. In this experiment the number of ferritin particles per micrometer of plasma membrane was 5.4 ± 1.2 (based on 175 ferritin particles counted from sections of eight cells). (For intracellular WGA-Ft of the same experiment, see below Table IV, experiment *D*).

SLOW CLEARANCE OF WGA-FT FROM CELL SURFACES IS NOT DUE TO ITS RECYCLING TO THE PLASMA MEMBRANE: Having established that the chitobiose wash is effective in removing cell surface WGA-Ft, we proceeded to



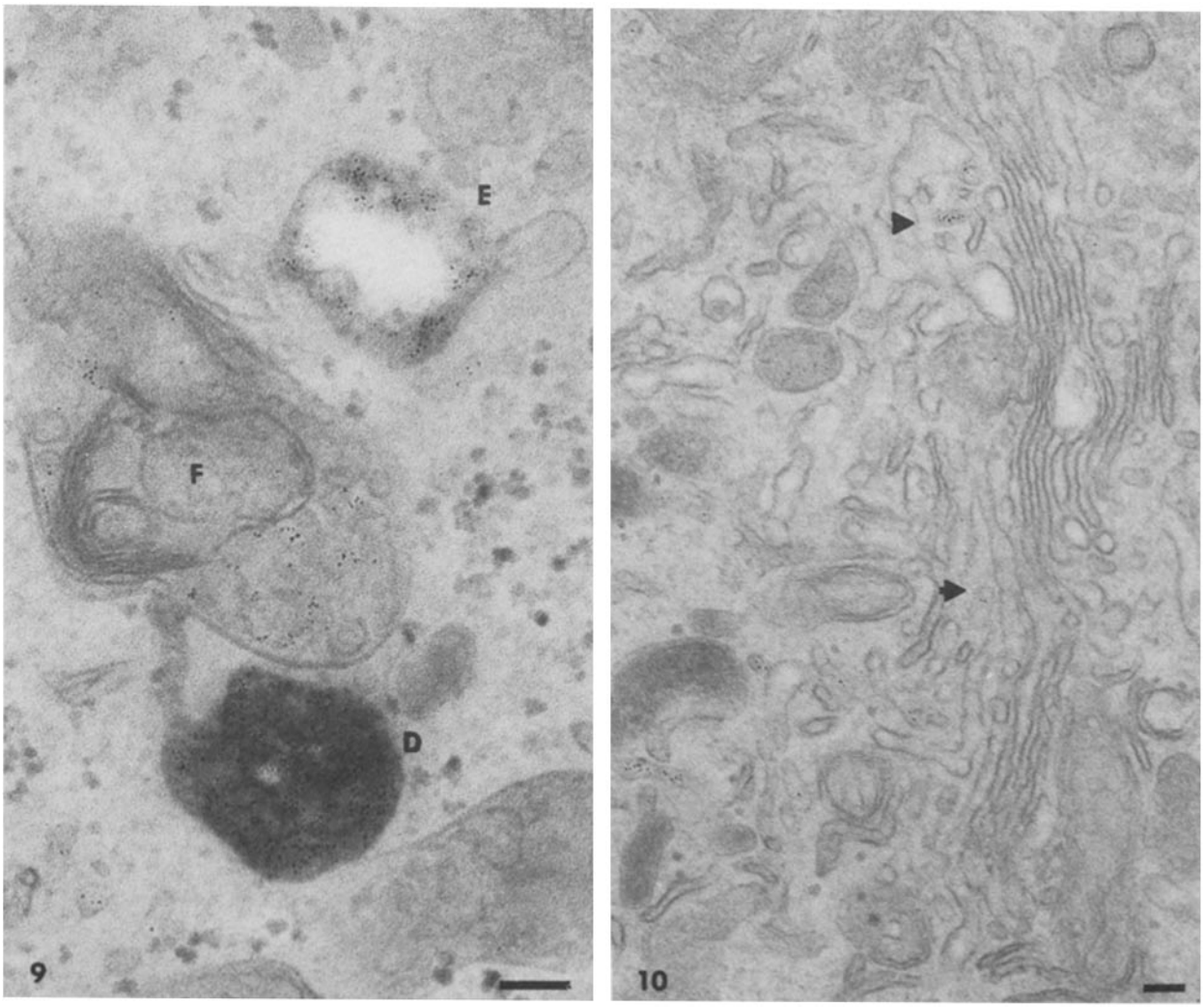
FIGURES 4–6 Fig. 4: Cells incubated with WGA-Ft and HRP as in Fig. 2. Arrow: endosome with HRP stain and ferritin. Arrowheads: doubly labeled vesicles connected with endosome; unstained. × 70,000. Fig. 5: Cells treated with WGA-Ft and HRP as in Figs. 2 and 4. Arrow: doubly labeled endosome. Arrowheads: vesicle-tubules connected with endosome contain only ferritin; unstained. × 61,000. Fig. 6: Cells treated with WGA-Ft and HRP as in previous figures stained with uranyl. Arrow: doubly labeled endosome. Arrowhead: "straight, fuzzy, lamellar" edge of endosome as originally described in receptosomes by Willingham, et al. (12). × 78,000.



FIGURES 7 and 8 Fig. 7: Cells treated with WGA-Ft and HRP as in Figs. 2–6. Anastomosing tubules and vesicles contain only ferritin; unstained. × 77,000. Fig. 8: Cells incubated with WGA-Ft for 15 min at room temperature, washed, incubated in full culture medium with 5 mg/ml HRP for 15 min at 37°C, washed, and incubated for 105 min at 37°C in full culture medium before fixation and stain with DAB. Arrow: coated vesicle (pit?). Arrowheads: coated vesicles (pits?) connected with smooth walled tubules; stained with uranyl. × 88,000.

an experiment designed to test whether internalized WGA-Ft is recycled to the cell surface. In this experiment sister cultures were incubated with WGA-Ft in buffer for 15 min at room temperature, washed with buffer, incubated in culture medium for 15 min at 37°C, and washed with chitobiose to remove surface-bound WGA-Ft; after the chitobiose wash, cells were incubated in culture medium for 2 h at 37°C before fixation and processing for electron microscopy (see below in experiment *E* in Table IV). The number of ferritin particles per micrometer of plasma membrane was 3.9 + 1.1 (based on 198 ferritin particles counted from electron micrographs taken from sections of 11 cells), or 90% of the initial bound WGA-Ft. The difference in ferritin counts between those cells and those fixed immediately after the chitobiose wash was not statistically significant by Student's *t* test. The concentration of intracellular WGA-Ft was similar in cells incubated for 15 min at 37°C and in those incubated at 37°C for an additional period of 2 h after the chitobiose wash (see below, Table IV, and compare ferritin per square micrometer between experiments *D* and *E*). In conclusion, under the conditions of the preceding three experiments, the chitobiose wash was effective in removing surface WGA-Ft and there was no recycling of internalized WGA-Ft to the cell surface in cells incubated at 37°C for 2 h after the chitobiose wash.

ENDOCYTOSIS OF WGA-FT: In Table I, the numbers of all labeled compartments are summarized; in Table II, labeled compartments are expressed per 100 μm^2 of cytoplasm. After incubation of cells with WGA-Ft for 15 min at room temperature, there was low but significant endocytosis of WGA-Ft in vesicles, tubules, and endosomes (Table II). Maximum labeling of WGA-Ft of vesicles and tubules, and of Golgi-associated vesicles and tubules, was observed in cells incubated for 15 min at 37°C after their initial labeling with WGA-Ft at room temperature (experiment *B*, Table II). The number of lysosomes labeled with WGA-Ft progressively increases with time (experiments *B*, *C*, *D*, Table II). Thus, three trends are observed in compartments labeled with WGA-Ft. (*a*) Labeled vesicles and tubules and Golgi-associated vesicles and tubules showed a peak after cells were incubated for 15 min at 37°C; (*b*) The number of labeled lysosomes progressively increases with time. (*c*) Endosomes with WGA-Ft showed an early peak (15 min at room temperature) followed by some decrease in numbers (15 and 30 min at 37°C), and by a second increase of their number (2 h at 37°C) to about the same level



FIGURES 9 and 10 Fig. 9: Cells treated with WGA-Ferritin for 15 min at room temperature, washed, and incubated in full culture medium with 5 mg/ml HRP at 15 min before fixation and incubation in DAB. *E*, doubly labeled endosome, *F*, lysosome labeled with ferritin, *D*, doubly labeled lysosomes; unstained. × 90,000. Fig. 10: Cells treated with WGA-Ft for 15 min at room temperature, washed, and incubated in full culture medium with 5 mg/ml HRP for 2 h at 37°C before fixation and incubation with DAB. Arrowheads: ferritin in vesicles and tubules at the *trans* face of the Golgi apparatus referred to in the text as Golgi vesicles; ferritin is not present in cisterns of the Golgi apparatus; unstained. × 52,000.

observed at the beginning of the experiment.

In Table III, compartments are presented according to their content of markers for fluid phase endocytosis (HRP), adsorptive endocytosis (WGA-Ft) labels, or of both labels (double label). Since the initial labeling of cells was done only with WGA-Ft, 100% of labeled compartments in each of the four categories contained only ferritin (Table III). In cells incubated for 15 min at 37°C with HRP in the medium, 83% of labeled vesicles and tubules contained only ferritin, 16% of labeled vesicles and tubules contained both labels while a negligible percentage of vesicles and tubules contained only HRP (Table III). Initially vesicles and tubules were computed separately; however, since similar values of labeling were seen in these two compartments and, quite often, a vesicle was continuous with a tubule, we treated these two compartments as a single one. In cells incubated for 15 min at 37°C, most Golgi-associated vesicles and tubules were labeled only with ferritin, while an interesting labeling pattern was observed in endosomes and lysosomes, most of which showed double labeling (94 and 80%, respectively, Table III). These observations are consistent with at least two hypotheses: either the membranes of the vesicles and tubules, most of which contain only WGA-Ft, fuse with the membranes limiting endosomes and lysosomes that contain both labels; or WGA-Ft is dissociated from its receptor at the endosome and/or lysosome and the two markers of fluid phase and adsorptive endocytosis join into a common pool. Actual connections between endosomes and vesicles or tubules containing both labels or only ferritin have been found (see Figs. 4 and 5). Finally, in experiment *D* (Tables I–III), cells incubated with WGA-Ft for 15 min at room temperature and washed were subsequently incubated for 105 min at 37°C in medium not containing HRP. With the exception of lysosomes, all compartments are predominantly labeled with WGA-Ft. The results of this experiment confirm that WGA-Ft undergoes endocytosis slowly and that HRP does not bind to WGA-Ft or to plasma membrane. We found that 4.0 ± 2.8% endosomes contained both labels, although cells were washed and incubated for 105

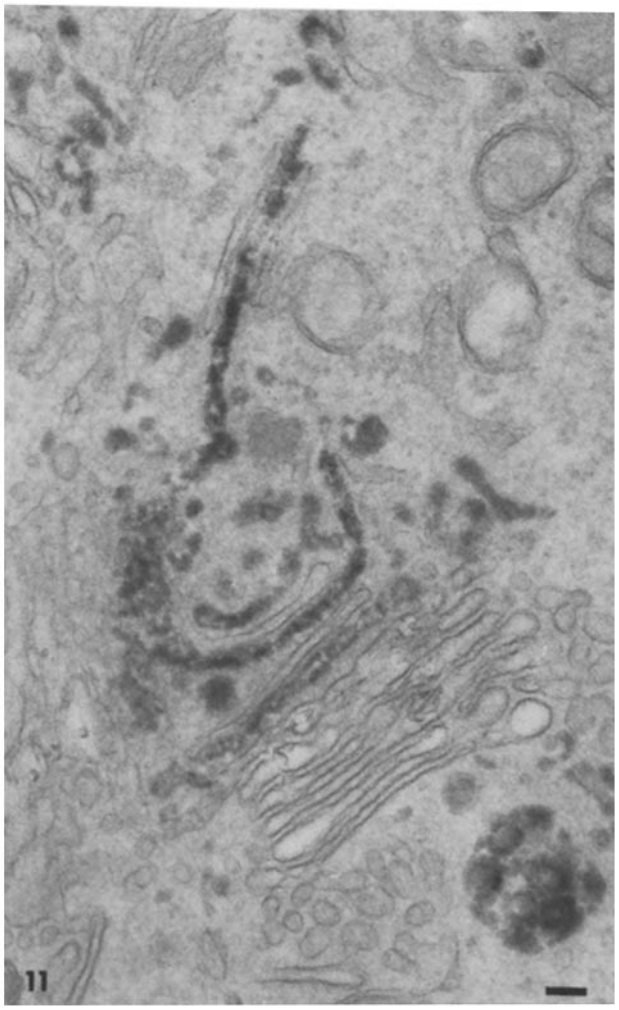


FIGURE 11 Cells incubated for 15 min at room temperature with 20 μg/ml WGA conjugated with HRP, washed, and incubated in full culture medium at 37°C for 2 h before fixation and incubation with DAB. Note intense peroxidase stain in cisterns of the Golgi; unstained. × 56,000.

min at 37°C in medium free of HRP; this indicates that HRP is not transported quickly from endosomes to another compartment(s).

DISTRIBUTION OF INTERNALIZED WGA-FT (TABLES IV AND V): Cells incubated with WGA-Ft in buffer for 1 h at 4°C showed no ferritin in endosomes and lysosomes and virtually no ferritin in vesicles or tubules adjacent to the cell surface. Therefore, ferritin counts in this experiment were not performed.

The results of five experiments in which endocytosis of ferritin was observed are summarized in Tables IV and V. In the first experiment, (*A*), cells were incubated with WGA-Ft in buffer for 1 h at 4°C, washed in cold buffer, and then incubated in culture medium for 5 min at 37°C before fixation and processing for electron microscopy. We observed that the sequential exposure of cells to 4°C, to room temperature, and then to 37°C, frequently resulted in a less than optimal preservation of the fine structure manifested by "swelling" of vesicles, etc. For that reason, in all subsequent four experiments, the initial "loading" of cells with WGA-Ft was carried out at room temperature (experiments *B*, *C*, *D*, and *E*). As illustrated in Table IV, comparable amounts and distributions of internalized WGA-Ft were observed in cells incubated with the ligand for 1 h at 4°C and then for 5 min at 37°C, and in cells incubated with the ligand for 15 min at room temperature (compare data between *A* and *B*, Table IV).

In experiments *B* and *C* sister cultures were used. In experiment *B*, cells were incubated with WGA-Ft in buffer for 15 min at room temperature, washed, and fixed for electron microscopy. In experiment *C*, cells, after an initial "loading" with WGA-Ft for 15 min at room temperature, were washed with buffer, and incubated in culture medium for 15 min at 37°C and then fixed for electron microscopy.

In experiments *D* and *E*, sister cultures were used; however, these experiments were done at another time than *A*, or *B* and *C*. Thus, while comparisons of absolute and relative values between *B* and *D* or *D* and *E* are valid, comparisons between *B* and *C* and *D* and *E* should take into account that these two groups of experiments were not done simultaneously with identical cultures. The last two experiments, *D* and *E*, were designed to investigate the amount and intracellular distribution of WGA-Ft by cells incubated in culture medium at 37°C for 2 h after the removal of surface WGA-Ft by chitobiose.

The results, summarized in Table IV justify the following conclusions: (*a*) Percentage distributions of WGA-Ft among the three principal compartments involved in its endocytosis, i.e., tubules and vesicles, (identified as vesicles), endosomes, and lysosomes show an early influx of WGA-Ft into endosomes and a later entry of WGA-Ft into lysosomes. For example, after 15 min at room temperature, cells had taken in 4.23 ± 0.31 of ferritin per square micrometer of cytoplasm, while at a later time, i.e., after an additional incubation of cells at 37°C for 15 min, the amount of intracellular ferritin rose to 30.54 ± 1.94 per square micrometer (compare *B* with *C*). In the same experiment, at the earliest time of endocytosis (*B*), 34.2 ± 2.9% of the total intracellular ferritin was in endosomes, while after 15 min at 37°C, endosomes contained

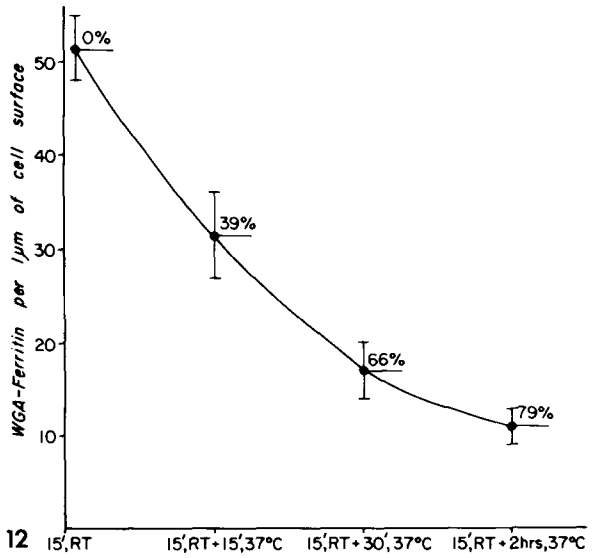


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FIGURE 12 Surface counts of ferritin in cells incubated with WGA-Ft for 15 min at room temperature (*15', RT*), or for 15 min, 30 min and 2 h at 37°C after incubation with WGA-Ft for 15 min at room temperature and wash (*15', RT + 15', 37°C*), (*15', RT + 30', 37°C*), (*15', RT + 2 hrs, 37°C*); percentage values represent percentage clearance of cell surface WGA-Ft when compared with the value obtained at 15' RT (0%).

TABLE I

Number of Labeled Compartments Counted in Each Experiment in PC-12 Cells

Experiment	Vesicles and Tubules			Golgi Vesicles and Tubules			Endosome			Lysosome			Total number counted
	FER	HRP	DBL	FER	HRP	DBL	FER	HRP	DBL	FER	HRP	DBL	
A	91	0	0	1	0	0	25	0	0	5	0	0	122
B	629	2	123	102	0	3	1	0	16	8	0	32	916
C	365	4	59	41	0	1	0	0	18	8	6	59	561
D	557	0	6	73	0	0	71	0	3	184	0	36	930

Cells were incubated for 15 min at RT with WGA-Ft, washed, and fixed (*A*); after initial incubation of cells as in *A*, cells were washed and incubated at 37°C for 15 min (*B*), or 30 min (*C*) in medium containing 5 mg/ml HRP. In *D*, cells were incubated for 15 min at RT with WGA-Ft, washed, incubated for 15 min at 37°C in medium with 5 mg/ml HRP, washed, and incubated for 105 min at 37°C in medium. Six cells were counted per each experiment. *Fer*, ferritin; *DBL*, compartments labeled with ferritin and HRP.

TABLE II

Total Number of Labeled Compartments per 100 μm² Cytoplasm of PC-12 Cells

Experiment	Vesicles and tubules	Lysosomes	Golgi vesicles and tubules*	Endosomes	No. of Cytoplasm Counted
					μm
A	28.7 ± 5.2	1.5 ± 0.4	5.0 ± 4.5	7.4 ± 1.1	324
B	171 ± 24.5	8.5 ± 1.3	372 ± 1.3	3.6 ± 0.6	468
C	98.7 ± 23.1	16.2 ± 2.2	235 ± 38.7	4.0 ± 0.7	451
D	73.6 ± 8.9	26.7 ± 2.4	123 ± 18.7	9.0 ± 2.8	824

For description of experiments see legend of Table I. *Golgi vesicles and tubules were expressed per square micrometer area of the Golgi apparatus.

TABLE III

Percent of Each of Four Compartments Labeled with HRP, Ferritin, and HRP and Ferritin (Double Label) in PC-12 Cells

Experiment	Vesicles and tubules			Golgi vesicles and tubules			Endosomes			Lysosomes		
	Ferritin	HRP	Double label	Ferritin	HRP	Double label	Ferritin	HRP	Double label	Ferritin	HRP	Double label
A	100	0	0	100	0	0	100	0	0	100	0	0
B	83.4 ± 4.8	0.2 ± 0.2	16.2 ± 4.7	97.1 ± 1.7	0	2.8 ± 1.7	5.9 ± 6.8	0	94.1 ± 6.8	20.0 ± 8.5	0	80.1 ± 8.6
C	85.3 ± 4.0	0.9 ± 1.1	13.8 ± 3.6	97.6 ± 1.6	0	2.4 ± 1.6	0	0	100	10.9 ± 2.8	8.2 ± 5.8	80.8 ± 4.5
D	98.9 ± 0.4	0	1.1 ± 0.3	100	0	0	95.9 ± 2.8	0	4.0 ± 2.8	83.6 ± 4.2	0	16.3 ± 4.3

For description of experiments, see legend of Table I.

19.9 ± 2.9% of the total intracytoplasmic ferritin. (*b*) The amount of intracellular ferritin in cells incubated for 2 h at 37°C after the chitobiose wash (8.22 ± 2.40) is almost identical to the amount of ferritin counted at the end of the chitobiose wash (8.41 ± 1.47) (compare *D* and *E*, Table IV). Therefore, the PC12 cells do not lose any detectable amounts of intracellular WGA-Ft during the 2-h period at 37°C. This result, coupled with the previously presented counts of surface ferritin, strongly suggests that the intracellular traffic of WGA-Ft is unidirectional, i.e., from the cell surface to endosomes to lysosomes. (*c*) Although the total amounts of intracytoplasmic WGA-Ft are similar in cells at the end of the chitobiose wash (*D*, Table IV) and after 2 h at 37°C (*E*, Table IV), there are significant differences in the intracellular distributions of WGA-Ft; after incubation of cells for 2 h at 37°C there is a significant reduction of WGA-Ft in vesicles and endosomes while a significant increase of WGA-Ft in lysosomes is observed (Table IV, *D* and *E*). Surprisingly, a low but significant percentage of total WGA-Ft is found in endosomes even after incubations of cells at 37°C for 2 h after the chitobiose wash (6.8 ± 3.0% of total, Table IV). This observation strongly suggests that the passage of WGA-Ft from endosomes is not a transient and fast event. The alternative explanation, i.e., that there is a continuous, low level transport of WGA-Ft from the cell surface to endosomes is unlikely since there is no significant difference in the low levels of surface ferritin between the end of the chitobiose wash (5.4 ± 1.2 ferritin per micrometer) and the end of the 2-h incubation in culture medium at 37°C (3.9 ± 1.1 ferritin per micrometer).

In Table V we summarize the distributions of WGA-Ft per organelle sections and per square micrometer of organelle. Comparisons of number of ferritin particles per organelle section or per square micron of organelle show that during the early phase of endocytosis there is approximately a two- to four-fold increase of ferritin concentration in endosomes and lysosomes (Table V, *B* and *C*). Interestingly, approximately similar concentrations of ferritin were observed in endosomes (1,028 ± 210) and in lysosomes (1,124 ± 217) of cells incubated for 15 min at 37°C (Table V, *C*). Comparisons of ferritin concentration between vesicles and endosomes and lysosomes may not be entirely valid if most of the WGA-Ft is bound on the membrane of vesicles, while most of WGA-Ft is in the lumen of endosomes and lysosomes as electron micrographs strongly suggest (Figs. 6, 8, and 9).

Examination of the data obtained from the two experiments in which most of the surface WGA-Ft was removed by chitobiose (Table V, *D* and *E*) indicate the following: comparable concentrations of WGA-Ft were observed in vesicles in cells fixed immediately after the chitobiose wash (488 ± 38) and in cells incubated in culture medium at 37°C for 2 h,

(575 ± 258). Since most of the surface WGA-Ft had been removed by the chitobiose wash, and there was no ligand in the incubation medium during the 2-h period, the finding of a considerable percentage of WGA-Ft in vesicles at this time is consistent with either one or both of the following two interpretations: one, there is a slow rate of fusion of endosomes and lysosomes with vesicles carrying WGA-Ft from the plasma membrane; two, many of the WGA-Ft containing vesicles represent carriers of WGA-Ft from endosomes to lysomes. The second interpretation is enhanced by the finding in experiment *E* (2 h at 37°C) of a decreased concentration of WGA-Ft in endosomes (171 ± 47/μm^2) in comparison to concentration of WGA-Ft in endosomes in cells incubated for 15 min at 37°C (355 ± 52) (compare *D* and *E* in Table V). At these two time points, the concentration of WGA-Ft in lysosomes is similar (649 ± 155/μm^2 in *D*, and 509 ± 99 in *E*, Table V).

Areas of vesicles, endosomes, and lysosomes containing WGA-Ft and of negative (WGA-Ft free) endosomes and lysosomes are shown in Table VI. To maximize the accuracy, reproducibility, and speed of the quantitative studies, counts of ferritin particles were done on electron micrographs from sections of cells that were not stained with lead or uranyl. We observed that in these micrographs the ferritin densities stand out with greater clarity than in stained sections. However, while we could identify ferritin-free endosomes and lysosomes, the recognition of ferritin-free vesicles and tubules was less precise. For that reason we did not count the ferritin

TABLE IV

Endocytosis of WGA-Ft by PC12 Cells

Experiment	NR cells	NR EM graphs	μm^2	NR Ft	Ft/μm^2	Percentage of total WGA-Ft in each organelle compartment: Vesicles	Golgi vesicles	Endosomes	Lysosome
(*A*) 1 hr 4°C-wash-5 min 37°C-fix	8	100	494	1,472	2.98 ± 0.52	44.1 ±4.6	0	38.4 ± 6.9	17.4 ± 5.2
(*B*) 15 min RT-wash-fix	6	72	388	1,644	4.23 ± 0.31	55.0 ± 3.4	0.7 ± 0.5	34.2 ± 2.9	10.1 ± 2.9
(*C*) 15 min RT-wash-15 min 37°C-fix	6	72	403	12,305	30.54 ± 1.94	60.4 ± 4.1	2.2 ± 0.6	19.9 ± 2.9	17.5 ± 3.5
(*D*) 15 min RT-wash-15 min 37°C-chitobiose-fix	9	118	911	7,670	8.41 ± 1.47	43.1 ± 3.5	4.1 ± 1.5	24.0 ± 5.4	28.7 ± 4.7
(*E*) 15 min RT-wash-15 min 37°C-chitobiose-2 h 37°C-fix	8	100	530	4,356	8.22 ± 2.40	22.9 ± 4.6	0.4 ± 0.2	6.8 ± 3.0	69.8 ± 6.6

Experiments *A*, *B*, and *C*, and *D* and *E* were done separately. In *B* and *C*, and *D* and *E* sister cultures were used. Chi-square tests showed significant difference in percentage distribution of WGA-Ft between *B* and *C*, and *D* and *E* ($P < 0.05$), but no difference between *A* and *B*. In all experiments the first incubation of cells was done with buffer containing WGA-Ft. *NR, EM,* electron microscope; *Ft,* ferritin.

TABLE V

Endocytosis of WGA-Ft by PC12 Cells

Experiment	WGA-Ft per sectioned organelle: Vesicle	Golgi vesicle	Endosome	Lysosome	WGA-Ft per square micron of organelle: Vesicle	Golgi vesicle	Endosome	Lysosome
(A) 1 h 4°C-wash-5 37°C-fix	3.86 ± 0.22	0	20.2 ± 2.3	13.3 ± 3.4	340 ± 75	0	249 ± 58	176 ± 73
(B) 15 min RT-wash-fix	6.90 ± 0.54	1.83 ± 0.40	18.2 ±2.2	21.0 ± 7.0	1,140 ± 164	‡	249 ± 22	539 ± 327
(C) 15 min RT-wash-15 min 37°C-fix	6.88 ± 0.19	4.44 ± 0.52	97.2 ± 15.1	59.8 ± 8.3	1,556 ± 257	934 ± 141	1,028 ± 210	1,124 ± 217
(D) 15 min RT-wash-15 min 37°C-chitobiose-fix	3.17 ± 0.10	2.40 ± 0.15	25.1 ± 3.0	41.6 ± 5.7	488 ± 48	490 ± 46	355 ± 52	649 ± 155
(E) 15 min RT-wash-15 min 37°C-chitobiose-2 h 37°C-fix	3.69 ± 0.22	3.40 ± 1.66	14.8 ± 4.4	29.4 ± 2.8	575 ± 258	‡	171 ± 47	509 ± 99

* Same experiments as described in Table IV.

‡ No points on positive Golgi vesicles.

By Student's *t* test there was significant difference between *B* and *C* in WGA-Ft per endosome and lysosome, and in WGA-Ft per square micrometer of endosome.

TABLE VI

Percent of Total Cytoplasmic Area Occupied by Endosomes, Lysosomes, and WGA-Ft Containing Vesicles

Experiment	WGA-Ft vesicles	Golgi vesicles	Negative endosomes	WGA-Ft endosomes	Negative lysosomes	WGA-Ft lysosomes	All WGA-Ft
(*A*) 1 h 4°C-wash-5 min 37°C-fix	0.39 ± 0.03	0.0	0.04 ± 0.02	0.46 ± 0.08	0.42 ± 0.10	0.29 ± 0.11	1.14 ± 0.12
(*B*) 15 min RT-wash-fix	0.20 ± 0.03	<0.01	0.12 ± 0.04	0.58 ± 0.06	0.89 ± 0.22	0.08 ± 0.04	0.87 ± 0.06
(*C*) 15 min RT-wash-15 min 37°C-fix	1.19 ± 0.18	0.07 ± 0.02	0.10 ± 0.04	0.44 ± 0.12	1.93 ± 0.63	0.45 ± 0.11	2.15 ± 0.25
(*D*) 15 min RT-wash 15 min 37°C-chitobiose-fix	0.74 ± 1.01	0.07 ± 0.04	0.0	0.57 ± 0.28	1.69 ± 0.18	0.34 ± 0.05	1.76 ± 0.33
(*E*) 15 min RT-wash-15 min 37°C-chitobiose-2 h 37°C-fix	0.33 ± 0.09	<0.01	0.09 ± 0.03	0.32 ± 0.08	0.64 ± 0.25	1.13 ± 0.23	1.79 ± 0.34

negative vesicular and tubular compartments.

The data on Table VI are consistent with the following four conclusions: (*a*) the percentage of the total cytoplasmic area occupied by the four WGA-Ft containing compartments (all WGA-Ft) represent 1–2% of the entire cytoplasm; (*b*) there is doubling of the total WGA-Ft area in cells incubated for 15 min at 37°C (compare *B* with *C* in all WGA-Ft columns); (*c*) the WGA-Ft containing endosomes show virtually no changes during the various stages of endocytosis (column WGA-Ft endosomes, Table VI), while the compartment of positive vesicles, and lysosomes shows a significant three- to five-fold expansion or decrease (columns of WGA-Ft vesicles and WGA-lysosomes, Table VI); (*d*) in all five experiments, there is an inverse relationship between positive and negative lysosomes; in the last two experiments in which endocytosis was stopped at the end of the chitobiose wash (*D*), or was allowed to proceed for 2 h after the chitobiose wash (*E*), there is a clear inverse relationship between the areas occupied by positive vesicles and lysosomes.

The negative endosomes occupy a very low, and probably insignificant, percentage of the total cytoplasmic area, while negative lysosomes occupy a significant part of the cytoplasm of PC12 cells. Since we did not perform serial sections, conclusions on negative lysosomes are tentative since the distribution of WGA-Ft may vary in sections of lysosomes.

DISCUSSION

Wheat Germ Agglutinin

Since the original report by Nagata and Burger on the isolation, purification, and crystallization of wheat germ agglutinin, numerous studies have described the chemical and biological properties of the lectin (27, 28). Wheat germ agglutinin binds specifically to *N*-acetylglucosamine, its derivative sugars such as di-*N*-acetylchitobiose, and to *N*-acetylneuraminic acid, which are present in plasma membranes of mammalian cells including neurons and synaptic membranes (29).

Unlike low density lipoprotein particles, asialoglycoproteins, α^2-macroglobulin, and other ligands with few surface receptors of limited specificity, WGA binds to a large number of heterogeneous "receptors" with terminal *N*-acetylglucosamine, di-*N*-acetylchitobiose, and *N*-acetylneuraminic acid (7, 9–13, 30). This limitation of wheat germ agglutinin as a probe of cell surfaces and of endocytosis is balanced by the fact that cells have more than one class of surface receptor, to a host of physiologic and pathologic ligands. Therefore, the quantitative study of adsorptive endocytosis of a ligand with numerous surface "receptors," such as wheat germ agglutinin, reflects probably quite faithfully events constantly occurring at the plasma membrane and in endocytic compartments. Furthermore, wheat germ agglutinin, at concentrations used in these experiments is not toxic to neurons in vivo and in vitro (18, 33–35).

Wheat Germ Agglutinin as a Probe of Neuronal Plasma Membranes and Endocytosis

Since the original studies by Schwab et al. and by us on the sensitivity of wheat germ agglutinin as a tracer of neuronal connections, wheat germ agglutinin labeled with [^{3}H], [^{125}I], or HRP has been used in a number of studies of neuronal connectivity in which orthograde and retrograde transport routes have been exploited (31–34). However, the cellular, subcellular, and quantitative aspects of the interactions between wheat germ agglutinin and neurons have not yet been elucidated. We have shown that WGA-HRP undergoes endocytosis in neuronal GERL, both in vivo and in vitro (4, 34, 35). Furthermore, in NGF-stimulated PC12 cells there was a tenfold increase of WGA-HRP that had undergone endocytosis in GERL in comparison to internalized WGA-HRP by PC12 cells not exposed to NGF (18). Lavail et al., using [^{125}I]WGA, have found by ultrastructural autoradiography that the lectin is transported in axons within a peripheral submembranous band of cytoplasm containing elongated cisternae of the smooth endoplasmic reticulum (36).

Endocytosis of WGA-Ft and WGA-HRP

Horseradish peroxidase, ferritin, colloidal gold, fluorescent reagents and radioactive isotopes are the most frequently used morphologic markers for ligands studied in receptor-mediated and adsorptive endocytosis. A question of considerable interest is whether the marker molecule modifies the endocytosis or detection of a given ligand. Most likely the most faithful and sensitive morphologic display of a ligand is provided by the quantitative ultrastructural autoradiography of a [^{3}H] or [^{125}I]-labeled ligand. The two limitations of the approach, resolution and false localizations produced by radioactivity scatter from adjacent heavily labeled sources can be overcome by the methods pioneered by Salpeter and colleagues (37). However, these methods are time-consuming and require computer analysis (38). For these reasons, ferritin is a reasonable alternative to autoradiography as it offers precision of localization and quantitation.

Since most of our previous work was done with HRP or radiolabeled ligands we decided to compare WGA-Ft and WGA-HRP (3–7, 18, 34, 35). The use of WGA-Ft and WGA-HRP gave comparable qualitative results with one striking exception; while we found extensive localization of WGA-HRP in cisternae of the Golgi apparatus and in adjacent vesicles, WGA-Ft was found only in vesicles at the *trans*-aspect of the Golgi apparatus but not in Golgi cisternae (compare Figs. 10 and 11; also see reference 18 on endocytosis of WGA-HRP by NGF-treated PC12 cells).

There are at least four explanations for the observed differential distribution of WGA-HRP and WGA-Ft in the region of the Golgi apparatus. First, WGA-Ft, because of the small size of ferritin, has been overlooked in Golgi cisternae. In view of the extensive quantitative studies conducted (we counted a total of 27,447 particles in 344 electron micrographs) we believe that WGA-Ft was not overlooked.

Second, the diffusion of the enzymatic product of HRP may have amplified a minute amount of WGA-HRP in Golgi cisternae. Since the introduction by Graham and Karnovsky of the DAB method for the light and electron microscopic detection of HRP the question of the distance of diffusion of the reaction product from its origin is uncertain. To answer this question Courtoy et al. studied the diffusion of HRP in a system involving cationic ferritin, rabbit antiferritin antibody, and HRP-labeled anti-rabbit antibody (39). In this system, which is not comparable to ours, DAB staining was found at >100 nm from ferritin clusters (39). In unpublished experiments we studied the diffusion of DAB in two systems involving concanavalin A-ferritin and HRP, or cationized ferritin-antiferritin antibody-HRP labeled antiantibody. We found diffusion distances of DAB of 14–55 nm. Therefore,

we do not believe that the diffusion of the HRP reaction product explains the differential localization of WGA-HRP and WGA-Ft in the Golgi cisternae, which measure several micrometers in length. Furthermore, the molar ratios of ferritin or HRP to WGA was almost identical in both conjugates (1:1).

Third, the covalent conjugation of the large ferritin molecule (molecular weight 500–500,000; iron core dimensions 5.5–6-nm in diam) with the relatively small wheat germ agglutinin molecule (molecular weight 18,000 for each of the two identical subunits) may have blocked, by steric hindrance or other mechanisms, a part of the WGA molecule essential for binding to cell surface receptor(s) targeted for endocytosis in the cisternae of the Golgi apparatus.

Finally, a fourth explanation for the differential endocytosis of WGA-Ft and WGA-HRP may be related directly to HRP. This enzyme glycoprotein, particularly rich in mannoside residues, may be preferentially targeted and/or retained by membranes rich in mannose receptors (41).

Co-Localization of Markers of Fluid Phase and Adsorptive Endocytosis in Endosomes

Analysis of the percent of each of the four counted compartments according to internalized label, i.e., HRP, ferritin or both labels (double) revealed three important trends. First, when HRP was present in the medium during the entire experiment, 94–100% of labeled endosomes contained both labels, while a smaller percentage of vesicles and tubules contained both labels (13–16%) (Table III, *B* and *C*); in these experiments (Tables I–III), the initial "loading" of cells with WGA-Ft was performed at room temperature for 15 min; under these conditions 34% of internalized WGA-Ft was in endosomes (*B*, Table IV). Therefore, the finding that upon subsequent incubations with HRP for 15 min or 30 min 97% of endosomes were doubly labeled indicates that this organelle is the recipient of both fluid phase and adsorptive endocytosis; furthermore, it is apparent that endosomes prelabeled with WGA-Ft are available for the subsequent endocytosis of HRP. Finally, the total endosome pool seems to participate in the endocytosis, since the finding of unlabeled or negative endosomes was negligible (Table VI). Second, when HRP was present in the medium only during the first 15 min of incubation at 37°C and cells were subsequently incubated in medium not containing HRP, only 4% of endosomes were doubly labeled while 96% of endosomes contained only ferritin. Third, irrespective of the duration of incubation of cells with HRP, 97–100% of labeled vesicles and tubules in the vicinity of the Golgi apparatus showed only ferritin.

In their 1982 review of receptor-mediated transport of IgG across the intestinal epithelium of the neonatal rat, Rodewald and Abrahamson presented an electron micrograph (their Fig. 13) of an apical vacuole containing both cationic ferritin and HRP (17). Our electron micrographs, Figs. 4 and 5, show virtually identical large vesicles, or endosomes-receptosomes, with fusing tubules, two of which contain only WGA-Ft (Fig. 5) and one containing HRP and WGA-Ft, as in the Rodewald and Abrahamson study (17). According to the previous authors, the apical vacuoles, which probably correspond to the endosomes-receptosomes of the present study, are sites of internal sorting of membrane bound and fluid phase markers. In conclusion, we have confirmed in a cultured cell that lacks the polarity of the in situ epithelial cell, that endosomes are recipients of both markers of fluid phase and adsorptive endocytosis.

Endocytosis of WGA-Ft in Golgi Vesicles

The recognition that an overwhelming percentage of vesicles and tubules in the vicinity of the Golgi apparatus have only WGA-Ft (Table III) suggests that a special class of endocytic vesicles and tubules, originating either directly from the plasma membrane or from endosomes-receptosomes, aggregate near the Golgi apparatus. The Golgi vesicles contained only 2–4% of WGA-Ft (Table IV). The functional significance of this small endocytic compartment is unknown; Willingham and Pastan have suggested that the Golgi system of vesicles is involved in receptor retrieval and recycling (11).

WGA-Ft Is Not Recycled to Plasma Membrane

Recycling of internalized receptors to the plasma membrane is considered to be a mechanism for the retrieval and reutilization of receptors and other plasma membrane moieties (40, 42). We demonstrated that most of WGA-Ft does not bind irreversibly to its "receptors" since 86% of surface bound WGA-Ft is washed off with chitobiose. Furthermore, we have shown that there is no increase of WGA-Ft on cell surfaces after the chitobiose wash and subsequent incubation of cells at 37°C for 105 min. Finally, virtually identical amounts of internalized WGA-Ft were found in cells right after the chitobiose wash and 105 min later (Table IV, *D* and *E*). All these data indicate that WGA-Ft is not recycled back to the cell surface. These data, however, do not imply that the "receptors" to which WGA-Ft is bound noncovalently are not recycled: to the contrary, most of these numerous and heterogeneous WGA "receptors" probably recycle back to the plasma membrane. We base this hypothesis on three lines of evidence; (*a*) In HeLa cells, a major portion of the surface [^{125}I]-labeled glycoproteins were internalized concurrently with wheat germ agglutinin. The electrophoretic profiles of internalized [^{125}I]-labeled proteins in these cells exposed to WGA were similar to those in plasma membranes isolated from control untreated cells (30). This observation is consistent with the conclusion that the wheat germ agglutinin-induced endocytosis of lactoperoxidase-iodinated plasma membrane proteins is a nonselective process (30). (*b*) Wheat germ agglutinin, in concentrations used in these experiments (20 μg/ml), is not toxic to neurons and PC-12 cells (18, 34, 36); and (*c*) extrapolating from studies we conducted with the d-galactose binding toxic lectin ricin, the number of WGA-Ft "receptors" per cell should be of the order of 5–8 × 10^8 (7). We would like to argue that the most efficient reconstitution of plasma membranes during the massive and nonselective endocytosis of ligand-receptor complexes is by receptor recycling (40, 42). Recycling of plasma membrane receptors has been shown to occur in two studies in which antireceptor antibodies were available (13, 43). This direct evidence for receptor recycling has implicated the so-called compartment for uncoupling of receptor from ligand system of vesicles that morphologically are quite similar to the tubulo-vesicular profiles of this study (Figs. 7 and 8) (13, 43). Receptosomes or endosomes have also been implicated in the retrieval of the receptor for its recycling to the plasma membrane and in the processing of the ligand to lysosomes (12, 14). Our findings are consistent with the hypotheses proposing compartment

for uncoupling of receptor from ligand or endosomes-receptosomes as sites for the uncoupling of receptor from ligand, receptor retrieval, and processing of the ligand to lysosomes; both types of organelles were labeled with WGA-Ft during the entire course of the experiment (Figs. 4–8, and Tables IV and V).

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