

## INTERNALIZATION OF LECTINS IN NEURONAL GERL

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### ABSTRACT

Conjugates of ricin agglutinin and phytohemagglutinin with horseradish peroxidase (HRP) were used for a cytochemical study of internalization of their plasma membrane "receptors" in cultured isolated mouse dorsal root ganglion neurons. Labeling of cells with lectin-HRP was done at 4°C, and internalization was performed at 37°C in a culture medium free of lectin-HRP. 15–30 min after incubation at 37°C, lectin-HRP-receptor complexes were seen in vesicles or tubules located near the plasma membrane. After 1–3 h at 37°C, lectin-HRP-receptor complexes accumulated in vesicles and tubules corresponding to acid phosphatase-rich vesicles and tubules (GERL) at the trans aspect of the Golgi apparatus. A few coated vesicles and probably some dense bodies contained HRP after 3–6 h of incubation at 37°C. Soluble HRP was not endocytosed under the conditions of this experiment or when it was present in the incubation medium at 37°C. Internalization of lectin-HRP-receptor conjugates was decreased or inhibited by mitochondrial respiration inhibitors but not by cytochalasin B or colchicine. These studies indicate that lectin-labeled plasma membrane moieties of neurons are endocytosed primarily in elements of GERL.

Considerable information concerning the mobility and distribution of plasma membrane proteins has been gained with the use of various ligands. Plasma membrane receptors of lymphocytes to anti-immunoglobulin antibodies or lectins undergo redistribution (patching and capping) and endocytosis (internalization) after exposure to the appropriate ligand (13, 32). Immunoglobulin-anti-immunoglobulin (Ig-anti Ig) complexes of lymphocyte plasma membranes segregate and internalize together with a significant percentage of lactoperoxidase iodinated plasma membrane proteins (14); in plasma cells, the Ig-anti-Ig complexes internalize in vesicles in the Golgi zone (2). In this paper, we report studies on the endocytosis (internalization) of neuronal plasma membrane "recep-

tors" for ricin (Ric) and phytohemagglutinin (PHA) labeled with horseradish peroxidase (HRP). We have shown by peroxidase and acid phosphatase cytochemistry that these two lectin-HRP conjugates internalize in vesicles and tubules corresponding to the acid phosphatase-rich component of the Golgi zone, i.e., GERL as defined by Novikoff et al. (4, 22–24), coated vesicles and dense bodies, which play a primary role in the uptake of soluble HRP and other proteins in neurons and other cells, are secondarily involved in the endocytosis of plasma membrane lectin-receptor complexes (1, 5, 9, 10, 17, 18, 28, 29, 31).

The mechanism and significance of the internalization of lectin receptors into GERL are unknown. It is tempting to speculate that plasma

membrane lectin-receptor complexes are endocytosed into GERL for digestion or eventual reutilization (recycling) (26).

## MATERIALS AND METHODS

### *Cell Cultures*

15-day-old mouse embryo dorsal root ganglia were dissected and transferred to a medium composed of 30 parts of horse serum, 58 parts of Earle's medium and supplementary glucose to a final concentration of 1%. The ganglia were placed on a collagen-coated plastic cover slip and grown in a 35-mm Falcon Petri dish (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) in a CO<sub>2</sub> incubator or in a roller tube (15, 19). 1-2 wk after the original explantation, cultures containing isolated neurons or small clusters of neurons were selected for incubation with Ric-HRP or PHA-HRP conjugates.

### *Lectins*

*Ricin agglutinin.* The agglutinin was isolated from a crude extract of *Ricinus communis* beans which was subsequently adsorbed onto glutaraldehyde-insolubilized stromata of rabbit erythrocytes and subsequently eluted with a glycine buffer (13). *Phaseolus vulgaris* agglutinin (PHA) was obtained from Difco Laboratories (Detroit, Mich.) and was used after dialysis against 0.15 M sodium chloride.

### *Conjugation of Lectins with HRP*

Lectins were covalently coupled with HRP with the two-step method utilizing glutaraldehyde as the cross-linking agent. The procedures employed have been described in detail elsewhere (3, 12); conjugates with 1:1 molecular ratios of lectin:HRP were used.

### *Preparation of Cells for*

#### *Lectin-HRP Binding*

Cells grown on plastic cover slips or strips were thoroughly washed with Earle's balanced salt solution (BSS) to remove the culture medium. In two experiments, washing of cells was followed by incubation for 1 h at 36°C in Earle's BSS or in Earle's BSS with 1% bovine serum albumin (BSA). This treatment did not affect the subsequent surface or intracellular demonstration of Ric-HRP. After washing, or after incubations in Earle's BSS, cells were incubated for 1 h at 4°C at concentrations of 1, 10, and 100 µg/ml of Ric-HRP or PHA-HRP. Dosage is expressed as micrograms of lectin per milliliter. The cells were subsequently washed in cold (4°C) Earle's BSS (three changes every 1-2 min) and were either fixed in paraformaldehyde-glutaraldehyde for 15-30 min at room temperature, or incubated in full culture medium at 37°C for 5 min, 30 min, 1, 3, 6, 24, and 48 h and then fixed in paraformaldehyde-glutaraldehyde (1% paraformaldehyde, 1% glutaraldehyde in 0.15 M phosphate

buffer pH 7.3 plus calcium chloride 0.02%). After fixation, cells were washed in Earle's BSS and stained for peroxidase (16). Usually, cells were stored overnight in 0.2 M cacodylate buffer, pH 7.35 with 5% sucrose, before staining for 10 min at room temperature with diaminobenzidine tetrahydrochloride (DAB) according to the method of Graham and Karnovsky (16).

### *Inhibition Studies with Sugars*

Ricin at a concentration of 100 µg/ml was incubated for 1 h at 37°C in Earle's BSS containing 0.1 M *D*-galactose,  $\alpha$ -lactose, *N*-acetyl-*D*-galactosamine, or  $\alpha$ -methyl-*D*-mannoside. Subsequently, the solution was kept at 4°C for 12 h. Cell cultures were washed in Earle's BSS containing appropriate sugars, and incubations with the lectin were performed in the presence of 0.1 M concentrations of the appropriate sugar.

### *Metabolic Inhibitors*

Cells, after incubation at 4°C for 1 h with the lectin-HRP, were washed in Earle's BSS containing the metabolic inhibitor and incubated at 37°C for 3 h in full culture medium containing the inhibitor. The following metabolic inhibitors or other agents were used: Na azide (1-10 mM), oligomycin (50-100 µg/ml), Na cyanide (1-2 mM), antimycin A (70-140 µg/ml), (carbonyl cyanide paratrifluoromethoxyphenylhydrazone (FCCP, 50 µg/ml), colchicine ( $1 \times 10^5$  M), cytochalasin B (2 mM) in dimethylsulfoxide (DMSO).

### *Cytochemistry*

The cytochemical reaction for HRP was done according to Graham and Karnovsky with DAB as substrate (16); the acid phosphatase reaction was performed according to Novikoff (22, 23), with disodium salt of CMP (cytidylic acid) as substrate. Incubations for acid phosphatase were carried out for 2-3 h at room temperature. Elmiskop 1 and 1A electron microscopes were used. Thin sections (silver to gold interference colors) were examined at 80 kV, and thick (0.5-1 µm) sections were examined at 100 kV. Thin sections were counterstained with uranyl salts or with lead and uranyl salts. Thick sections of cells already stained with DAB were not counterstained with lead or uranyl salts.

## RESULTS

Under these cell culture conditions, isolated large neurons grew over a substrate of fibroblasts and Schwann cells. Under phase optics, these neurons appeared as spherical or oval cells with pale nuclei, well identified nucleoli, and granular cytoplasm (Fig. 1). One or two long and tortuous processes often originated from these neuronal perikarya; these processes could be traced over many micrometers and were free of lateral short

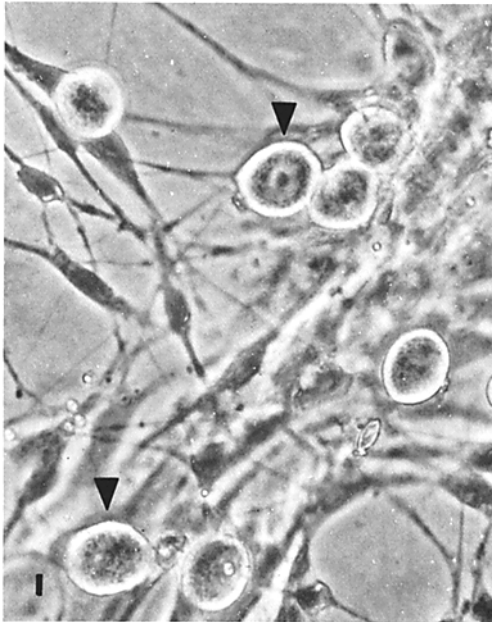


FIGURE 1 15-day-old embryo mouse DRG's, 1 wk after explantation. Neurons appear as isolated spherical cells with birefringent edges growing on top of collagen substrate, fibroblasts, and Schwann cells (arrowheads).  $\times 400$ .

(spines) or long branches. In the electron microscope, these neurons were characterized by large nuclei with dispersed chromatin and prominent nucleoli (Fig. 2). The perikaryon had numerous groups of flattened smooth cisterns usually occupying a juxtannuclear segment of the cytoplasm (Golgi zone) (Fig. 3). Numerous microtubules and intermediate filaments traversed the Golgi zone (Fig. 3). Dense bodies, mitochondria, ribosomal rosettes, and rough endoplasmic reticulum (ER) filled the cytoplasm. A few multivesicular bodies were observed. Most ribosomes were in polysomal groupings, but well organized rough ER appeared especially in the periphery of the neurons. A narrow rim of cytoplasm (about  $500 \text{ \AA}$ ) immediately underneath the plasma membrane of these neurons was composed of amorphous, vaguely fibrillar material; microtubules were not observed in this narrow ectoplasmic region. Frequently, neurons were surrounded by a thin rim of satellite cell cytoplasmic processes. Smooth vesicles isolated or in clusters, with or without dense cores, resembling synaptic vesicles were not seen in the perikarya. In these preparations, growth cones were identified, but synapses were not found. Coated

vesicles were seen, especially in the Golgi zone. These neurons did not contain cytochemically demonstrable endogenous peroxidase.

#### Incubation with Lectins

Both Ric-HRP and PHA-HRP gave similar results. When cells were fixed immediately after 1-h incubation at  $4^{\circ}\text{C}$  in Earle's BSS containing 10 or  $100 \mu\text{g}$  of lectin-HRP and stained for 10 min for HRP with DAB, a continuous, peripheral, plasma membrane layer of oxidized DAB was seen (Fig. 4). Dense bodies and other cytoplasmic sites did not stain with DAB. Cells incubated with  $1 \mu\text{g}/\text{ml}$  of Ric-HRP or PHA-HRP did not acquire positive membrane staining.

#### Internalization

After the original incubation at  $4^{\circ}\text{C}$  with Ric-HRP or PHA-HRP, cells were washed in  $4^{\circ}\text{C}$  Earle's BSS and transferred into full culture medium *without* lectin-HRP, and incubated at  $37^{\circ}\text{C}$  for 5 min, 15 min, 30 min, 1, 2, 3, 6, 24, and 48 h. Cells were subsequently fixed and stained with DAB. Incubations for 5 min and 15 min did not result in easily detectable intracytoplasmic stain. After 30 min at  $37^{\circ}\text{C}$ , peripheral intracytoplasmic staining was seen while plasma membrane staining was diminished or absent (Fig. 5). Early sites of lectin-HRP internalization were vesicles or tubules,  $0.1\text{--}1 \mu\text{m}$  in largest dimension (Fig. 5); coated vesicles were not identified among these early sites of lectin-HRP internalization; the oxidized DAB-osmium black precipitates filled entirely the lumen of these vesicles or tubules, the limiting membrane of which was seen with difficulty. In the space between the DAB-positive tubules vesicles, ribosomes were not present.  $0.5\text{--}1 \text{ h}$  after incubation at  $37^{\circ}\text{C}$  of initially labeled neurons ( $4^{\circ}\text{C}$ ), vesicles or tubules near the innermost (concave or trans) area of the Golgi apparatus took up the peroxidase stain (Fig. 6). Maximum staining for lectin-HRP of the tubules or vesicles near the Golgi apparatus was achieved after incubations at  $37^{\circ}\text{C}$  for 2–3 h. The vesicular stain was seen after 24- and 48-h incubations, but the organization of the elements in the Golgi zone was disrupted and the neurons, especially 48 h later, had significant signs of "degeneration" characterized by dispersed ribosomes and ER, swollen mitochondria, and clumping of nuclear chromatin. At doses of 10 or  $100 \mu\text{g}/\text{ml}$  of Ric-HRP or PHA-HRP, these changes were seen invariably after 48

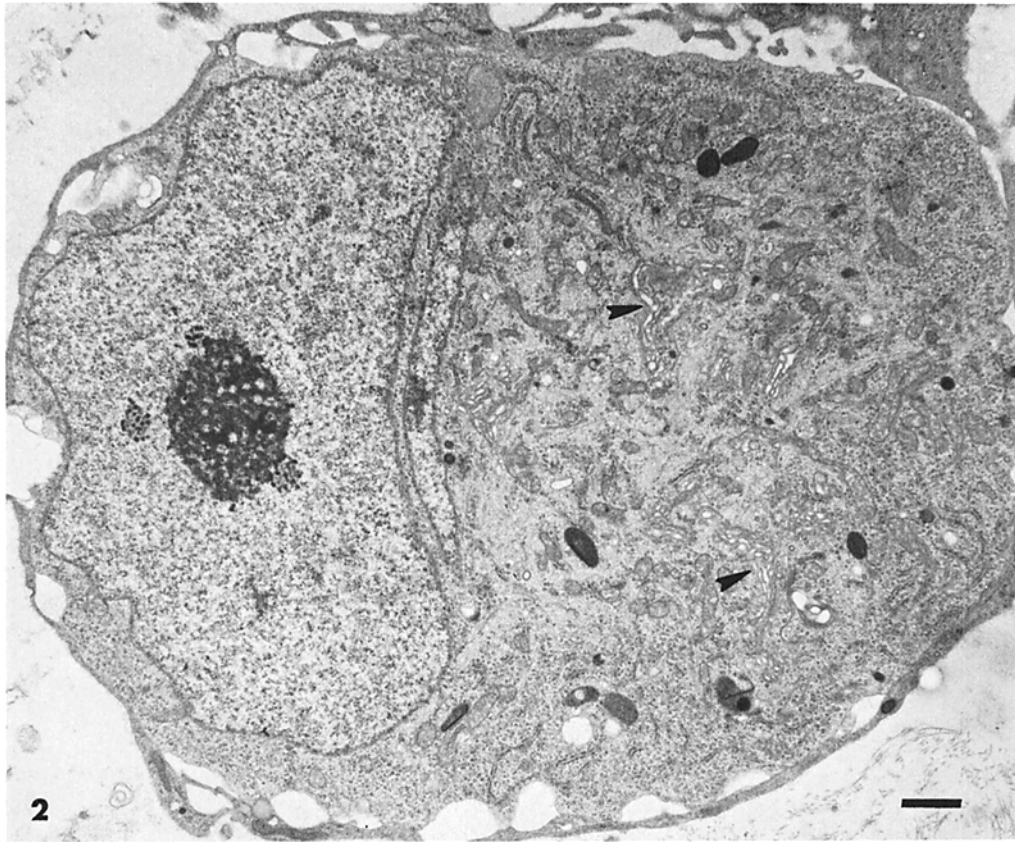


FIGURE 2 Electron micrograph of isolated neuron. Golgi elements (arrowheads). Bar, 1  $\mu\text{m}$ .  $\times 8,000$ .

h. 3–6 h after incubation at 37°C, a few coated vesicles and dense bodies became positive for HRP (Figs. 7 and 8).

#### Surface Stain

In these experiments, patching or capping of surface HRP-lectin stain was not observed; localized surface stain could be missed in thin-sectioned, voluminous cells. Studies of cell surfaces with the scanning electron microscope might be helpful in establishing or excluding the presence of patches or caps of lectin-receptor conjugates. Surface stain disappeared after 15–30 min incubations at 37°C.

In order to define more precisely the internalization site(s) of Ric-HRP complexes, we compared the peroxidase- and acid phosphatase-staining in thin and thick (1- $\mu\text{m}$ ) sections (Figs. 8–10) and compared the results with those of Novikoff et al. (23). In 1- $\mu\text{m}$  sections, internalized plasma membrane receptor-Ric-HRP complexes ap-

peared in the form of clusters of vesicles, or as elongated curving linear densities (Fig. 8). In many instances, the unstained cisterns of the Golgi apparatus were visible while the peroxidase staining at the inner or trans element of the Golgi apparatus was obvious (Fig. 8). Occasionally, irregular polyhedral patterns of peroxidase stain were seen which resembled similar profiles seen by Novikoff et al. in acid phosphatase-stained sections of mouse dorsal root ganglia, and in our acid phosphatase-stained cultured dorsal root ganglion neurons (DRG) (Figs. 8–10). In our preparations, the acid phosphatase stain was in the form of elongated tubular profiles, interrupted by polygonal stained areas (Fig. 9). Similar staining patterns were observed with internalized Ric-HRP (Fig. 8).

Inhibition studies are summarized in Table I. Although precise measurement of the degree of inhibition of the internalization is not possible, the qualitative studies with various metabolic inhibi-

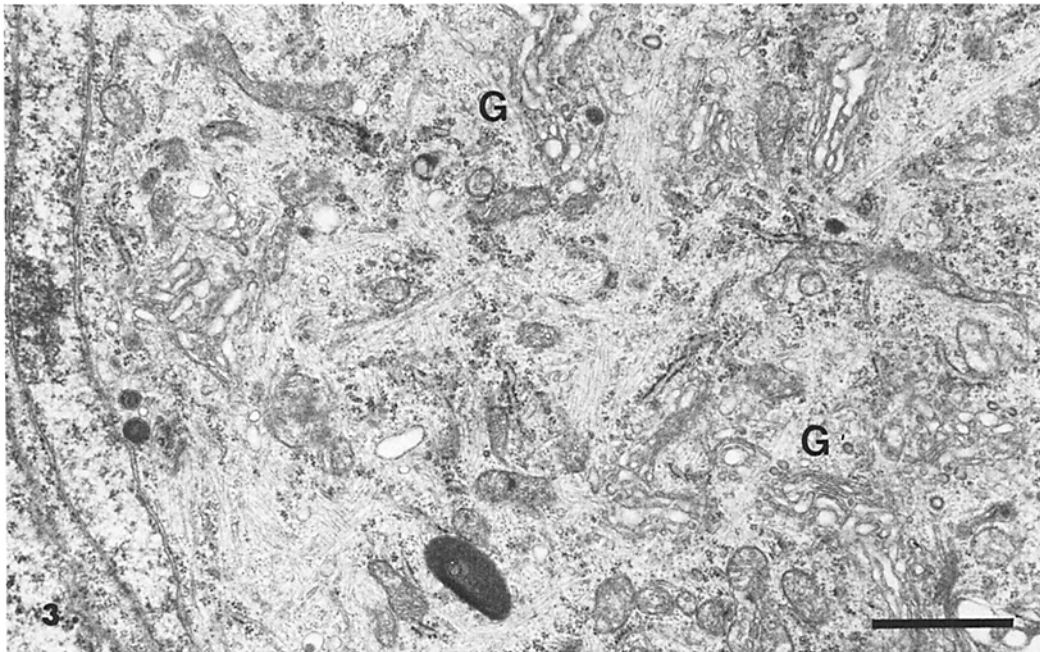


FIGURE 3 Detail from Fig. 2. Golgi apparatus (G). Bar, 1  $\mu\text{m}$ .  $\times 19,000$ .

tors suggest that only FCCP, a potent uncoupler of oxidative phosphorylation, produced an unequivocal inhibition of Ric-HRP or PHA-HRP internalization. Oligomycin (blocks mitochondrial ATPase), Na azide, Na cyanide, and antimycin A (inhibitors of mitochondrial respiration) did not produce a severe inhibition of lectin-HRP internalization. Perhaps sufficient reserves of endogenous ATP account for the failure of some of these agents to inhibit the internalization of lectin-HRP. The studies with the metabolic inhibitors were performed in consultation with Dr. Anthony Scarpa of the Department of Biochemistry and Biophysics, University of Pennsylvania.

#### Controls

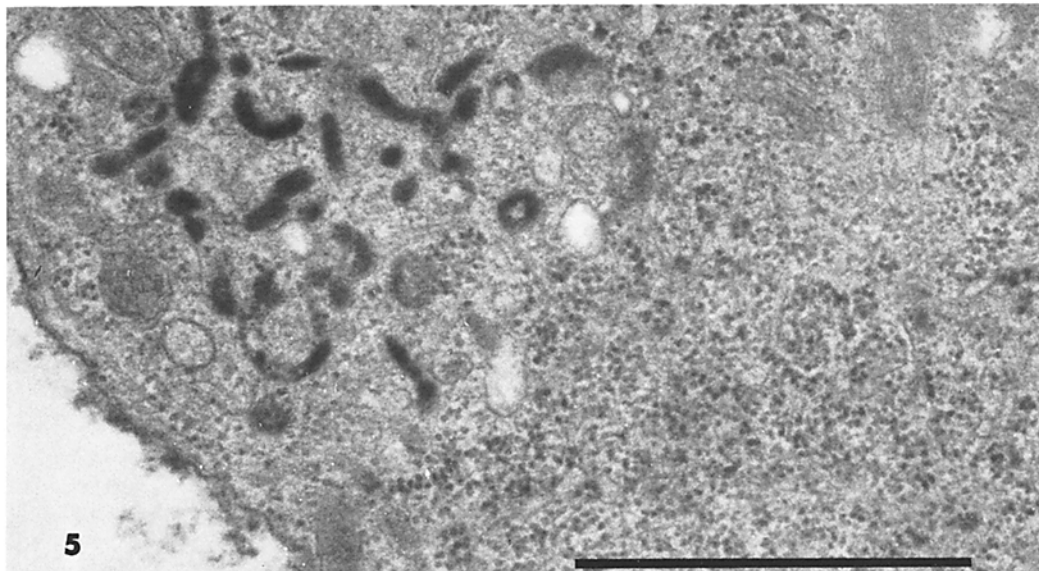
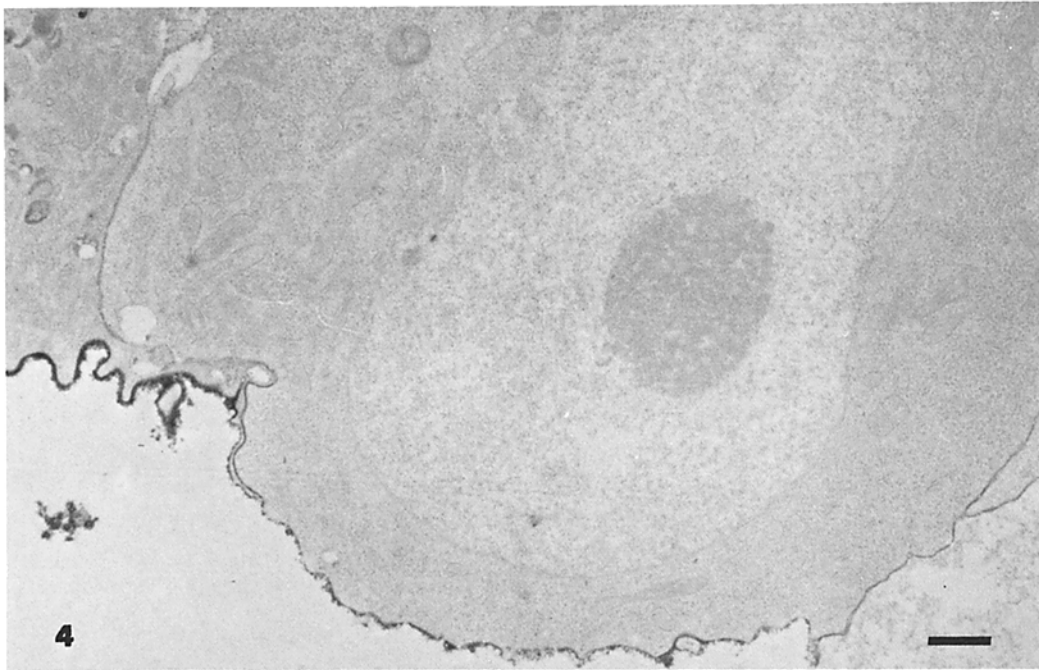
The following controls were used: soluble HRP (Sigma Grade VI, Sigma Chemical Co., St. Louis, Mo.) and "activated" HRP, i.e., HRP after exposure to glutaraldehyde and filtration in a G25 Sephadex column (3). When soluble HRP was incubated at 4°C with unfixed cells and the preparation then was washed and incubated at 37°C for 1–3 h, surface or internal stain for HRP was not seen. When "activated" HRP was incubated with neurons at 4°C and the cells then were incubated at 36°C for 3 h, only surface stain of oxidized DAB was seen.

DRG's incubated for 3 h in full culture medium containing HRP at concentrations of 1 or 10 mg/ml showed surface stain only occasionally (adsorption?). Internal stain of HRP was not observed in GERL, coated vesicles, or dense bodies.

*Inhibition with sugars:* In a previous study with lymphocytes, we noted that the surface binding of PHA-HRP was not inhibited by the sugar *n*-acetyl-*d*-galactosamine while binding of Ric-HRP was inhibited by *d*-galactose- or *d*-galactose-containing saccharides (12). In this study, we examined the inhibitory effect of *d*-galactose,  $\alpha$ -lactose, *N*-acetyl-*d*-galactosamine, and  $\alpha$ -methyl-*d*-mannoside on the binding of Ric-HRP on the plasma membrane of cultured DRG's. After incubation with  $\alpha$ -lactose, there was decreased binding, as judged by fainter DAB precipitates, but no complete inhibition of the stain. Incubations in the presence of the other sugars did not significantly alter the surface stain of lectin-HRP. These results suggest that Ric-HRP, in addition to showing affinities with *d*-galactose-containing molecules, binds to other moieties of the plasma membrane, such as polyelectrolytes (6).

#### DISCUSSION

The concept that cisternal and tubular elements near the inner (concave, trans) aspect of the Golgi

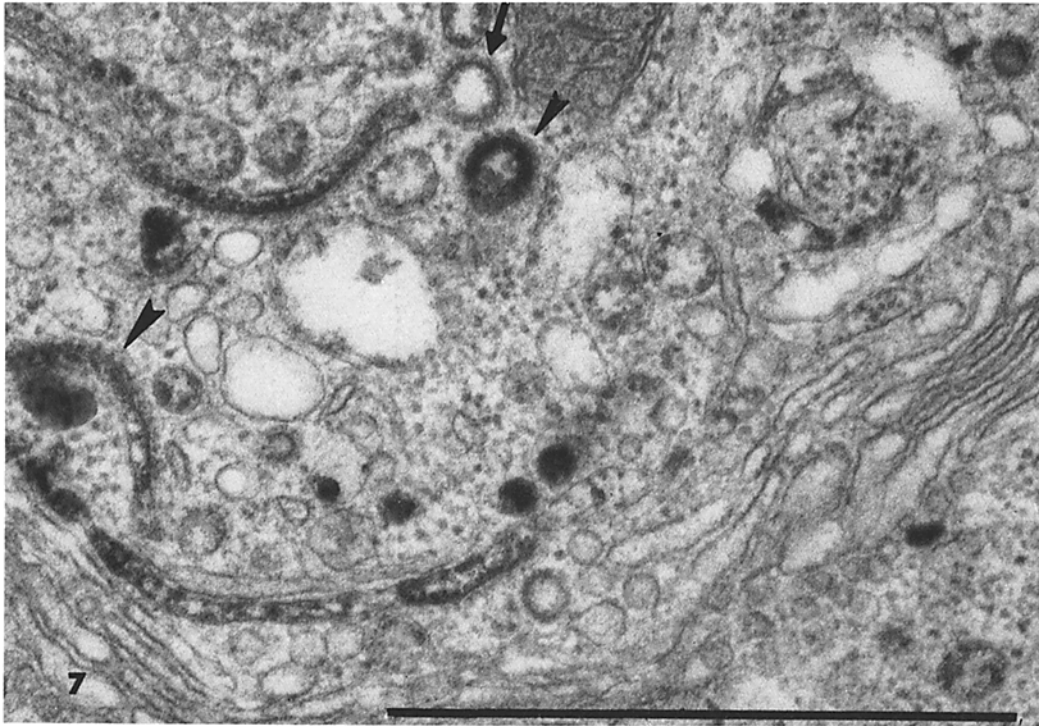
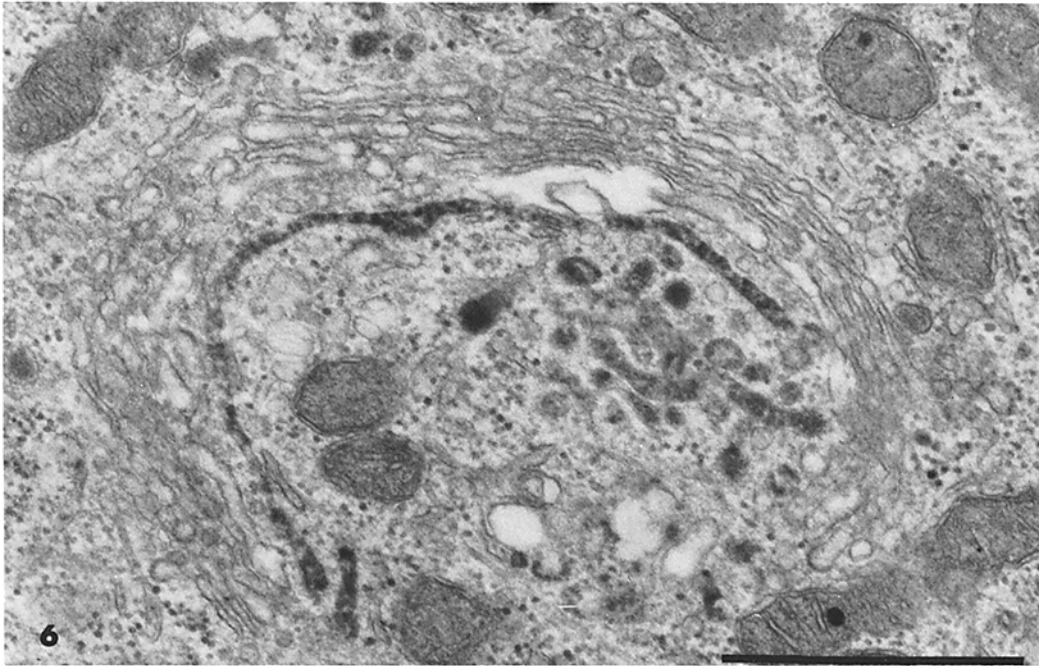


**FIGURE 4** Cultured mouse DRG neuron, incubated with 100  $\mu\text{g/ml}$  Ric-HRP for 1 h at 4°C, washed, fixed, and stained only with DAB. Bar, 1  $\mu\text{m}$ .  $\times 8,500$ .

**FIGURE 5** DRG neuron labeled with Ric-HRP at 4°C, washed and incubated in full medium at 37°C for 30 min. Note DAB-positive vesicles or tubules. Bar, 1  $\mu\text{m}$ .  $\times 53,000$ .

apparatus form a separate unit (GERL) originated from the cytochemical studies of Novikoff and collaborators (4, 22-24). Neuronal GERL stains with acid phosphatase while the cis- and trans-elements of the Golgi zone (8) are negative for

acid phosphatase; certain elements of the Golgi apparatus are positive for a number of diphosphatases (4, 22-24). In our material, a comparison between the peroxidase stain of internalized lectins and the acid phosphatase stain performed



**FIGURE 6** DRG internalization of Ric-HRP (2 h); DAB stain is present in vesicles and one elongated tubule (cistern?) at inner (concave) aspect of Golgi zone. Bar, 1  $\mu\text{m}$ .  $\times$  41,000.

**FIGURE 7** Internalization of Ric-HRP in DRG (3 h); coated vesicle, about 1,000  $\text{\AA}$  in diam., without internal oxidized DAB (arrow). Somewhat larger coated vesicle or cistern with internal DAB precipitates (arrowheads). Bar, 1  $\mu\text{m}$ .  $\times$  85,000.

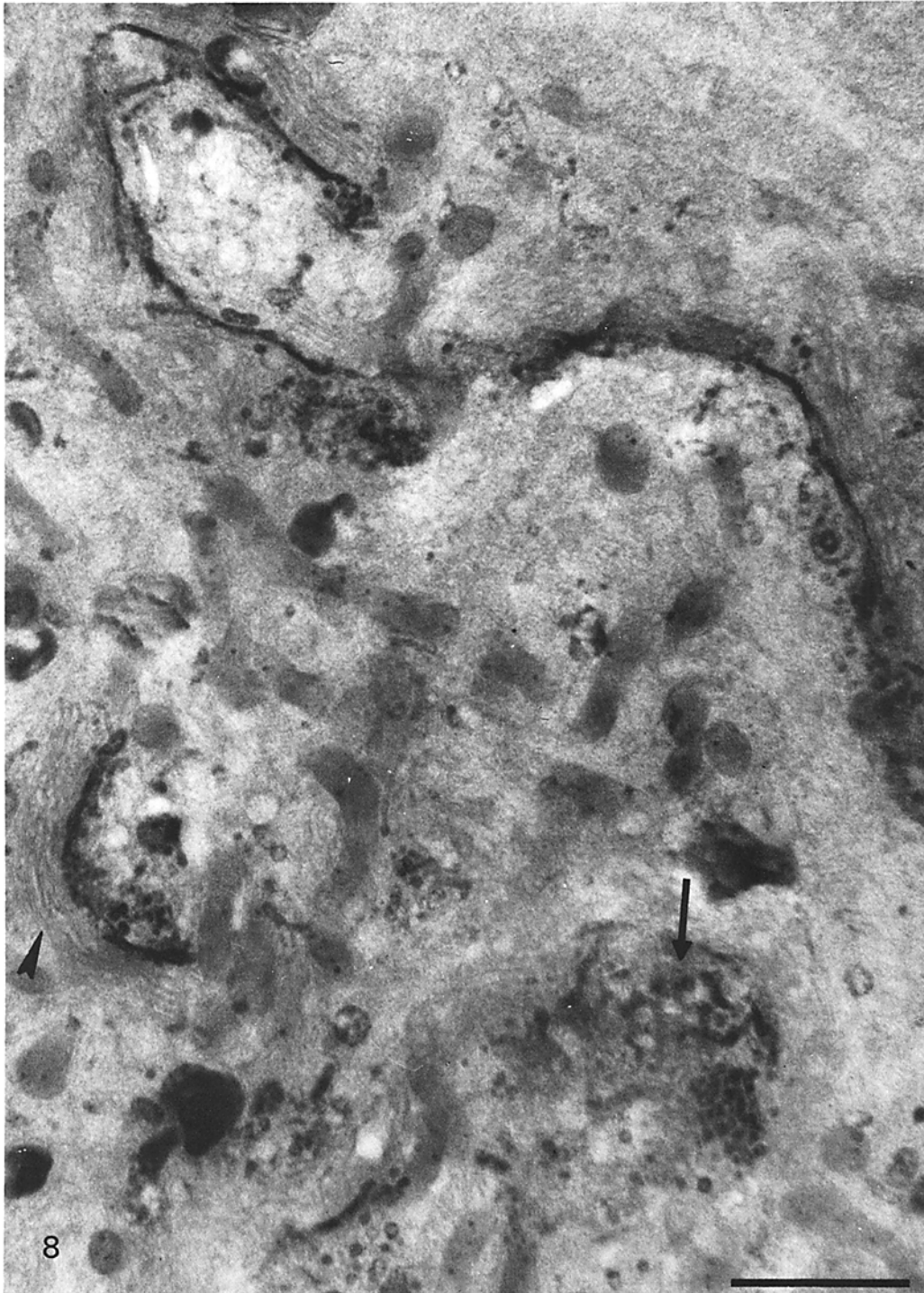


FIGURE 8 DRG internalization of Ric-HRP (3 h). 1- $\mu$ m thick section examined at 100 kV in Elmiskop 1A. Stained only with DAB. Unstained Golgi apparatus (arrowhead). Note adjacent stained vesicles and elongated profiles of GERL. Pentagonal or hexagonal profiles (arrow). Bar, 1  $\mu$ m.  $\times$  26,000.





FIGURE 9 DRG. Acid phosphatase. Thick (1- $\mu\text{m}$ ) section. GERL. Cf. Fig. 8, especially zones of polygonal profiles (arrow). Bar, 1  $\mu\text{m}$ .  $\times 45,000$ .

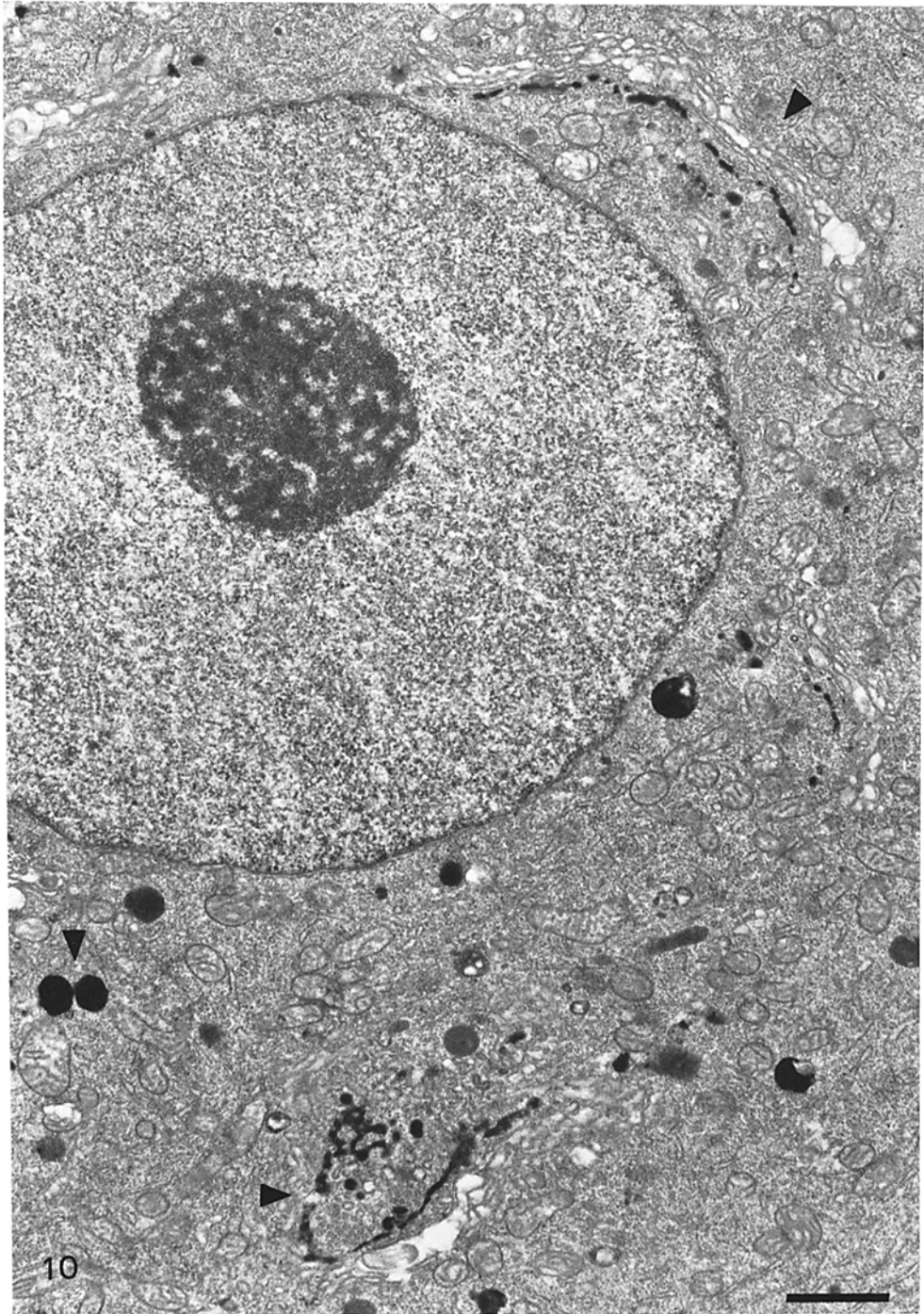


FIGURE 10 DRG. Acid phosphatase. Thin section. GERL and dense bodies show reaction product (arrowheads). Cf. Figs. 6 and 7. Bar, 1  $\mu$ m.  $\times$  14,000.

TABLE I  
Inhibition Experiments on PHA-HRP, Ric-HRP Internalization (1 h at 36°C)

	Internalization	Concn
Control	+++	—
Na Azide	+ to +++	1 or 10 mM
Oligomycin	± to ++	50 or 100 µg/ml
NaCN	+ to ++	1 or 2 mM
Antimycin A	+	70 or 140 µg/ml
FCCP	0	50 µg/ml
Colchicine	++	1 × 10 <sup>-4</sup>
Cytochalasin B	++	2 mM

Na Azide, Oligomycin, NaCN, antimycin A, and FCCP: two exp. Colchicine and cytochalasin B: one exp. 100 µg/ml of lectin used.

on thick and thin sections shows that these stains are remarkably similar (cf. Figs. 6–10). The present experiments are supportive of the concept of GERL and suggest that plasma membrane-bound agents may be processed in a different fashion than soluble substances, such as free HRP, which, in neurons and in other cells, is endocytosed in coated vesicles, dense bodies, and multivesicular bodies (1, 9, 10, 17, 18, 27, 31). The reasons for the differences in the uptake and intracellular distribution of soluble HRP, vis-a-vis lectin-bound HRP, are not clear, and several possibilities have been suggested, such as failure of fusion of lectin-containing phagocytic vacuoles with lysosomes, or inactivation of lysosomal enzymes by the lectin (7, 11). It was noticed by Edelson and Cohn that in peritoneal macrophages Con A was taken up by pinocytotic vesicles which failed to fuse with lysosomes (7); in that study, Golgi elements did not stain with Con A-HRP (7); therefore no general conclusions can be drawn from our experiments involving the uptake of Ric-HRP or PHA-HRP by neurons. Furthermore, Ric and PHA have toxic or other effects on plasma membranes and cells. Preparations of Ric contain unavoidable traces of ricin toxin which, in extremely low amounts, induces a profound inhibition of protein synthesis (25). PHA and other lectins are bivalent or multivalent agents which cross-link exposed plasma membrane “receptors” and induce alterations of proteins not only at the outer, but also at the inner surface of plasma membranes (30). Furthermore, the lack of complete inhibition of the stain produced by Ric-HRP or PHA-HRP by appropriate sugars strongly suggests that these compounds have affinities with several plasma membrane moieties, a notion already introduced by biochem-

ical studies (6). Ric or PHA receptors are certainly not analogous to hormone or drug receptors which are characterized by specificity of binding, finite number of binding sites, the triggering of a specific biologic response, and other criteria.

Lectin-HRP conjugates may accentuate endocytosis, and in that regard our findings may be analogous to the occasional endocytosis of HRP into tubules of the Golgi zone which has been reported in cultured neurons after strychnine stimulation (18), in absorptive intestinal cells (5), and in somatotrophs and mammatrophs of rat pituitary after stimulation with N<sup>6</sup>-monobutyryl cyclic AMP (9, 27); thus, the differences in the uptake of soluble HRP and lectin-HRP conjugates between neurons and other cells may be only quantitative. On the other hand, lectin-HRP-plasma membrane complexes may be endocytosed by a mechanism and route which are different from those operating in the uptake of soluble HRP. Lectin-HRP conjugates bind on plasma membranes, while soluble HRP does not. In the case of soluble HRP, we may be observing the vesicular (luminal) transport of a soluble protein; with lectin-HRP, we may be studying plasma membrane endocytosis or “flow.”

The lectins used in these experiments have significant and lethal (ricin) biologic effects, and the evoked cellular response may be of pathologic significance.

Finally in our culture system, neurons are isolated, whereas in organotypic cultures or in tissue, neurons have established specific contacts with other neurons and possibly with glial cells. Increased mobility of cell surface receptors has been demonstrated in dissociated chick embryo retinal neurons (20, 21). Thus, the phenomena we have studied may be relevant to, and may occur more often during development and organogenesis.

Lectin-HRP was noted in a few coated vesicles and dense bodies 3 h after internalization (37°C). It seems that lectin-HRP appeared in coated vesicles and dense bodies after the peripheral vesicles tubules and GERL became positive. We feel that the transport of lectin-HRP from GERL into dense bodies (lysosomes) is analogous to the proposed origin of lysosome, including dense bodies, from GERL in neurons (23). In the present experiments, GERL appears to be a direct recipient of internalized plasma membrane lectin-receptor complexes.

The implications of the unusual pattern of endocytosis of neuronal plasma membrane-bound substances are unknown at present. Future experi-

ments with lectins and with other compounds with affinities for neuronal plasma membranes will clarify the significance of the endocytosis of plasma membrane into GERL.

The authors wish to thank Drs. Alex and Phyllis Novikoff for useful discussions of this work.

This work was supported by U. S. Public Health Service grants, NINCDS 05572-12 and 10648-04; by grant 889-B2 from the National Multiple Sclerosis Society, and by Delegation Generale a la Recherche Scientifique et Technique research grant DGRST 72-70298 (France).

Dr. Nicholas K. Gonatas is a Josiah Macy, Jr. Fellow.

Received for publication 4 August 1976, and in revised form 19 November 1976.

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