VISUALIZATION OF CONCANAVALIN A-BINDING SITES
WITH SCANNING ELECTRON MICROSCOPY

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Plant lectins, because they bind to specific carbohydrate moieties, can be used to investigate the glyco-components of the cell surface. When considering the distribution, number, or mobility of lectin-binding sites, a distinction must be made between a natural distribution and a redistribution induced by binding with multivalent ligands (2, 4, 5, 8, 14, 15). In some cell types the induced redistribution results in the phenomenon of "capping." Taylor et al. (14), on the basis of immunofluorescent studies, introduced the term capping to refer to the progressive movement and clumping of receptor-ligand complexes (e.g. binding site-lectin complexes) to form an aggregate of these com-

plexes, which is referred to as a "cap." The cap may subsequently be endocytosed or shed, leaving the cell surface denuded of its receptor molecules (11, 16). Concanavalin A, a tetravalent lectin from jackbean (*Canavalis ensiformis*), which binds specifically to alpha-methyl-D-glucopyranoside, alpha-methyl-D-mannopyranoside, and beta-methyl-D-fructofuranoside (3), will induce capping in several cell types [e.g. lymphocytes (6, 7, 12, 15, 17) and L-cell]. In localizing concanavalin A-binding sites as an indication of the amount and distribution of alpha-methyl-D-glucopyranoside, alpha-methyl-D-mannopyranoside, and beta-methyl-D-fructofuranoside (or similar) groups on the cell surface, the total binding site population should be observed, and conditions defined to distinguish between native and induced distribution.

Previous studies have, for technical reasons, been limited in their description of the topographical distribution of binding sites. Localization of binding sites on the total exposed surface has not been well defined, particularly with respect to regional specializations such as microvilli and ruffles. In general, only fragments of a surface, or surfaces distorted by drying have been observed. Scanning electron microscopy (SEM), however, provides an effective means for observing overall distribution of lectin-binding sites, including their location with respect to surface specializations. A method for visualizing concanavalin A (Con A)-

binding sites with SEM is presented here, along with observations on their behavior.

MATERIALS AND METHODS

Cells

ERYTHROCYTES AND LYMPHOCYTES: Whole peripheral rat blood was obtained from etherized rats by cardiac puncture using a heparinized syringe. The blood was diluted 12 times in 0.9% NaCl. 10-ml aliquots were pelleted by centrifugation in a clinical centrifuge (International, CI, International Equipment Co., Needham, Mass.) and pelleted cells were resuspended in 10 ml 0.9% NaCl. This procedure was repeated three times. Blood cells were then mounted on an 11 × 22-mm piece of cover glass which had been heavily coated with carbon in a vacuum evaporator: the cover glass was placed at an angle in a conical centrifuge tube, carbon side up, and the blood suspension, diluted 3 times in 0.9% NaCl, was centrifuged for 10 min at 120 g. The blood cells adhering to the cover glass were then used for the demonstration of Con A-binding sites. Both erythrocytes and lymphocytes were observed in these preparations. Lymphocytes were also separated from buffy coat preparations, identified by light microscopy, and mounted as above.

L-929 CELLS: L-929 cells were plated onto 22 × 22-mm cover glasses in 30-mm plastic petri dishes (Falcon Plastics, Division of BioQuest, Oxnard, Calif.). Cover glasses had been washed in ethanol and/or nitric acid before use. Cells were grown at 37°C in Nutrient Mixture F-12 (Grand Island Biological Co., Grand Island, N. Y.), pH 7.2, supplemented with 10% fetal calf serum and antibiotics (50 U/ml penicillin, 50 μg/

FIGURE 1   Rat erythrocyte incubated in solutions of Con A and hemocyanin (from *Busycon canaliculatum*) as described in the text. The cylindrical profiles (*h*) on the cell surface are the hemocyanin molecules within the Con A-hemocyanin complexes which label Con A-binding sites. The distribution of label on erythrocytes is apparently two-dimensionally random and remains dispersed regardless of the temperature or duration of incubation. Short sequences of aligned hemocyanin molecules are observed, but do not predominate [e.g. two sequences including the labeled molucle (*h*)]. × 25,000. *Insert:* hemocyanin molecules. Both rectangular and circular profiles are seen. × 95,000.

FIGURE 2   Rat erythrocyte incubated in Con A in the presence of alpha-methyl-D-glucoside, a competitive inhibitor of Con A binding, which precludes binding of Con A-hemocyanin complexes. Note that the membrane is smooth and devoid of bound molecules. × 25,000.

FIGURE 3   Normal rat lymphocyte. Though the surface is covered with irregular protrusions (microvilli, *mv*), note that the membrane itself is smooth (arrows). The uropod (*u*) is a characteristic feature of lymphocytes. × 12,500.

FIGURE 4   Rat lymphocyte exposed to Con A and hemocyanin at 20°C for 30 min as described in the text. This lymphocyte has the same morphological features as the control preparation (Fig. 3). However, note the bound hemocyanin molecules (*h*) within Con A-hemocyanin complexes, which identify Con A-binding sites. The complexes are distributed over the entire surface of the cell except for the periphery near the substrate. *u*, uropod; *mv*, microvilli. × 14,300.
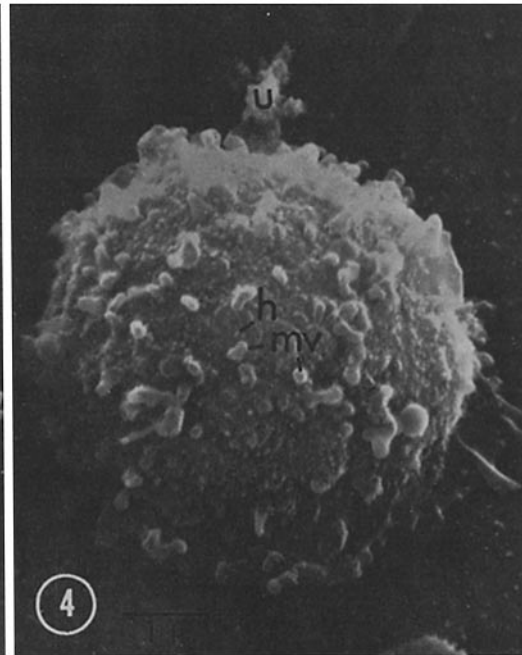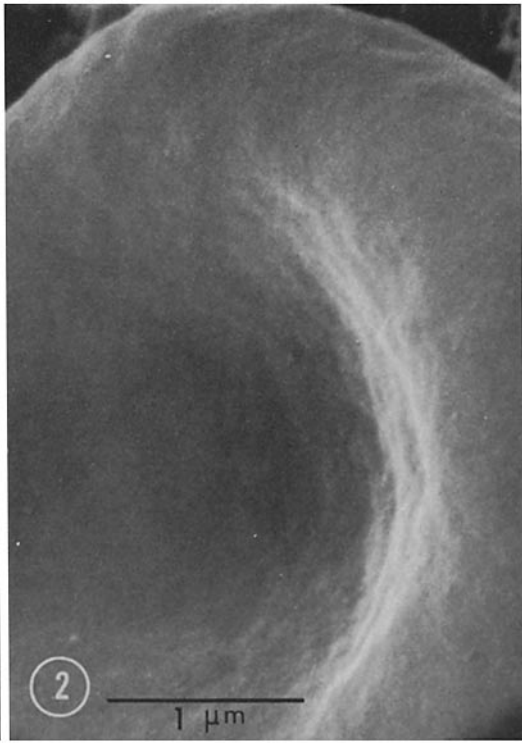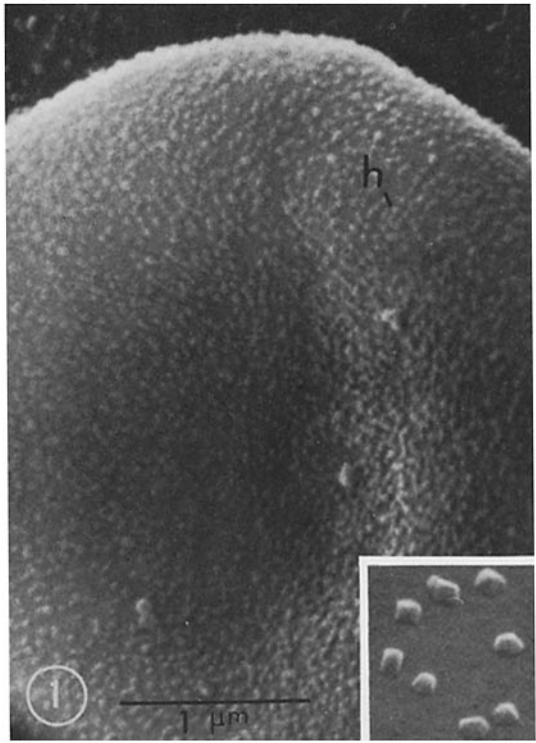
ml streptomycin, and 2.4 µg/ml amphotericin B) (Grand Island Biological Co.), in a high humidity incubator under an atmosphere of 5% CO_2-95% air. Cells were used for experimentation when they were still sparse (9.68 × 10³ cells), but not earlier than 36 h after plating.

Demonstration of Concanavalin A-Binding Sites

As shown by Smith and Revel (13), hemocyanin from *Busycon canaliculatum* binds directly to Con A, thus labeling Con A-binding sites. Therefore, erythrocytes, lymphocytes, and L-cells were incubated in Con A solution, washed, incubated in hemocyanin solution, washed, and then prepared for SEM. Lyophilized Con A was obtained from Calbiochem, San Diego, Calif. An initial sample of *Busycon canaliculatum* hemocyanin was obtained from Dr. Richard Rodewald of Dr. Morris Karnovsky's laboratory; hemocyanin was subsequently prepared according to the method of Karnovsky (6), and further purified with a Sephadex G-500 column.

EXPERIMENTAL CELL INCUBATIONS

ERYTHROCYTES AND LYMPHOCYTES: Erythrocytes and lymphocytes (mounted on carbon-coated cover glasses) were treated in parallel experiments at 4°C, 20°C, and 37°C according to the following steps: (a) incubation for 10 min in 2 ml of Con A solution (100 or 200 µg Con A/ml of 0.9% NaCl); (b) wash with 0.9% NaCl; (c) incubation for 10 min in 2 ml of hemocyanin solution (190 µg hemocyanin/ml 0.9% NaCl); (d) wash with 0.9% NaCl. Cells were then immediately prepared for SEM or allowed to incubate at the appropriate temperature for various time intervals up to 2 h after initial exposure to Con A solution.

L-929 CELLS: L-929 cells were treated with Con A and hemocyanin in a manner identical to the above procedure except that Con A (100 µg/ml) and hemocyanin (190 µg/ml) solutions were made up in nutrient mixture F-12 (without serum), pH 7.2, and cells were washed and incubated in nturient mixture F-12 (without serum), pH 7.2

CONTROL CELL INCUBATIONS

Erythrocytes and L-929 cells were exposed to Con A alone or to hemocyanin alone by substituting an incubation in saline or media for one in hemocyanin solution or Con A solution, respectively. Cells were also incubated in Con A in the presence of alpha-methyl-D-glucoside (75 mM), a competitive inhibitor of Con A binding (3).

Preparation for Scanning Electron Microscopy

In preparation for SEM, cells were fixed at 37°C for 15 min with 3% glutaraldehyde in 50% Puck's saline G (Grand Island Biological Co.), buffered with 0.05 M cacodylate, pH 7.4. After a brief but thorough wash with Puck's saline G, cells were postfixed for 15 min with 1% osmium tetroxide buffered with 0.2 M cacodylate, pH 7.4, and rapidly dehydrated through a series of graded concentrations of acetone. Preparations were then dried by the critical point method using liquid CO_2, as described by Porter et al. (9) (Sorvall apparatus, Ivan Sorvall, Inc., Newtown, Conn.), and coated in a vacuum evaporator with carbon (100 Å) and then with gold (100 Å). Cells were then examined and photographed at 20kV or 30kV with a Cambridge S4 stereoscan electron microscope.

OBSERVATIONS

Individual Con A molecules cannot be discerned with SEM because their widest dimension is 90 Å (10), and the best resolution with the Cambridge S4 SEM is 150 Å. However, hemocyanin from *Busycon canaliculatum*, which binds directly to Con A (13), can be identified with SEM because it is a cylindrical molecule, 350 Å in diameter. Hemocyanin, therefore, can be used to label Con A-binding sites.

Erythrocytes

Con A-binding sites can be clearly demonstrated on rat erythrocytes. The normal erythrocyte membrane is smooth and the cell surface lacks protrusions. After exposure to Con A and hemocyanin the erythrocyte surface is covered with the cylindrical profiles of hemocyanin molecules within the Con A-hemocyanin complexes (see Fig. 1). Hemocyanin molecules can be recognized by their characteristic size and shape: cylinders, 350 Å in diameter. The complexes are bound to the cell surface in a disperse, two-dimensionally random distribution, independent of the temperature or duration of incubation. It should be noted, however, that short sequences of aligned hemocyanin molecules are observed in sufficiently densely labeled preparations (Fig. 1). It is not known whether this arrangement of marker molecules is a function of the Con A-binding sites or of the hemocyanin molecules. The specificity of the Con A-hemocyanin labeling is demonstrated by the three control incubations, which, in each case, show erythrocyte membranes smooth and devoid of bound molecules (Fig. 2).

Lymphocytes

Con A-binding sites can also be identified on lymphocytes. Fig. 3 shows a normal lymphocyte

from whole peripheral rat blood. Though the normal cell surface is covered with irregular protrusions (microvilli), it can be seen that the membrane surface itself is smooth. A trailing uropod, a characteristic feature of lymphocytes, can also be identified. A lymphocyte with bound Con A-hemocyanin complexes is shown in Fig. 4. The label is distributed over the entire exposed membrane except for the periphery of the exposed surface adjacent to the substrate.

L-929 Cells

L-929 cells cap when they are allowed to incubate at 37°C for 2 h after initial exposure to Con A. By observing the distribution of Con A-hemocyanin complexes after incubations of less than 2 h, a sequence of redistribution suggestive of movement can be described (see diagram, Fig. 5).

A normal L-929 cell (Fig. 6) is flat with centrally located microvilli. The membrane is smooth and devoid of particulate material; the surface lacks small irregularities. The cell periphery, in part or in total, extends into pseudopodia, the edges of which ruffle.

The sequence of redistribution of Con A-hemocyanin complexes, as depicted in Fig. 5 and Figs. 6–11, is based on L-929 cells whose total peripheries are extended into pseudopodia. Varia-

tions with different cell morphologies will be discussed below. The native distribution of Con A-hemocyanin complexes, determined by incubating cells at 4°C (Fig. 7), was apparently random and remained dispersed over the total cell surface, including microvilli and ruffles, regardless of the duration of the incubation. When the temperature is raised to 37°C, or the incubation is done at 37°C, a progressive redistribution of Con A-hemocyanin complexes occurs as they clump and move to form a cap. Initially, label withdraws from the cell periphery as clusters of Con A-hemocyanin complexes form (Fig. 8); ruffles are denuded first. These clusters further aggregate to form a band of label at the periphery of the centrally located region of microvilli (Fig. 9). Microvilli usually lose their label before the loss of label from the surrounding surface area. Con A-hemocyanin complexes continue to clump and the distribution of label becomes more localized; eventually a cap is formed (Figs. 10 and 12). The process of capping denudes the membrane, at least temporarily, of Con A-binding sites (Fig. 11). Work is in progress to define the temporal and spatial aspects of the reappearance of Con A-binding sites.

A variation in this pattern is seen when only a portion of the cell periphery is extended into

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FIGURE 5 Distribution and induced redistribution of Con A-hemocyanin complexes (suggestive of movement) observed on L-929 cells. Refer to the text for incubation procedures.

FIGURE 6 Normal L-929 cells. The cell is flat with centrally located microvilli (mv). The whole periphery of the cell extends into pseudopodia, the edges of which ruffle (r). × 1,320.

FIGURE 7 Native distribution of Con A-binding sites on L-929 cells. L-929 cells were incubated at 4°C in Con A and hemocyanin solutions. Binding site distribution on both the unperturbed cell surface and on protrusions such as microvilli (mv) is apparently random and remains dispersed regardless of the duration of incubation. h, hemocyanin label. × 16,800.

FIGURE 8 L-929 cell incubated at 37°C for 30 min in Con A and hemocyanin solutions. Label "moves" away from the edge of the cell (direction indicated by the arrow) toward the periphery of the region containing microvilli. Distinct clumps of label have formed (c-h), though individual molecules are still apparent (i-h). Label is still apparent on microvilli. × 15,525.

FIGURE 9 L-929 cell incubated at 37°C in Con A and hemocyanin solutions for 1 h. Label has aggregated in a band (b) around the periphery of the centrally located region of microvilli (mv) and is less apparent toward the periphery of the cell (p). × 7,935.

FIGURE 10 L-929 cell incubated at 37°C in Con A and hemocyanin solutions for 2 h. Label has become localized in a cap (c) which appears to be partially extruded from the membrane. × 3,500.

FIGURE 11 L-929 cell incubated at 37°C in Con A and hemocyanin solutions for 2 h. The region of the membrane adjacent to the cap (see Fig. 10) is shown here; it is devoid of bound molecules as is the rest of the cell surface. × 15,525.
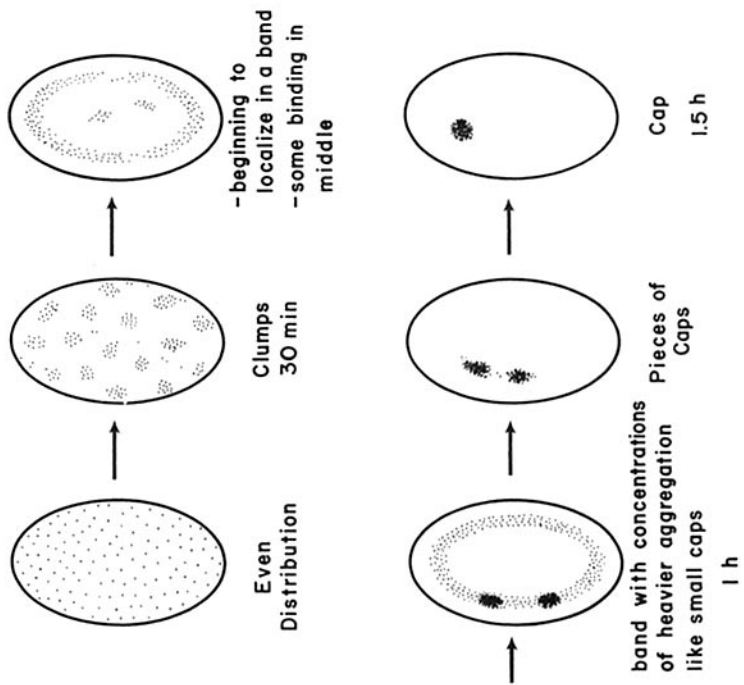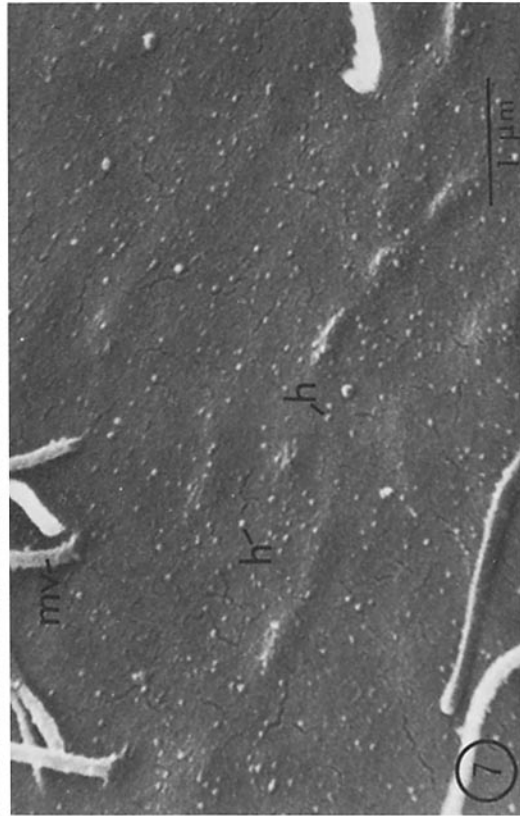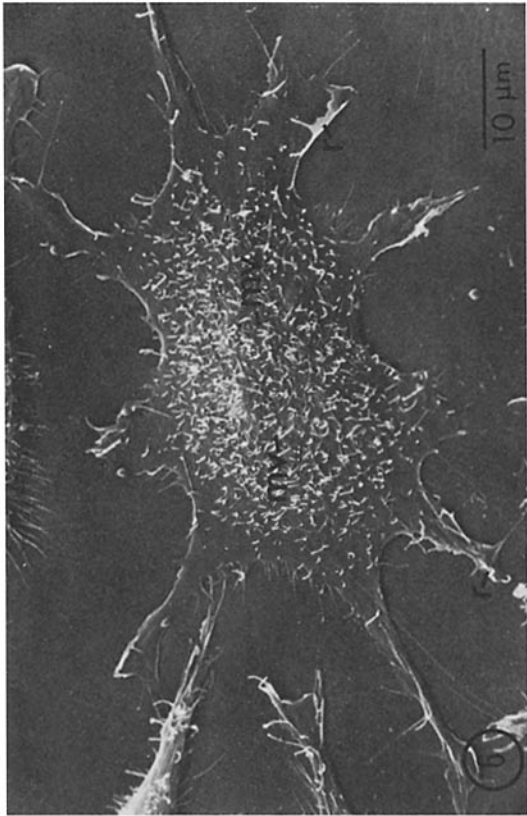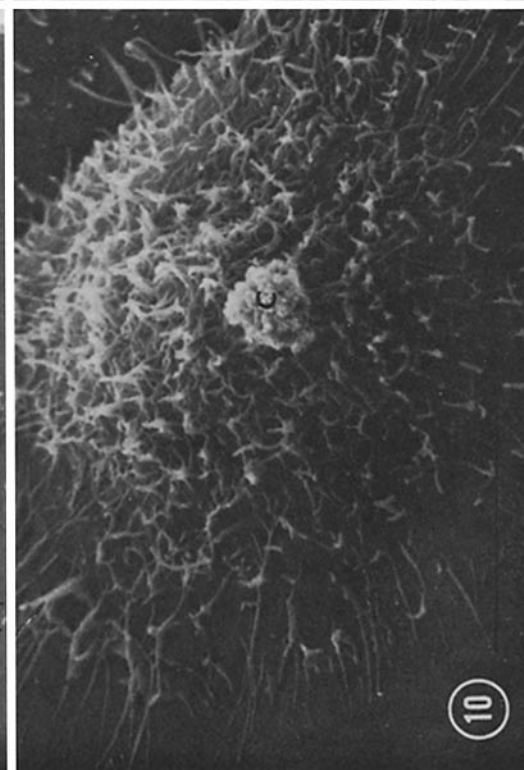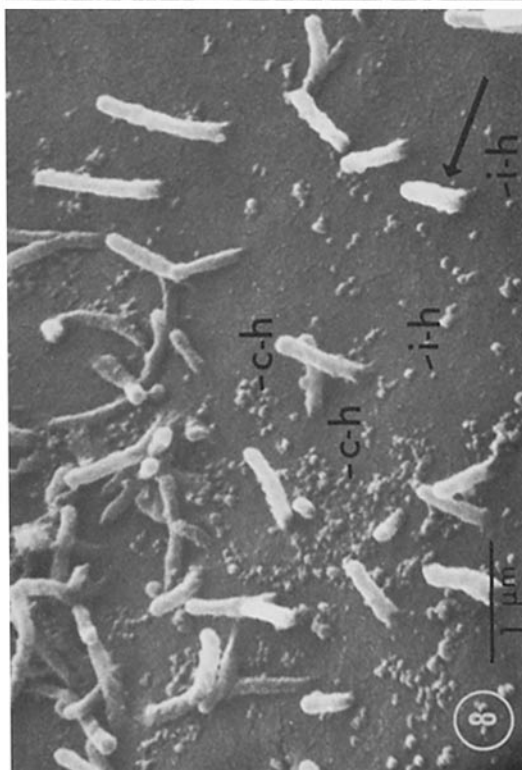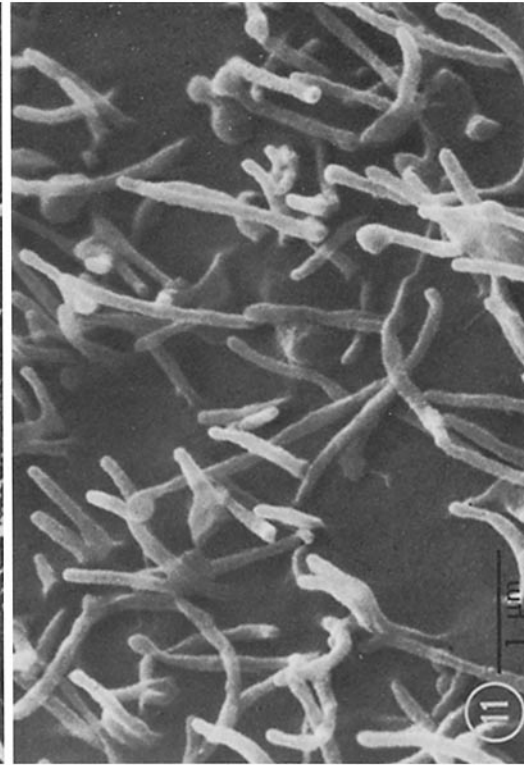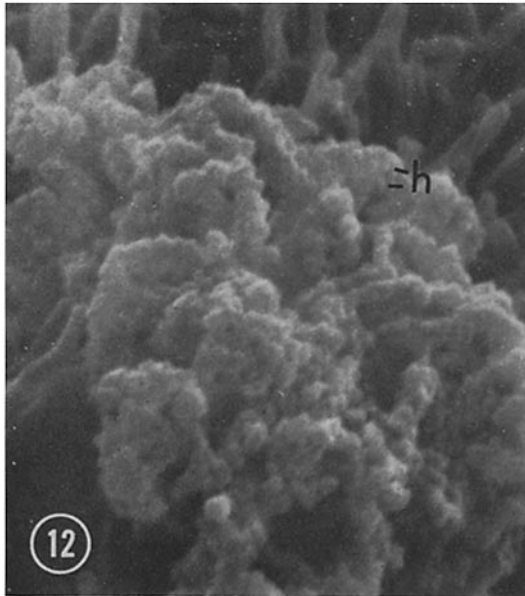
FIGURE 12 Cap on a L-929 cell incubated at 37°C in Con A and hemocyanin solutions for 2 h. Individual hemocyanin molecules (h) can be discerned within the aggregate. × 23,400.

pseudopodia. Then, the band is discontinuous, with segments localized only in regions of the cell periphery from which the pseudopodia extend. Such a variation also affects the location of the cap, which is usually formed away from the extended pseudopodia. If the cell is bipolar with opposite ends extended in pseudopodia, two caps may form.

DISCUSSION

Utilization of SEM to visualize Con A-hemocyanin complexes has several advantages over the methods which employ light microscopy (fluorescence studies) and transmission electron microscopy (TEM) (thin sections, replicas of freeze-etch preparations and other replica techniques). These advantages are important in considering the distribution of Con A-binding sites.

(a) SEM provides a more effective means of visualizing the whole surface of a cell than serial thin sectioning and subsequent reconstruction from TEM images. Ultrastructural reconstructions do not always provide the unambiguous surface contours revealed by SEM, and they require much more work.

(b) SEM provides the opportunity to examine any area of the exposed cell surface. This area can

be oriented with respect to the rest of the cell and, further, can be compared to similar areas on other cells (an advantage over freeze-etching and other replica techniques). In addition, most of the surface area can be viewed without visual distortion of area due to the curvature of the specimen (via rotation and tilting of the specimen).

(c) The use of the critical point drying technique in the preparation of the SEM specimens avoids the distortion of surface morphology which occurs when the cell preparations are air dried. The change in surface topography which results from the surface tension (2,000 lb/in²) (1) during air drying affects the observed distribution of binding sites.

SEM, therefore, provides an effective means to observe the distribution of bound hemocyanin molecules over the exposed surface area of the cell, and then to correlate this distribution with the surface topography and its functional implications. It can be readily discerned whether an area of membrane is representative of other cells in the field. This is necessary for identifying a localized or discrete binding pattern and, further, for observing a change in the distribution of sites with respect to time, as in the phenomenon of capping.

A general pattern of redistribution suggestive of movement (clumping, band formation, and capping) has been observed on L-929 cells. This pattern varied predictably depending on the extent to which the cell periphery extended into pseudopodia. To the extent that pseudopodia are a reflection of cell motility, these results suggest that directed cell movement influences the pattern of redistribution of Con A-hemocyanin complexes.

Hemocyanin labeling can be applied to the observation of other lectin-binding sites or antigenic sites. As suggested by the work of Karnovsky and co-workers, hemocyanin can be conjugated chemically to specific antibodies (4) or other lectins before incubation. Consequently, the distribution of these sites can be effectively visualized with SEM. It should be noted, however, that the resolution of closely packed binding sites is prevented by the size of the hemocyanin molecule. For example, the greatest dimension of Con A is 90 Å in diameter (10) and, therefore, one hemocyanin molecule (350 Å × 500 Å) could cover several closely juxtaposed Con A molecules and/or binding sites. A fine analysis of binding site juxtaposition would, therefore, require a TEM study using a ferritin-labeled ligand.

In conclusion, utilization of SEM with a hemo-cyanin-labeling technique allows effective observation of the distribution of membranous receptor molecules (e.g. Con A-binding sites) with respect to cell morphology. This technique, complemented with a study using ferritin labeling (which would provide greater resolution of binding site juxtaposition), would provide definitive data on binding site distribution.

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