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A SCANNING ELECTRON MICROSCOPE STUDY OF CONCANAVALIN A RECEPTORS ON RETINAL ROD CELLS LABELED WITH LATEX MICROSPHERES

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Con A-methacrylate microsphere conjugates prepared by a two-step glutaraldehyde reaction were used to label Con A-binding sites on bovine rod photoreceptor cells for visualization by scanning electron microscopy. A dense distribution of markers was observed on the surface of the rod outer segment, the inner segment, and the synaptic region. Disk membranes also appear to be heavily labeled with the Con A-microsphere conjugates. The Con A inhibitor, α-methyl mannoside, inhibited the binding of the conjugate to the surface of these visual cells.

INTRODUCTION

Vertebrate photoreceptors are highly differentiated cells with respect to both structure and function (1, 2). The rod outer segment (ROS)[1] located at one end of this elongated cell consists of an assembly of stacked membranous disks surrounded by a plasma membrane. This specialized organelle is the site for the detection of light and the resulting decrease in membrane permeability to sodium ions (3). A narrow cilium connects the outer segment to the inner segment and the adjoining nuclear region where the metabolic and biosynthetic machinery of the rod cell is located. At the opposite end of the cell is found the synaptic region which functions to relay electrical signals from the rod cell to other neurons of the retina.

Numerous studies on the composition and organization of ROS have been carried out in an effort to gain insight into the molecular mechanism of visual excitation (4, 5). The major protein component in ROS membranes is the visual pigment, rhodopsin (6–9). Using a variety of biophysical techniques, it has been shown that rhodopsin is densely packed in the ROS disk membranes (10), oriented with its chromophore in the plane of the membrane (11) and free to undergo translational diffusion (12). Energy transfer studies (13) suggest that rhodopsin is approximately 75 Å long and of sufficient length to traverse the lipid bilayer membrane. In support of this view, Jan and Revel (14), using peroxidase-labeled antibodies, have shown that antigenic sites on rhodopsin are exposed on both sides of the membrane. Immunochemical labeling experiments (14, 15) have also revealed the presence of rhodopsin on ROS plasma membranes as well as on the disk membranes. Hemocyanin-antibody complexes used as markers for rhodopsin (16) were seen by scanning electron microscopy (SEM) to be densely distributed on the surface of ROS.

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[1] Abbreviations: ROS, rod outer segment; SEM, scanning electron microscopy; Con A, concanavalin A.

Recently, Steinemann and Stryer (17) have shown that the plant lectin, concanavalin A (Con A), binds to the carbohydrate unit of rhodopsin (18). This binding site appears to be exposed on the surface of ROS disk membranes (17, 19). In this paper, the distribution of Con A receptors on the entire surface of bovine photoreceptor rod cells and ROS membranes is reported. New reagents consisting of polymeric microspheres (20) bonded to Con A are used as visual markers for SEM.

METHODS

Preparation of Retinas, Photoreceptor Cells and Rod Outer Segments

Retinas were dissected under dim red light from cattle eyes maintained on ice in the dark. Several retinas were fixed in 0.125% glutaraldehyde-PBS for 1 hr at 25°C. After rinsing in PBS, the retinas were placed in 0.1 M glycine-PBS for 1 hr. Several tissue slices, approximately 2 X 2 mm, were used in the labeling of retina tissue. Photoreceptor cells and cell fragments were obtained by shaking the remaining fixed retina tissue in PBS for 1 min.

ROS were prepared from unfixed retina by the procedure of McConnell (21) as modified by Papermaster and Dreyer (22). In order to minimize disruption of the ROS, however, the homogenization steps were omitted. After centrifugation on the sucrose gradient, ROS were washed twice by dilution in PBS followed by centrifugation at 12,000 X g for 10 min. For some experiments, ROS were fixed in 0.125% glutaraldehyde-PBS for 30 min at 25°C. The ROS were then washed in PBS and resuspended in 0.1 M glycine-PBS for 1 hr.

Preparation of Concanavalin A-Microsphere Conjugates

Copolymer methacrylate latex spheres, approximately 300 Å in diameter (20), were generously provided by S.P.S. Yen and Dr. A. Rembaum. Derivatization of the spheres with diaminoheptane was carried out as previously described (20). Con A (Miles-Yeda, three times crystallized) was conjugated to the derivatized spheres by the two-step glutaraldehyde procedure. 0.5 ml of 25% aqueous glutaraldehyde (Polysciences) was added to a 10 ml solution of latex (8 mg/ml) in 0.01 M sodium phosphate buffer, pH 7.0. After stirring for 1 hr at 25°C, the activated spheres were dialyzed against three changes of 0.01 M phosphate buffer, pH 7.0 (1 liter each) at 4°C over a period of 18 hr. To 10 ml of the activated spheres were added 10 ml of Con A (7 mg/ml) in 0.1 M phosphate, pH 7.0, containing 0.1 M glucose and 1×10^{-3} M NaN_3. The reaction was allowed to proceed with stirring for 12 hr at 25°C. Unbound Con A was separated from the Con A-microsphere conjugate by centrifugation on a discontinuous sucrose gradient as previously described (20). The conjugate was then dialyzed extensively against buffer containing 0.15 M NaCl, 5×10^{-4} M $MnCl_2$, 5×10^{-4} M $CaCl_2$, and 2×10^{-3} M HEPES, pH 7.4. Large aggregates were removed by centrifugation at 1,000 g for 10 min. Final conjugate concentration was 8–10 mg/ml.

[^{125}I] Con A prepared by the lactoperoxidase procedure (23) was used to determine the amount of Con A which was bonded to the spheres (20). Aliquots of the reaction mixture at various times were removed and washed in 0.1 M glycine, 0.1 M acetate, pH 4.5, by repeated centrifugation (20,000 X g 30 min). The amount of [^{125}I] Con A bound was determined by liquid scintillation counting.

Labeling Studies

Retina tissue, photoreceptor cells, or ROS were suspended in 0.1 ml of Con A-microsphere conjugates for 0.5–1 hr at 25°C. In control experiments, α-methyl mannoside at a final concentration of 0.01 M was added either initially to inhibit or after 0.5 hr to reverse the specific binding of the Con A conjugate. Excess reagent was removed by either rinsing the tissue in PBS or washing the photoreceptor cells or ROS by repeated centrifugation. In some experiments, labeling was carried out on ROS which had been sedimented onto glass cover slips (20).

Preparation of Specimens for SEM

Tissue samples or cells adsorbed onto glass cover slips were fixed in 1.25% glutaraldehyde-PBS solution at 25°C for 1 hr and subsequently postfixed in 1% OsO_4-0.1 M collidine buffer for 1 hr. Dehydration was carried out through a graded series of ethanol solutions. Samples were critical point dried from Freon 13 and subsequently coated with gold-palladium. An ETEC Autoscan scanning electron microscope was used to examine the specimens.

RESULTS

Coupling of Concanavalin A to Latex Microspheres

Polymeric microspheres consisting of hydroxyethyl methacrylate, methacrylic acid, methylmethacrylate, and ethylene glycol dimethacrylate (20) were coupled to Con A by a two-step glutaraldehyde reaction. In the initial step, free amino groups on diaminoheptane-derivatized spheres were activated with glutaraldehyde; in the second step, amino groups on Con A were reacted with the activated spheres after the excess glutaraldehyde had been removed. In a limited study the extent of the bonding of Con A at a concentration of 3.5 mg/ml to the microspheres was measured as a function of time. As shown in Table I, the coupling reaction proceeds slowly at 25°C, and pH 7.

TABLE I. Covalent Bonding of Con A to Glutaraldehyde-Activated Microspheres at 25°C, pH 7.0

Time (hr)	mg Con A per mg Latex
3	0.031
7	0.067
12	0.136
23	0.173

Localization of Concanavalin A Receptors

Retina. When glutaraldehyde-fixed retina tissue was treated with Con A-microsphere conjugates, the outer segment surface of the rod photoreceptor cells was found to be heavily labeled with microspheres (Fig. 1a). Exposure of the retina to light, prior to fixation and labeling, had no effect on the distribution of the markers. Retina tissue which was incubated with conjugate in the presence of the Con A inhibitor, α-methyl mannoside, displayed only a few particles on the surface of the outer segments (Fig. 1b).

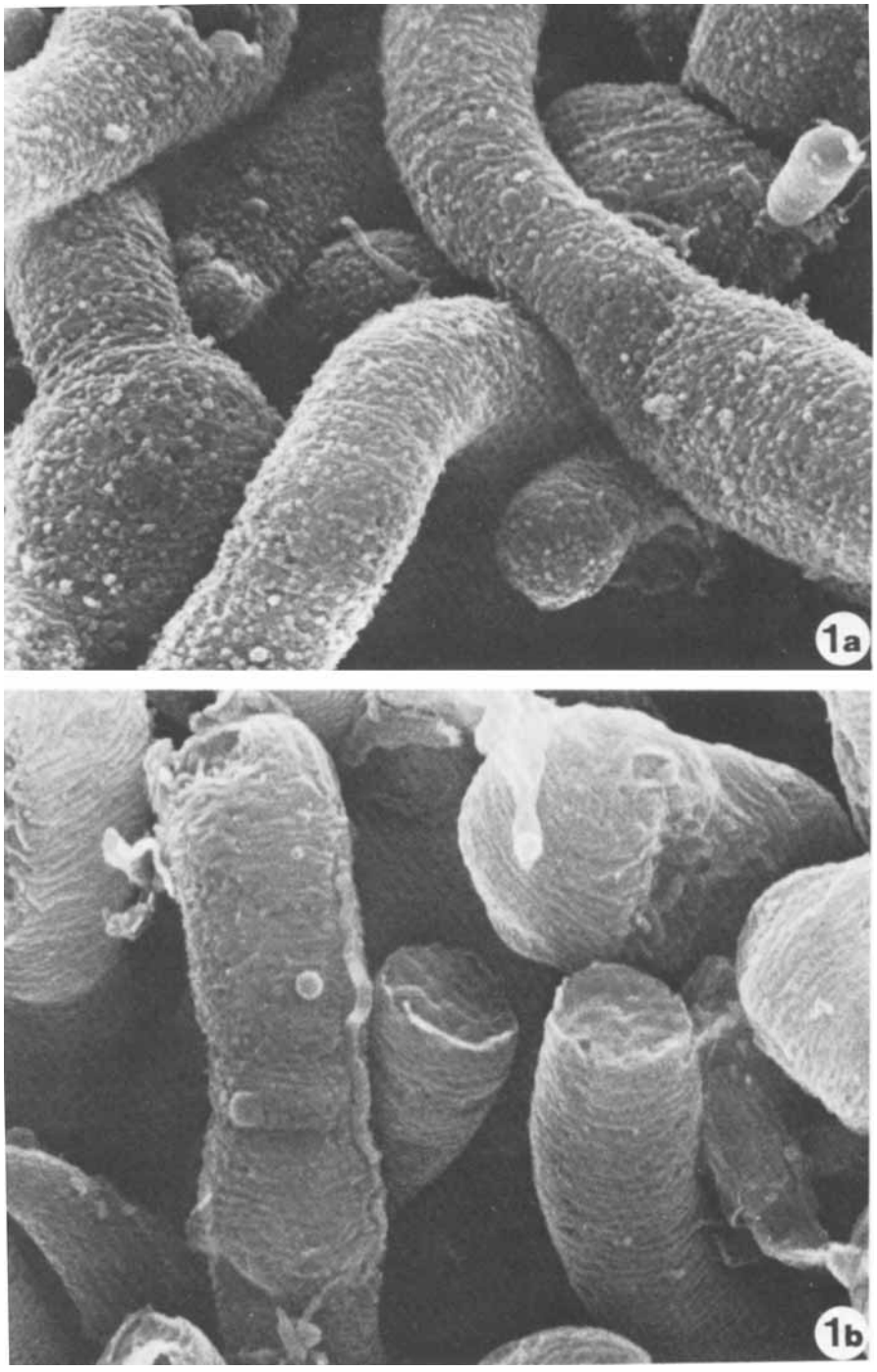


Fig. 1. Scanning electron micrographs of rod outer segments in bovine retina. (a) Tissue samples fixed with glutaraldehyde and treated with Con A-microsphere markers. The surface of the ROS are heavily labeled with the microspheres. (b) Control. Tissue sample treated with Con A-microsphere markers in the presence of 0.01 M α-methyl mannoside. Only a few microspheres are bound to the ROS. × 21,600.

Isolated photoreceptor cells. The distribution of Con A receptors along the entire length of photoreceptor cells was examined on fixed cells dissociated from retina tissue. The use of this preparation containing isolated cells and cell fragments avoids the problem of reagent penetration which may be encountered when tissue specimens are labeled. A scanning electron micrograph of a photoreceptor cell labeled with Con A-microsphere conjugates is shown in Fig. 2a. Microsphere markers ranging in diameter from 400–500 Å as seen under the SEM were densely packed on the surface of the synaptic region, the inner segment, and the outer segment of the rod cell. The cilium connecting the inner and the outer segment was less heavily labeled and in some cases completely devoid of markers.

The labeling of Con A receptors was not affected by the presence of 0.01 M galactose (Fig. 2b). However, if 0.01 M α-methyl mannoside was present during incubation with the Con A-microsphere conjugates, only a few spheres were present on the cell surface (Fig. 2c). Addition of α-methyl mannoside to photoreceptor cells labeled with the Con A-microsphere markers resulted in a partial reversal of the conjugate binding (Fig. 2d).

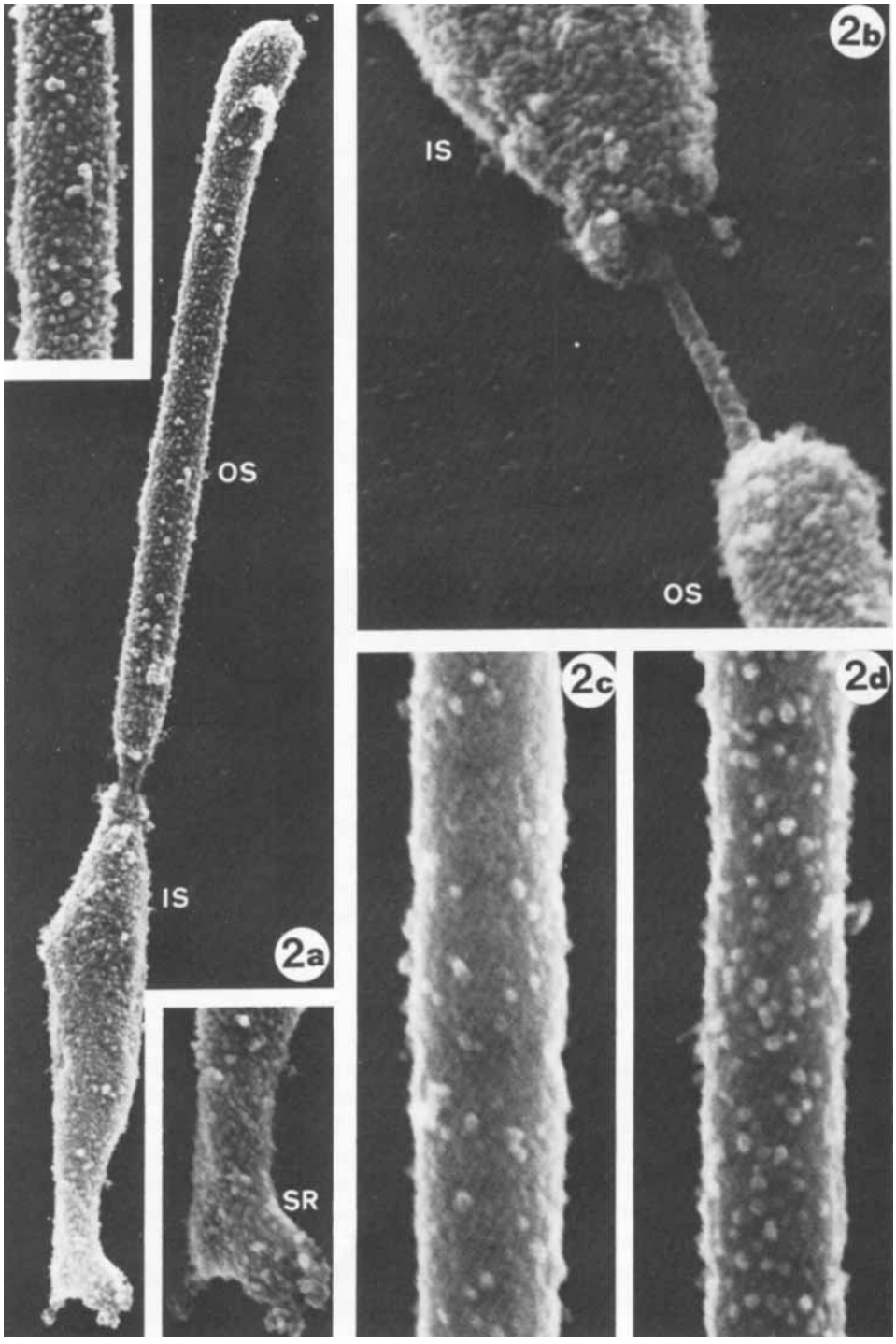
Rod outer segments. In order to confirm that the Con A binding sites are tightly associated with the surface membrane, ROS purified on sucrose gradients and thoroughly washed were used in some labeling experiments. The same dense packing of microspheres seen on retinal photoreceptor cells was observed when unfixed or fixed ROS were treated with Con A conjugates.

In addition to isolated ROS, disk structures having a diameter approximately equal to the width of the outer segment, 0.75 μm, were present either as isolated units or as large aggregates. When incubated with Con A-microsphere conjugates, the surface of these disk membranes was also specifically tagged (Figs. 3a, b).

DISCUSSION

Polymeric microspheres, previously used as markers for antigens on red blood cells (24) and lymphocytes (20), were coupled to Con A by a two-step glutaraldehyde reaction. These conjugates were used to specifically label Con A receptors on bovine retinal rod cells. As visualized by SEM, a dense packing of markers was found on the surface of the outer segment, the inner segment, and the synaptic region of rod cells which had been prefixed with glutaraldehyde. The surfaces of ROS disks were also heavily labeled. Due to the relatively large size of the markers, 400–500 Å diameter, any fine organization of Con A receptors, if present, could not be resolved by this technique. Likewise, any redistribution of receptors (28) on unfixed ROS membranes which may have occurred as a result of labeling with a multivalent marker was not detectable.

The presence of Con A receptors on the outer surface of both ROS disk and plasma membranes raises the following questions related to the organization of components in ROS membranes. Is the carbohydrate unit of rhodopsin oriented so as to serve as the Con A-binding site on both membrane systems? Are there Con A-binding sites on the inner or cytoplasmic side of the ROS plasma membrane and on the inner surface of the disks? Related to this, Steinemann and Stryer (17) have shown in quantitative studies that one Con A monomer is bound per retinal group on vesicles derived from disk membranes. Vesicles composed only of lipids from disk membranes do not bind Con A (19). On the other hand, electron microscope studies indicate that disks are formed by the pinching off of the invaginated ROS plasma membrane (25). Hence, Con A receptors exposed on


OS
IS
2a
SR
IS
OS
2b
2c
2d

the external surface of ROS plasma membranes may be expected on the inner surface of disk membranes if an alteration or reorganization of membrane components does not occur during disk formation. Further biochemical and electron microscope studies should help answer these questions.

Finally, in principle, the technique of affinity density perturbation, originally formulated by Wallach et al. (26) and subsequently modified by Lim et al. (27), can be used in conjunction with these Con A-microsphere conjugates to separate ROS plasma membranes from disk membranes for biochemical analysis. In this method, outer segments in retina tissue can be labeled with the Con A-microsphere conjugates and subsequently isolated from contaminating membranes by conventional procedures. Subsequently, the purified ROS can be disrupted by physical means such as homogenization. Isopycnic centrifugation of these membranes on a continuous density gradient should separate unlabeled disk membranes from the ROS plasma membranes which are denser due to the bound Con A-microsphere complex. Biochemical characterization of these membranes would aid in understanding the molecular nature of visual excitation.

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Fig. 2a. Isolated bovine rod photoreceptor cell treated with Con A-microsphere markers. A dense labeling with microspheres is seen on the outer segment (OS), the inner segment (IS), and the synaptic region (SR). Some microsphere aggregates can be seen on the cell surface. × 8,900. Insets, × 18,000.

Fig. 2b. Photoreceptor cells treated with Con A-microsphere markers in the presence of 0.01 M galactose. Cilium connecting the outer and inner segment is shown. × 28,500.

Fig. 2c. Control. Photoreceptor cell treated with Con A-microsphere conjugate in the presence of 0.01 M α-methyl mannoside. A few particles are present on the surface of the ROS. × 31,300.

Fig. 2d. Photoreceptor cell incubated with Con A-microsphere markers for 30 min; subsequently, α-methyl mannoside (0.01 M) was added and incubation was continued for 30 min. Under these conditions, α-methyl mannoside is shown to partially reverse the binding of the Con A conjugate. × 29,450.

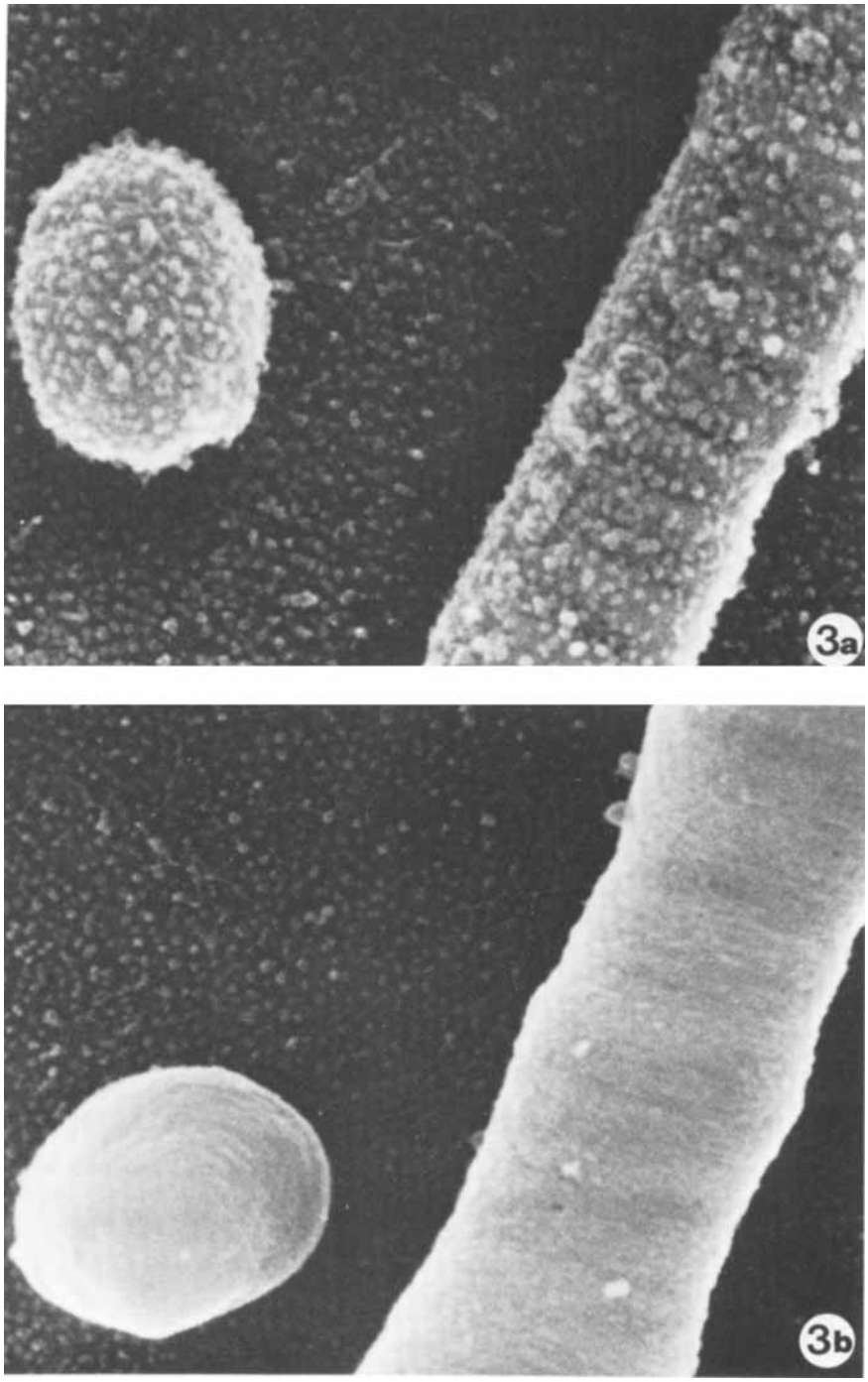

Fig. 3. ROS purified by sucrose density centrifugation. (a) ROS and disk treated with Con A-microsphere markers. (b) ROS and disk treated with Con A-microsphere markers in the presence of 0.01 M α-methyl mannoside. × 36,000. Labeling was carried out on ROS which first had been sedimented onto glass cover slips. Nonspecific binding of Con A-microspheres to the glass surface is evident on the background.

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