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Detection of Cholesterol in Cell Membranes by Use of Bacterial Toxins

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A method is described for the detection of cholesterol in membranes from erythrocytes, mycoplasmas, and bacterial cells by a ferritin-labeling technique. Membranes treated with cereolysin, a bacterial hemolysin which specifically binds to cholesterol, and then treated with ferritin-antitetanolysin, were specifically ferritin-labeled for cholesterol. A similar antigen-antibody system, streptolysin O-ferritin-antistreptolysin, was also used successfully with erythrocyte membranes. There was an uneven distribution of ferritin in erythrocyte membranes suggesting that the distribution of cholesterol may not be entirely random. *Mycoplasma gallisepticum* was intensely labeled, but *Acholeplasma laidlawii* with or without cholesterol in the membranes was not labeled, suggesting an unusual location for cholesterol in *A. laidlawii* membranes. As controls, two of three species of bacterial membranes lacking cholesterol were not ferritin-labeled.

Although the chemical composition of isolated cell membranes can be determined with relative ease, it has proved consistently difficult to elucidate the organization of the constituent lipids and proteins and to understand their arrangement and distribution in the broad plane of the membrane. One approach to this problem is to use cytolytic bacterial toxins as specific markers for chemical constituents of the membrane. Previous work in this laboratory (8) has shown that when natural or synthetic membranes are treated with the hemolytic α-toxin of *Staphylococcus aureus*, six-membered ring-shaped aggregates of α-toxin (12*S*) formed on the surface of the membrane, sometimes in a rectilinear arrangement. Since similar rings of α-toxin, formed in the absence of membranes, were arranged in hexagonal close packing, it is possible that the rectilinear pattern indicates a rectilinear distribution of a particular membrane component to which the α-toxin is attached. Remsen, Watson, and Bernheimer (18) pretreated preparations of erythrocyte membranes with trypsin, lipase, Pronase, and phospholipase C and found that phospholipase C and, to a lesser extent, Pronase prevented the formation of rings on subsequent treatment with α-toxin. These results were interpreted as evidence for an ordered rectilinear arrangement of a membrane component, perhaps a phospholipid.

It seemed to us that other bacterial toxins with cytolytic activity, such as streptolysin O from group A streptococci, could be used as markers for particular membrane constituents. Several species of bacteria produce hemolytic, oxygen-labile lytic toxins (lysins) similar to streptolysin O: e.g., tetanolysin from *Clostridium tetani* and cereolysin from *Bacillus cereus*. These lysins are antigenically related since they are neutralizable by hyperimmune horse antistreptolysin or antitetanolysin (although not neutralizable by human antistreptolysin), and they also share the property of being irreversibly and specifically inhibited by low concentrations of cholesterol and structurally related sterols. The lytic activity of streptolysin O is dependent upon the presence of cholesterol in the membranes of mammalian cells, and cholesterol appears to be the site to which streptolysin O attaches (2, 9, 15). Bernheimer and Davidson (3) showed that parasitic mycoplasmas which have cholesterol in their membranes were susceptible to streptolysin O, but saprophytic mycoplasmas (*Acholeplasma laidlawii*) containing no cholesterol and bacterial protoplasts which lack cholesterol were resistant to lysis (1).

The specificity of streptolysin O-type toxins for cholesterol suggested that they might be useful tracers for cholesterol in membrane

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studies although, unlike *S. aureus* α-toxin, no electron microscope studies have demonstrated streptolysin O attached to membranes. In this paper evidence is presented that ferritin-labeled antistreptolysin or antitetanolysin can be employed as specific markers for cholesterol in erythrocyte, mycoplasma, and bacterial membranes treated with cereolysin or streptolysin. Electron microscope studies with appropriate controls demonstrate that ferritin-labeled antibody attaches specifically only to erythrocyte and parasitic mycoplasma membranes containing cholesterol and pretreated with cereolysin or streptolysin. Although no particular arrangement of the ferritin was seen, its distribution over the surface of the membranes was not always random.

MATERIALS AND METHODS

Rabbit erythrocyte membranes for agglutination. These were prepared from 20 ml of blood by the method of Dodge, Mitchell, and Hanahan (5). The membranes finally were suspended in 20 ml of hypotonic phosphate buffer.

Cereolysin. This was prepared as described by Bernheimer and Grushoff (4). The preparation contained 1.0×10^6 hemolytic units per ml and it was activated with an equal volume of 1% cysteine solution before use.

Horse antistreptolysin. This was "antistreptolysin globulins," 20,000 units/ml, batch no. 7, 19-9-47, Serum Institute, Carshalton, England.

Anti-horse rabbit serum. Serum from a rabbit immunized against horse serum was kindly provided by Z. Ovary. The lot bore the designation: rabbit 82, serum pool 26-II-57 ZO.

Ferritin conjugates. A batch of tetanus antitoxin (antitetanolysin), kindly supplied by W. C. Latham of the Massachusetts Public Health Biologic Laboratories, Boston, was found to have a titer of antistreptolysin of 5,000 units/ml. Since electrophoretic analysis of the antitetanolysin showed that albumin had been removed previously, it was not fractionated further, except for dialysis for 3 days against 0.02 M phosphate-buffered saline (PBS), *p*H 7.4, to remove the thiomersalate preservative.

Human myeloma antistreptolysin serum was kindly provided by E. Kjems of the Statens Seruminstitut, Copenhagen, Denmark. It contained 180,000 units of antistreptolysin per ml.

Normal horse serum was from the City of New York Department of Health and contained less than 32 antistreptolysin O units per ml.

The globulin fractions from human antistreptolysin and from normal horse sera were isolated by three precipitations with 33% saturated $(NH_4)_2SO_4$ at *p*H 7.8. After dialysis against PBS the protein content of globulin solutions was estimated at 280 nm in a Beckman DU spectrophotometer with human gamma globulin fraction II (Nutritional Biochemicals Corp., Cleveland, Ohio) as a standard.

Cadmium-free 2× crystalline ferritin (Nutritional Biochemicals Corp.) was conjugated, at 20 to 25 C, to the normal globulins, the antistreptolysin globulins, and the antitetanolysin globulins by the method of Sri Ram et al. (21) by using *p, p'*-difluoro-*m, m'*-dinitrodiphenylsulfone (General Biochemicals Corp., Chagrin Falls, Ohio) as coupling agent. After conjugation, the crude conjugate was centrifuged on a density gradient as described by Vogt and Kopp (22) to remove most of the unconjugated globulin. The free ferritin and ferritin-globulin conjugate layers were recovered. Remaining traces of unconjugated globulin were removed from the conjugate by three washes in PBS at $100,000 \times g$ for 3 hr. Removal of unconjugated antibody globulin was followed by titrating supernatant fluids for antistreptolysin activity. The final ferritin conjugate was suspended in PBS, sterilized by passage through a bacterial Millipore filter, and stored in sterile containers at 4 C. The conjugated horse antitetanolysin and human antistreptolysin had titers of 32 and 600 antistreptolysin units per ml, respectively.

Erythrocyte membranes. Rabbit blood was withdrawn by cardiac puncture and dispensed into 20 volumes of PBS. Erythrocytes were collected by centrifugation and washed three times in PBS. Washed cells were osmotically lysed by pipetting 1 ml of a dense cell suspension into either 40 ml of hypotonic phosphate buffer, *p*H 7.4 (5), or into distilled water (8). Hemoglobin-free membranes were harvested by centrifugation for 15 min at $15,000 \times g$.

Micrococcus lysodeikticus and Sarcina lutea membranes. Growth was in 200 ml of peptone-yeast extract broth for 24 hr at 30 C in a shaking incubator. The cells were collected by centrifugation, washed twice in 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, *p*H 7.5, and suspended in 40 ml of 0.1 M Tris buffer containing 1.5 M sucrose and 1 mg of lysozyme (Worthington Biochemical Corp., Freehold, N.J.). After 30 min at 20 to 25 C, 0.4 ml of 1 M $MgCl_2$ was added to stabilize the protoplasts (1). Membranes were obtained by osmotic lysis of the protoplasts in Tris buffer containing 10 mM $MgCl_2$.

Bacillus megaterium membranes. Growth was at 30 C for 24 hr in 200 ml of 2% peptone broth in a shaking incubator. Harvested cells were washed twice in Tris buffer and resuspended in 2 ml of Tris buffer containing 10 mM $MgCl_2$, 0.2 M sucrose, and 2 mg of lysozyme (24). After standing at 20 to 25 C for 45 min, the suspension, consisting of a mixture of protoplasts and cells, was pipetted into 50 ml of Tris buffer containing 10 mM $MgCl_2$. Protoplast membranes were collected by centrifugation and separated from whole cells by low-speed centrifugation.

Mycoplasma gallisepticum membranes. The organism was grown for 24 to 48 hr at 37 C in a modified Hayflick medium (10) containing 0.5% glucose and 0.05% phenol red in a shaking incubator. The cells were harvested and washed twice in PBS. In attempts to obtain membranes, the cells were suspended in 10 ml of 2 M glycerol for 10 min at 37 C and then pipetted rapidly into 100 ml of distilled water (19). Membranes were collected by centrifugation at $40,000 \times g$ for 30 min and washed twice in Tris buffer containing 10 mM $MgCl_2$.

A. laidlawii membranes. Strain A of *A. laidlawii*

was grown in the Tryptose broth medium described by Razin and Cleverdon (17) with 1% bovine serum albumin, fraction V (Calbiochem, Los Angeles, Calif. 90054), but without penicillin. In experiments where the medium was to be cholesterol-free, the albumin was defatted with diethyl ether before incorporation into the medium. In other experiments the medium was supplemented with cholesterol. Grade A cholesterol (Calbiochem) was recrystallized twice from ethanol before use. Twenty milligrams of crystalline cholesterol was dissolved in 1 ml of ethanol plus 1 ml of 10% (v/v) Tween 80 and sterilized by filtration before addition to 1 liter of the medium. Growth of *A. laidlawii*, with or without cholesterol, was in 500-ml volumes in 2-liter flasks incubated statically at 37 C for 24 hr. The organisms were harvested by centrifugation and washed once in PBS. Packed cells were suspended in 2 ml of PBS and pipetted rapidly into 100 ml of distilled water. Membranes thus obtained by hypotonic lysis were washed twice in Tris buffer containing 10 mM $MgCl_2$.

Ferritin-labeling of membrane preparations. Rabbit erythrocyte membranes were suspended, labeled, and washed in PBS by centrifugation at 10,000 × *g* for 15 min. For the mycoplasmal and bacterial membranes, Tris buffer containing 10 mM $MgCl_2$ was used in place of the PBS, and centrifugation was at 40,000 × *g* for 30 min.

Approximately 0.5 ml of test membrane preparation was mixed with 0.1 to 0.2 ml of activated cereolysin, and the mixture was allowed to stand for 30 min at 20 to 25 C. Unadsorbed cereolysin was removed from the preparations by two washes with 30 volumes of buffer per wash. The membranes were suspended in 1 ml of buffer, and 0.2 ml of ferritin-conjugated antiserum was added and allowed to react for 30 min. Finally, the membranes were washed twice to remove unadsorbed ferritin. In several experiments erythrocyte membranes were treated with cysteine-activated streptolysin (batch 73PRIII, kindly supplied by S. P. Halbert in 1958) and labeled with human antistreptolysin-ferritin conjugate.

To minimize nonspecific staining in the case of *M. gallisepticum*, the following modifications were made to the general procedure described above. *M. gallisepticum* cells, after harvesting and washing, were suspended in 1 ml of the unconjugated antitetanolysin for 30 min at 20 to 25 C. The cells were washed three times in buffer, treated with activated cereolysin, washed twice more, and treated with the ferritin conjugate. Nonspecific ferritin was removed by two alternate washes of the cells in saline and distilled water.

Controls. Controls were included in each labeling experiment for the various membrane preparations and were: (i) test system with cereolysin omitted, (ii) blocking with unconjugated antiserum, (iii) free ferritin, or (iv) ferritin-conjugated normal globulin in place of the immune conjugate.

Electron microscopy. The dilute specimen in phosphate or Tris buffer was placed either directly on Formvar-supported carbon-coated grids or mixed with stain prior to being put on the grid. Negative staining was done with 2% ammonium molybdate in distilled water, *p*H 5.2, for 1 min. The excess stain was removed with a piece of filter paper and the grids allowed to dry in a petri dish. Similar results were obtained with the two different methods applied in this work.

For thin sections, pelleted specimens were fixed with 2.5% glutaraldehyde in Kellenberger's Veronal acetate buffer, *p*H 6.1, for 1 hr and postfixed in 1% OsO_4 in the same buffer for 17 hr (11, 20). The postfixed specimens were embedded in 1% Noble agar and thereafter they were stained with 0.5% magnesium uranyl acetate. Blocks were dehydrated in an alcohol series, infiltrated, and embedded in Epon by the method of Luft (14). Sections were cut with a diamond knife on an LKB 4800A ultratome and placed on a Formvar carbon-coated grid. Sections were stained with uranyl acetate and lead citrate (7).

Both thin sections and negatively stained preparations were examined with a Siemens Elmiskop 1 operated at 80kv with a 35-μm objective aperture. A liquid nitrogen decontamination device was used.

RESULTS

Agglutination of erythrocyte membranes. To 1 ml of rabbit erythrocyte membranes was added 0.4 ml of cereolysin. After 30 min at 20 to 25 C the mixture was centrifuged at 12,000 × *g* for 10 min. After discard of the supernatant fluid, the sedimented membranes were suspended in 1 ml of 0.077 M NaCl and 0.067 M phosphate buffer, *p*H 7.0, and 0.01 ml of horse antistreptolysin was added to the suspension. After the suspension had stood overnight at 4 C followed by 60 min at 37 C, no agglutination was observed. Next, the mixture was centrifuged at 12,000 × *g* for 10 min. The supernatant fluid was discarded, the membranes were suspended in 1 ml of 0.077 M NaCl and 0.067 M phosphate, *p*H 7.0, and 0.05 ml of anti-horse rabbit serum was added to the suspension. After 60 min at 37 C followed by 48 hr at 4 C, the membranes had settled to the bottom of the tube leaving a clear supernatant fluid (tube no. 1 of Table 1), and the membranes in control tubes (tubes no. 2 to 8) remained suspended. A scheme qualitatively delineating what we believe to be the molecular basis for the observed agglutination is given in Fig. 1. The results encouraged us to perform further experiments utilizing ferritin-labeled antibody as a marker for cholesterol. A scheme explaining the rationale for using ferritin-labeled antibody is shown in Fig. 2.

Ferritin-labeling of erythrocyte membranes. Washed erythrocytes were osmotically lysed by addition of either distilled water or hypotonic phosphate buffer. The former treatment was found to yield a large proportion of unfragmented membranes which were less

liable to nonspecific ferritin-labeling than those prepared by use of hypotonic buffer. The intensity of ferritin-labeling in different test preparations was moderate or heavy (Fig. 3). The distribution of the ferritin was not ordered, nor was it entirely random, because clusters of ferritin were frequently seen. Similar labeling results were obtained with the streptolysin-antistreptolysin-ferritin (Fig. 5) and the cereolysin-antitetanolysin-ferritin system (Fig. 3). Streptolysin and, to a lesser extent, cereolysin caused the appearance of holes in the membrane (Fig. 4 and 6). In control experiments where cereolysin or streptolysin was omitted, or the blocking procedure with unconjugated antitetanolysin was applied, the amount of ferritin attached to the membrane was much less than in test preparations. In sectioned material, ferritin was visible on both surfaces of the test membranes, (Fig. 7) but few ferritin particles were seen in controls (Fig. 8).

TABLE 1. *Agglutination of erythrocyte membranes*

Tube no.	Cysteine-activated cereolysin (μliter)	0.5% Cysteine (μliter)	Horse anti-streptolysin (μliter)	Anti-horse rabbit serum (μliter)	0.85% NaCl (μliter)	Agglutination
1	40		10	50		+
2	40		10		50	0
3	40			50	10	0
4	40				60	0
5		40	10	50		0
6		40	10		50	0
7		40		50	10	0
8		40			60	0

Ferritin-labeling of bacterial membranes lacking cholesterol. Membranes in excellent condition were readily prepared by osmotic lysis of protoplasts of *M. lysodeikticus* and *S. lutea*. Neither test nor control membrane preparations became labeled with ferritin (Fig. 9 and 10).

Membranes from *B. megaterium* were frequently in poor condition and probably were not entirely free of cell wall and cytoplasmic debris. Ferritin-labeling was heavy and apparently completely nonspecific because the degree of staining was similar with or without cereolysin pretreatment. Similar results were obtained with the use of ferritin conjugates of normal horse globulins, confirming the nonspecificity of the labeling.

Ferritin-labeling of mycoplasmal membranes. Membrane preparations from *A. laidlawii*, grown with or without cholesterol in the medium, were obtained in excellent condition by osmotic lysis of cells. There was no ferritin-labeling in test preparations grown with or without cholesterol (Fig. 11 and 12) nor in control preparations untreated with cereolysin. Scattered molecules or groups of molecules of ferritin were occasionally seen after washing, but they do not specifically relate to presence or absence of cholesterol.

We first attempted to obtain membranes of *M. gallisepticum* by hypotonic lysis but found that large pieces of normal membrane were rarely obtained. In contrast, unlysed cell prep-

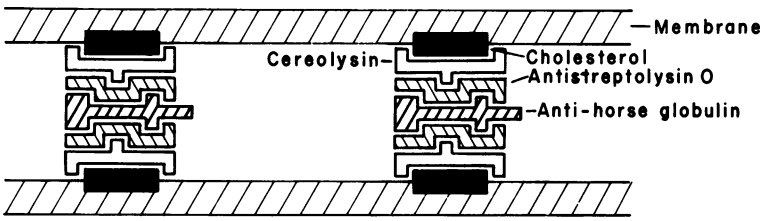


FIG. 1. *A hypothetical scheme for agglutination of membranes shown in results of Table 1. Cereolysin attaches specifically to cholesterol in the membrane. Horse antistreptolysin as antibody reacts with cereolysin and agglutination of the membranes occurs when antihorse globulin is added.*

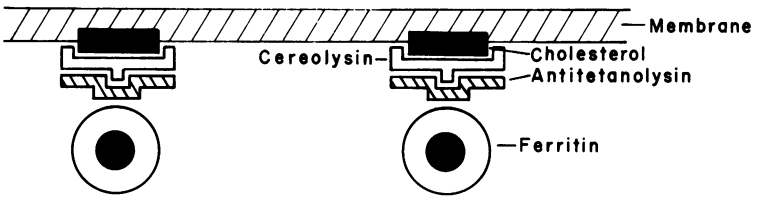
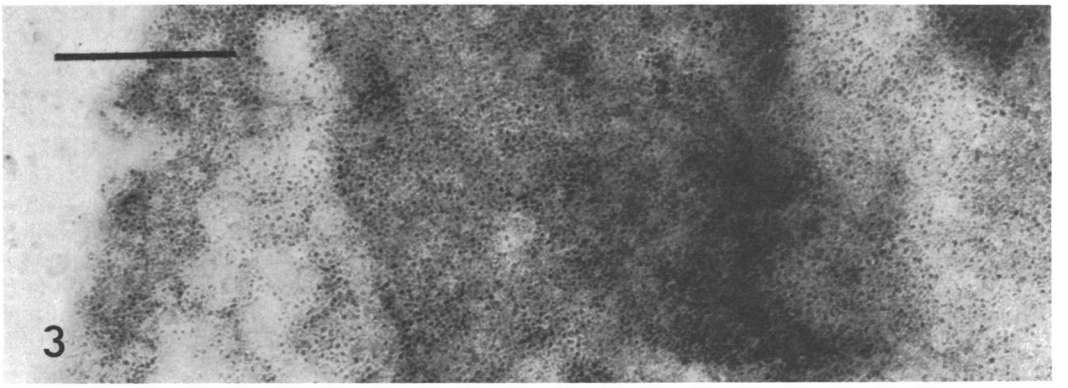
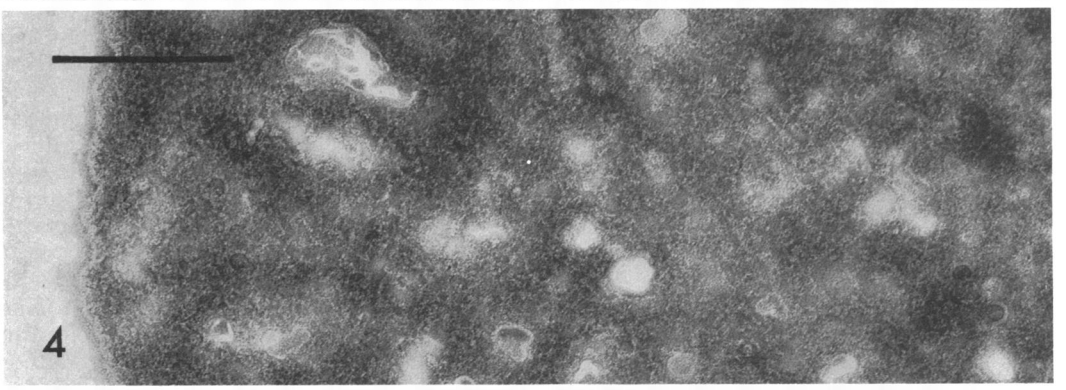
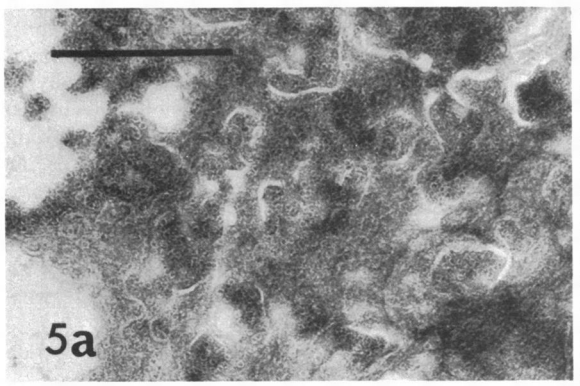
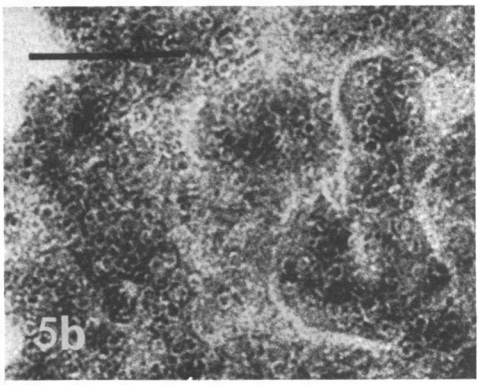


FIG. 2. *Scheme for labeling of membrane cholesterol by cereolysin and antitetanolysin conjugated to ferritin.*









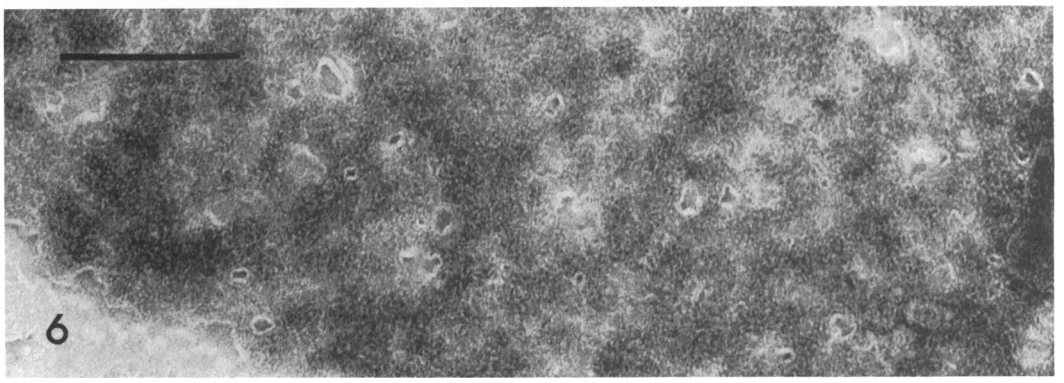
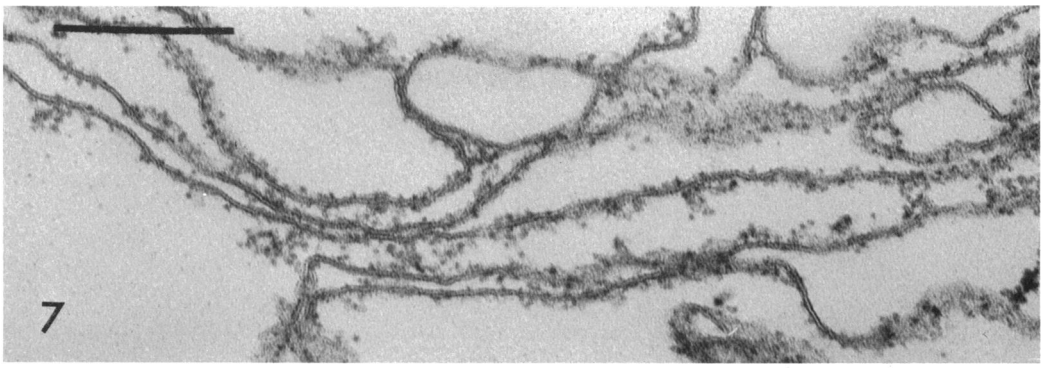


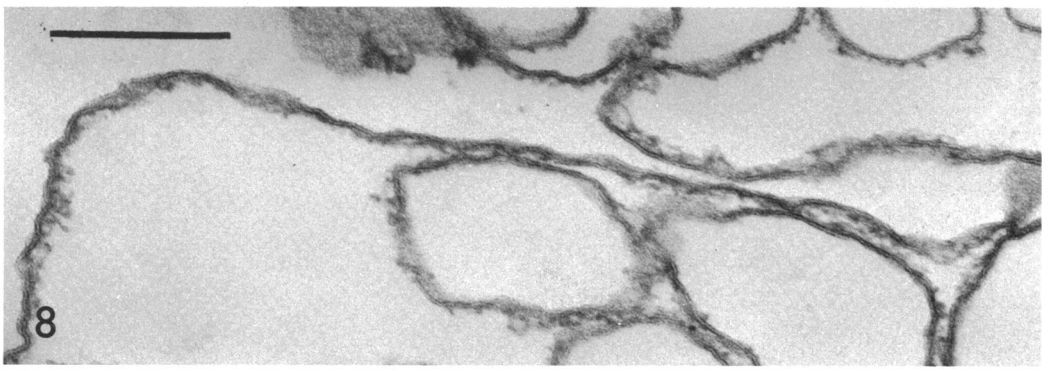
FIG. 3. *Portion of rabbit erythrocyte membrane treated with cereolysin, ferritin-antitetanolysin, and negatively stained with ammonium molybdate. The ferritin is seen to be somewhat unevenly distributed. In this and succeeding figures the bar indicates 0.25 μm, except in Fig. 5b where the bar indicates 0.1 μm.*

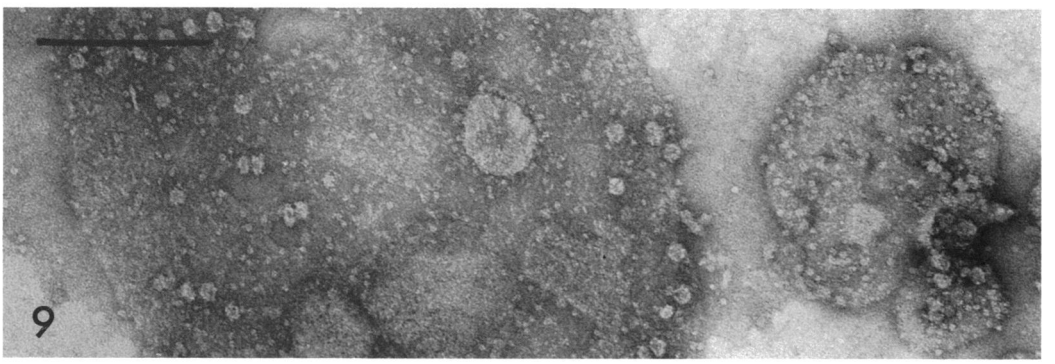
FIG. 4. *Portion of rabbit erythrocyte membrane treated as in Fig. 3 but exposed to unlabeled antitetanolysin prior to treatment with ferritin-antitetanolysin. Labeling has been blocked. Occasional holes or pits caused by cereolysin can be seen.*

FIG. 5. *Portion of rabbit erythrocyte membrane treated with streptolysin, ferritin-antistreptolysin, and negatively stained. Numerous holes or pits were produced by streptolysin. Labeling is present and is more readily seen in 5b which is a 2.2-fold enlargement of a portion of 5a.*

FIG. 6. *Portion of rabbit erythrocyte membrane treated with streptolysin, unconjugated ferritin, and negatively stained. No labeling is present.*







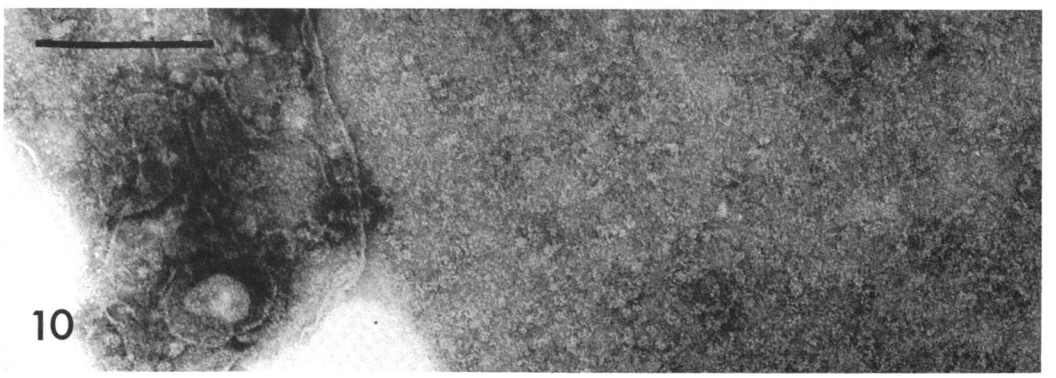


FIG. 7. *Section of rabbit erythrocyte membrane treated with cereolysin and ferritin-antitetanolysin and stained with magnesium uranyl acetate and lead citrate. Ferritin is present on both sides of the membranes.*

FIG. 8. *Section of rabbit erythrocyte membrane treated as in Fig. 7 but exposed to unconjugated antitetanolysin before treatment with ferritin-antitetanolysin. Labeling has been blocked.*

FIG. 9. *Portions of plasma membranes of Micrococcus lysodeikticus treated with cereolysin, ferritin-antitetanolysin, and negatively stained. No labeling is present.*

FIG. 10. *Portions of plasma membranes of Sarcina lutea treated with cereolysin, ferritin-antitetanolysin, and negatively stained. No labeling is present.*

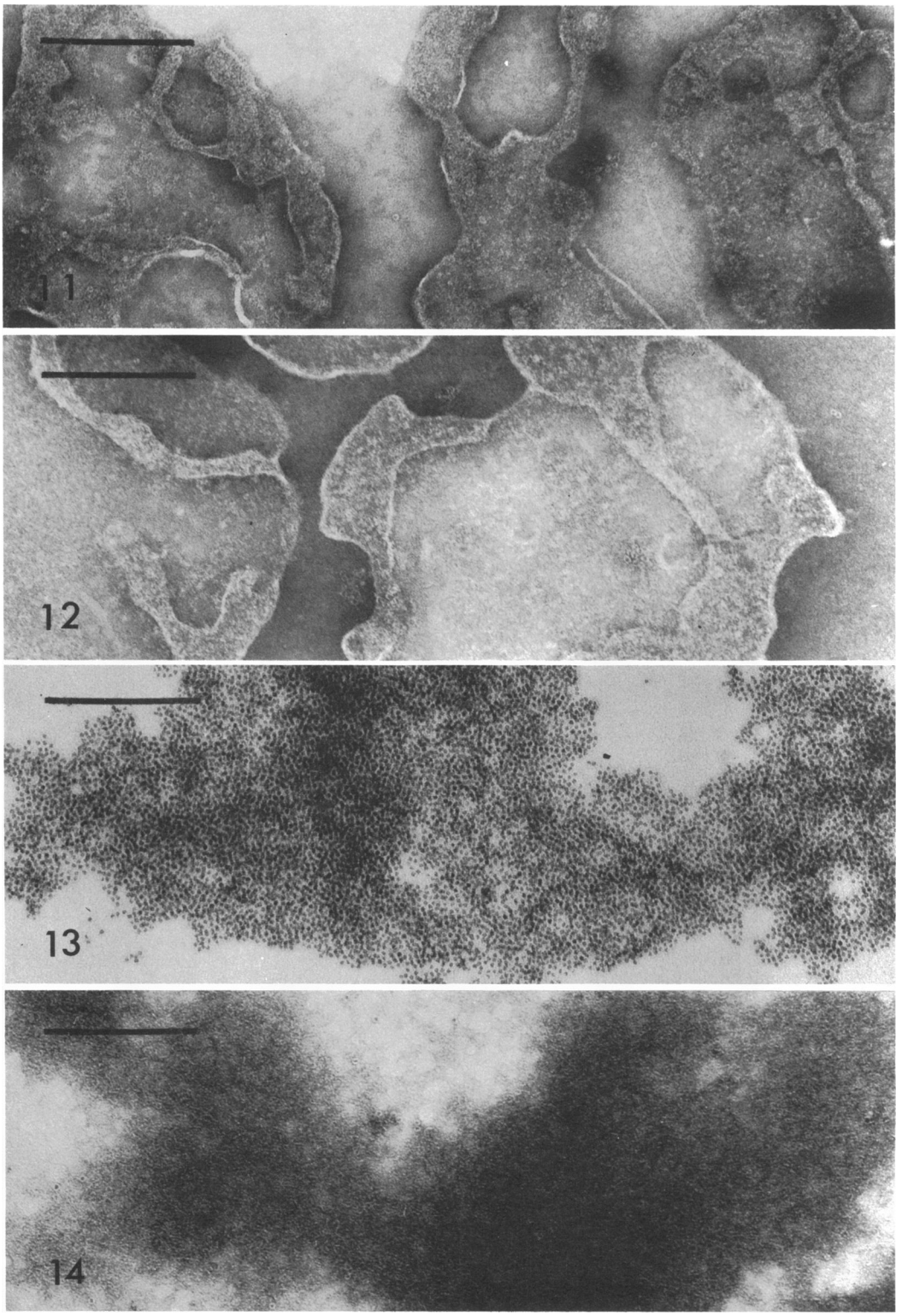


FIG. 11. *Portions of membranes from Acholeplasma laidlawii grown in presence of cholesterol. Membranes were treated with cereolysin, ferritin-antitetanolysin, and negatively stained. Very little ferritin is present.*

FIG. 12. *Portions of membranes from Acholeplasma laidlawii grown in absence of cholesterol. Membranes were treated with cereolysin, ferritin-antitetanolysin, and negatively stained. No labeling is present.*

FIG. 13. *Portion of membrane of Mycoplasma gallisepticum treated with cereolysin, ferritin-antitetanolysin, and negatively stained. Ferritin labeling its intense.*

FIG. 14. *Portion of membrane of Mycoplasma gallisepticum treated with ferritin-antitetanolysin and negatively stained. (Cereolysin treatment was omitted.) There is little or no ferritin labeling.*

arations contained a proportion of normal membrane fragments presumably derived by mechanical breakage of the fragile cells during handling. Nonspecific staining was reduced significantly by use of the latter type of membranes in place of osmotically prepared membranes and by alternate washes of ferritin-labeled membranes in water and PBS. Under these conditions, test preparations of *M. gallisepticum* membranes were heavily labeled and control preparations untreated with cereolysin were only weakly labeled (Fig. 13 and 14). In the majority of membranes the ferritin was uniformly distributed and closely packed but showed no particular arrangement.

The results of ferritin-labeling experiments are summarized in Table 2.

TABLE 2. *Intensity of ferritin-labeling with ferritin-antitetanolysin on cereolysin-treated membranes*

Membrane prepn	Test	Control[a]
Rabbit erythrocyte	++	±
Mycoplasma gallisepticum	+++[b]	±
M. laidlawii with cholesterol	±	±
M. laidlawii without cholesterol	±	±
Bacillus megaterium	+++[c]	+++[c]
Sarcina lutea	0	0
Micrococcus lysodeikticus	0	0

[a] Control membranes were prepared with nonimmune conjugate or with blocking procedure (see text for details).

[b] +++, Almost complete coverage with ferritin; ++, moderate coverage with ferritin; ±, a few ferritin particles visible; 0, no ferritin visible.

[c] Labeling found to be nonspecific.

DISCUSSION

The mammalian erythrocyte membrane is well established as a model system for studying the general properties of cell membranes. We have used it in an effort to elucidate the distribution of cholesterol in the broad plane of the membrane. The ferritin-labeling technique developed for the erythrocyte membrane was then applied to mycoplasmal cells containing cholesterol.

The role of cholesterol in membranes is thought to be structural. Studies with monomolecular films show that it has a membrane-condensing effect and may impose restrictions on the orientations of other lipids (13, 16). A number of chemically dissimilar compounds will attach to cholesterol in erythrocyte membranes, forming complexes, and causing lysis of the cells. Polyene antibiotics such as filipin specifically complex with cholesterol (12), reorienting it to produce pits or holes about 30 nm in diameter in rat erythrocyte membranes. Streptolysin O may act in an analogous manner since, according to Dourmashkin and Rosse (6), it produces holes about 50 nm in diameter in rabbit erythrocyte membranes. In our experience streptolysin O produced holes similar to those seen by Dourmashkin and Rosse, but when cereolysin was used instead of streptolysin holes were less frequently observed. Although streptolysin caused extensive damage to the structure of the membrane through the formation of holes, whereas cereolysin produced less drastic changes, the latter agent may be more useful in investigating membrane structure. The results obtained with ferritin-labeling of cereolysin- or streptolysin-treated membranes and the results of control experiments lead us to conclude that the distribution of cholesterol is specifically represented by the ferritin particles. The distribution of ferritin was not entirely random inasmuch as some areas of aggregation were found. Therefore, there may be areas of the erythrocyte membrane in which cholesterol is localized in relatively high concentrations. Sectioned material shows that labeling occurs on both inner and outer surfaces of the membrane.

Ferritin-labeling of cholesterol in membranes of *M. gallisepticum* was heavy and presumably reflected the relatively high content of cholesterol (6%) in the membrane (17). Although the percentage of cholesterol in rabbit erythrocyte membranes is similar, ferritin-labeling was generally less dense than that obtained with *M. gallisepticum*. To a small extent, nonspecific ferritin-labeling can account for some of the difference noted between the two membrane types, but it seems more probable that more of the mycoplasmal cholesterol is situated at the surface of the membrane, where it is readily accessible to the cereolysin, and so becomes labeled by ferritin-antitetanolysin.

In experiments with *A. laidlawii*, Weber and Kinsky (23) found that *A. laidlawii* was susceptible to lysis by filipin only when grown in the presence of cholesterol, and filipin had no effect on cholesterol-free cells. However, Bernheimer and Davidson (3) found that the presence or absence of cholesterol did not alter the insusceptibility of *A. laidlawii* to lysis by streptolysin O, although *M. gallisepticum* and *M. neurolyticum* with obligate requirements for cholesterol were susceptible to lysis. In the present study there was no ferritin-labeling of *A. laidlawii* cells grown with or without cholesterol and this suggests that cereolysin is unable to bind with cholesterol in these mem-

branes. The reasons for these anomalous results are not clear, but it may be that the cholesterol is incorporated into *A. laidlawii* membranes in a position accessible to the small filipin molecule but not to larger molecules such as cereolysin and streptolysin. *A. laidlawii* cholesterol may be unusual in its position or orientation as compared with the cholesterol in other membranes.

Bacterial membranes do not contain cholesterol, and membranes of *M. lysodeikticus* and *S. lutea*, as expected, failed to become labeled and therefore were useful as controls. Intense nonspecific labeling of *B. megaterium* appeared to be due to affinity between normal gamma globulin and an unidentified constituent of the membrane.

The method described has limitations. Its nature presupposes that the only cholesterol which will be labeled is that which is accessible to cereolysin or streptolysin. Moreover, the cereolysin or streptolysin themselves may bring about a redistribution of some of the membrane sterol so that the extent to which the ferritin mirrors the native location of cholesterol is not clear. Despite these objections the results appear to provide some insight into the distribution of cholesterol in a few types of membranes. It seems reasonable to suppose that the ferritin-labeling technique could be used to explore the distribution of cholesterol in a much wider variety of cellular and subcellular membranes.

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