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Spherosome Membranes

HALF UNIT-MEMBRANES

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ABSTRACT

Spherosomes are bounded by unusual single-line "membranes" which measure 2 to 3.5 nanometers in width, contrasted to the well known tripartite unit-membranes which measure 6 to 8.5 nanometers in over-all thickness. Juxtaposed externally (from the side addressing the hyaloplasm), two spherosomal membranes adjoin to form a thicker single line, but apposed internally (the sides that contact stored lipid) two single-line membranes touch to form a tripartite structure resembling a unit-membrane. Morphologically, we interpret the single-line membranes of spherosomes as half unit-membranes whose polar surfaces face the hyaloplasm and whose lipoidal nonpolar surfaces contact internal storage lipid.

Corroboration of this interpretation was shown biochemically by demonstrating the presence of membrane structural protein in peanut spherosomes. In addition, an immunological identity between membrane protein isolated from spherosomes of quiescent seeds and membrane protein extracted from the mitochondrial fraction of 10-day germinated seedlings was observed. We conclude that the atypical, single-line membranes bounding spherosomes are in fact biological membranes that correspond to half unit-membranes.

Spherosomes are intracellular oil-containing particles that have been observed by plant cytologists for over a half century (6), yet even with the advent of electron microscopy their cytological status remains somewhat uncertain. They are spherical bodies about 1 μ in diameter, are highly refractile in the light microscope, have an osmiophilic matrix, and are bound by unusual single-line membranes (25).

Since most biological membranes have been resolved into tripartite unit-membrane structures, the question arises, are the single-line membranes bounding spherosomes actually membranes? Indeed, the membranes bounding spherosomes are not visible following permanganate fixation (24) and have confused a great many microscopists using other fixatives (9). Perhaps the appellation "interfacial structure" (14) might have been a more appropriate choice, since interfacial structures similar to these single-line membranes exist around certain fat droplets of artificially prepared emulsions.

The purpose of this communication is to show that the single-line material bounding spherosomes is truly a biological membrane, probably a half unit-membrane. We show (*a*) that the two sides of the membrane are dissimilar, one surface being lipoidal and the other nonlipoidal, and when apposed from the lipoidal side will form a bimolecular leaflet of lipid identical morphologically to the unit membrane; (*b*) that structural protein exists in spherosomal membranes of peanuts; it differs from other peanut proteins but is very similar to structural protein from other sources; and (*c*) evidence for an immunological identity between protein of spherosomal membranes and protein from membranes of more familiar organelles in the mitochondrial fraction of germinating seedlings.

MATERIALS AND METHODS

One kilogram of peanut (*Arachis hypogaea* L.) seeds, Virginia 56R variety, was soaked in water for 2 hr, after which the testae were removed. The seeds, in 100-g lots, were homogenized in a Waring Blendor for 1 min with 300 ml of ice-cold 0.5 M NaCl in 50 mM tris-HCl buffer, pH 7.2. The homogenates were filtered through eight layers of cheesecloth and centrifuged at 30,000*g* for 20 min at 0 C. The creamy fat pads from the tops of the centrifuge tubes were removed with a spatula, combined, and rehomogenized in fresh buffered saline. The rehomogenized fat pad fraction was again centrifuged at 30,000*g* for 20 min at 0 C, and again the fat pads were removed and rehomogenized in fresh buffered saline. This process was repeated five more times, followed by three using distilled water instead of buffered saline solution. The final fat pad was our washed spherosomal preparation. A portion of the spherosomal preparation was defatted with hexane-acetone (11) and stored in a vacuum desiccator.

Mitochondrial fraction was prepared from peanut seedlings which were germinated for 10 days at room temperature. The seedlings were homogenized in five times their weight of 0.3 M sucrose containing 0.1 M phosphate buffer, pH 7.2, and filtered through eight layers of cheesecloth. The filtrate was centrifuged at 700*g* for 10 min to remove cell debris and larger particles. The supernatant was centrifuged at 20,000*g* for 20 min, after which the supernatant was discarded. The pellet was resuspended in fresh buffered sucrose, and the process was repeated two more times; the final pellet was the mitochondrial fraction. The pellet was defatted and dehydrated with hexane-acetone (11) and stored in a vacuum desiccator.

Structural protein was extracted from freshly prepared spherosomes by the method of Zahler *et al.* (26). Spherosomal preparation was homogenized with an equal volume of 1.4% acetic acid and allowed to set at room temperature overnight. The mixture was centrifuged at 30,000*g* for 20 min, and the fatty layer was discarded. A portion of the sample was lyophilized and stored in a vacuum desiccator.

Total soluble protein was obtained from testae-free peanuts by homogenization in 0.1 M phosphate buffer, pH 7.2, and

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centrifugation at 30,000*g* for 20 min. The clear solution between the fat pad and pellet was considered total soluble protein solution.

Protein concentration was determined by the method of Lowry *et al.* (12). Amino acid analysis was conducted on 6- and 20-hr protein hydrolysates according to the method of Moore and Stein (15). Antisera were prepared by Antibodies, Incorporated, Route 1, Box 1482, Davis, California according to their protocol, except that the membrane samples were solubilized in 0.3% sodium dodecyl sulfate containing 3% urea for injection into rabbits. Immunological studies were conducted by the double diffusion method of Ouchterlony (17) as previously described (7). Membrane proteins were solubilized by homogenization in Singh and Wasserman's preparation (21) with a tissue grinder.

Electron microscopic examination of spherosomes and ghosts were conducted as described previously (10). The term "ghost" is herein applied to spherosomes that were first im-

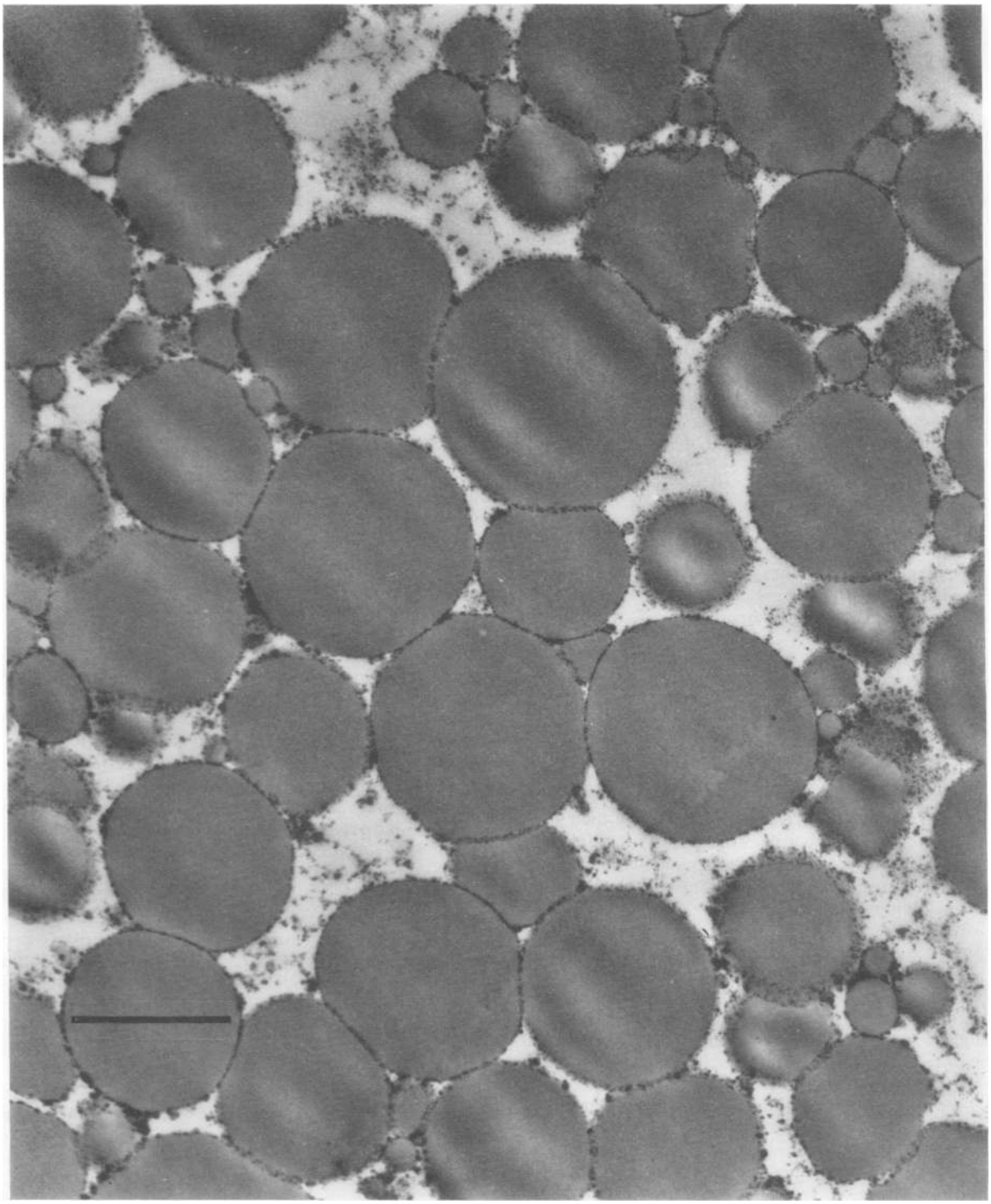

FIG. 1. Electron micrograph of isolated spherosomes from peanuts showing homogeneity of the spherosomal fraction. Note that no other organelles (other than spherosomes) are present. Bar represents 1 μ.

mobilized in agar, then defatted; the term "defatted spherosomes" denotes spherosomes which were first defatted (1) and then immobilized in agar. All materials were fixed with 6% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, containing 6% sucrose, followed by postfixation in 2% osmium tetroxide in 0.1 M phosphate buffer, pH 7.2.

RESULTS AND DISCUSSION

Fine Structure Studies. Thoroughly washed spherosomal fractions were free of any structures that possessed unit-membranes; they consisted only of lighter-than-water spherosomes which were bounded by atypical single-line membranes measuring 2 to 3.5 nm in width (Fig. 1). Other organelles that possessed unit-membranes were never observed in these preparations.

Membranes delimiting spherosomes were difficult to discern following osmium tetroxide fixation due to the osmiophilic nature of the spherosomal contents; however, single-line boundaries could easily be recognized around ghosts. Single-line boundaries of two closely appressed ghosts were often seen converging to form a slightly thicker single-line about 3 to 4 nm in breadth at the juncture where the two ghosts abut (Fig. 2).

Immobilized spherosomes, whether fixed before or after fat extraction (ghosts), appeared as circular objects. Unextracted, spherosomes appeared as circular areas with uniformly osmiophilic matrices; defatted, they appeared as circular electron-transparent areas bounded by single fine lines. Preparations of defatted spherosomes, however, contained very few circular profiles; instead, myriads of membranous elements, often resembling myelin, were present (Fig. 3). Examination at higher magnification showed that many of the membranous elements resembled unit-membranes (Fig. 4). Trelease (22) observed a similar phenomenon in isolated lipid bodies of corn embryos. Since tripartite structures were absent from both isolated spherosomes and ghost preparations (even when they abutted), the new configurations were believed to be caused by the collapse of defatted spherosomes—probably the result of apposition of two inner surfaces of spherosomal membranes.

We investigated this possibility by preparing a series of spherosomal fractions varying in lipid content. Spherosomes were homogenized for various time intervals in a biphasic mixture of diethyl ether and saline (1) and examined in the electron microscope. Centrifugation of partially defatted spherosomal preparations yielded two fractions, a fat pad and a pellet. As defatting progressed, the fat pad portion decreased in amount and the pellet size increased. Electron microscopic examinations of these two fractions showed that the fat pad consisted mainly of oil droplets interspersed with bits of membranous material, while the pellet fraction was largely composed of membranous material with occasional droplets of oil.

The membranous structures which were formed by the defatting of spherosomes were morphologically similar, if not identical, to typical unit-membranes. They measured approximately 8 nm in total width, with each dark line measuring

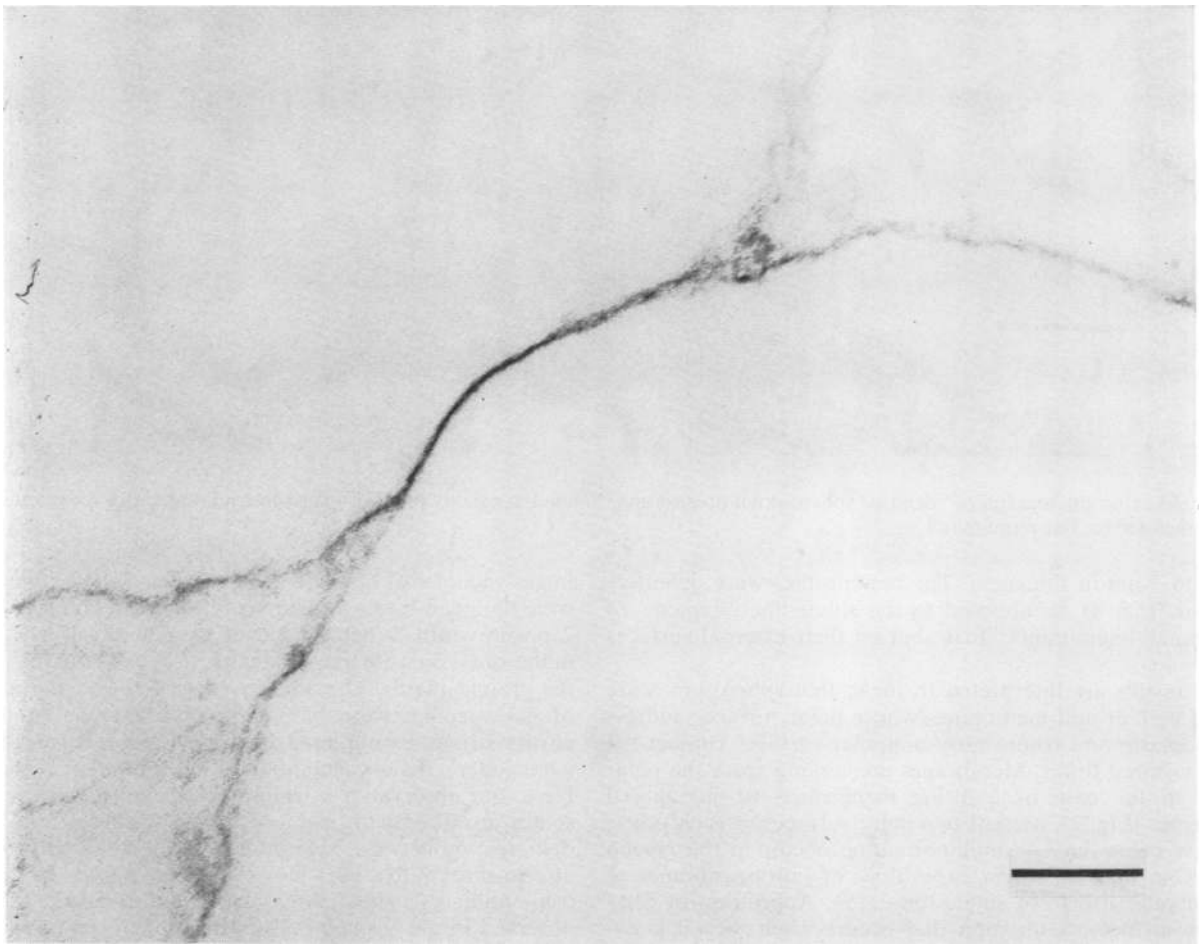

FIG. 2. Electron micrograph of two apposing spherosomal ghosts illustrating the single-line nature of spherosomal membranes juxtaposed externally. Bar represents 0.1 μ.

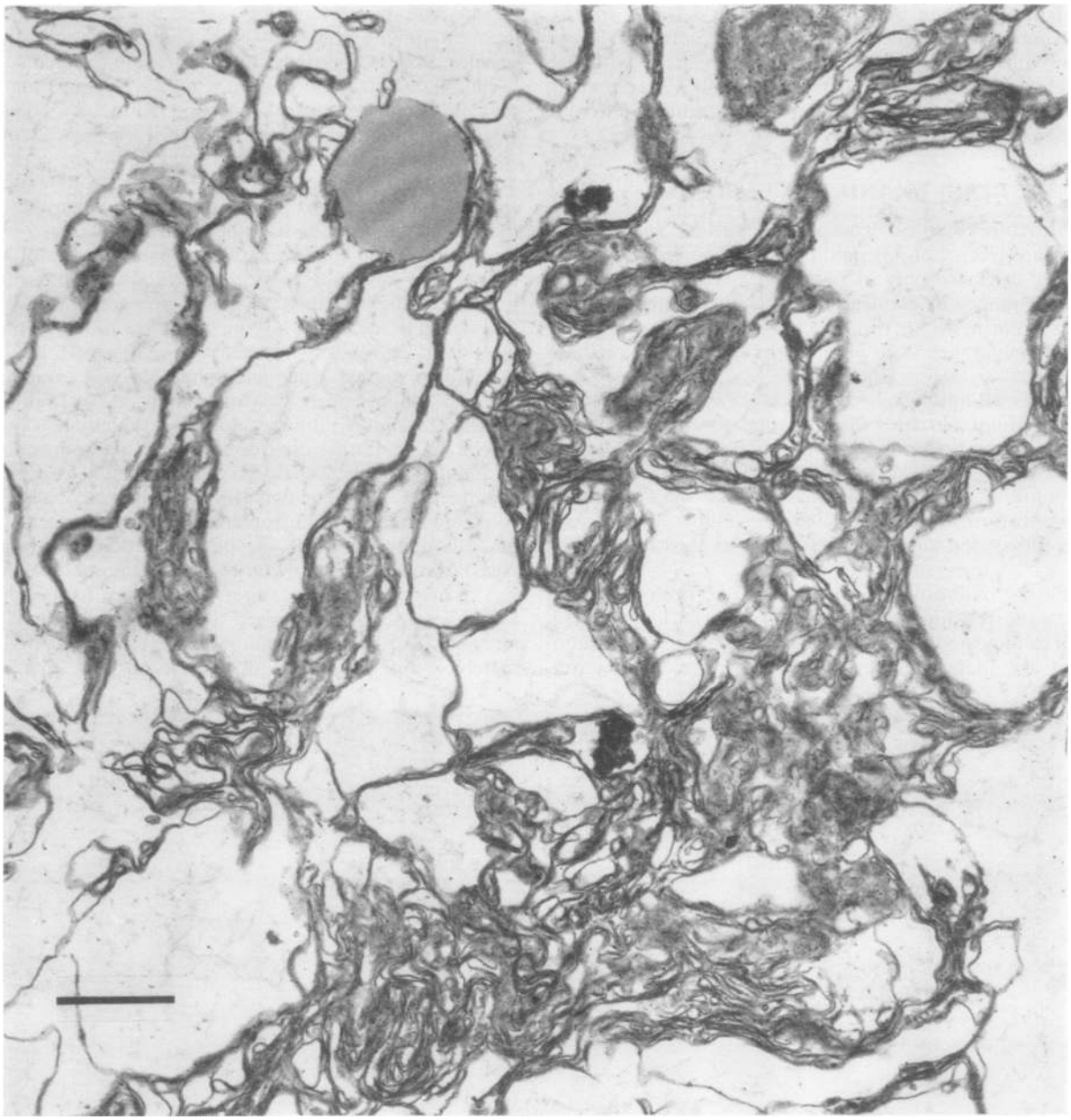

FIG. 3. Electron micrograph of "defatted spherosomal preparation." Note the paucity of oil droplets; instead, numerous membranous profiles manifest themselves. Bar represents 1 μ.

about 2 to 3 nm in thickness. The new profiles were definitely trilaminar (Fig. 4), as opposed to the single-line character of spherosomal "membranes" that abut on their external surfaces (Fig. 2).

These results are interpreted to mean that spherosomes are bounded by half unit-membranes whose polar surfaces address the hyaloplasm and whose fatty nonpolar surfaces contact the internally stored lipids. Membranes converging from the polar side, as in the case of abutting membranes of juxtaposed spherosomes (Fig. 2), present two polar surfaces to each other, forming a single line. A similar situation occurs in the myelin sheath of nerve cells, where appositions of unit-membranes of Schwann cells also form single lines (19). Apposition of fatty nonpolar surfaces, a situation that occurs when the oil is extracted from the spherosomal interior, produces bimolecular leaflets of lipid with a trilaminar structure.

Bowen and Jensen (3) have observed a similar phenomenon in gas vacuoles of blue-green algae. Gas vacuoles filled with gas were bounded by single-line membranes which measured about 2 nm in width. When devoid of gas, the algal cells contained numerous tripartite membranous structures in the matrix of the ground plasm. The authors suggested that the membranes of gas vacuoles were half unit-membranes and that the tripartite structures observed in the cytoplasm of gas-free algae were collapsed gas vacuoles. This interpretation is also applicable to our observations. Unlike gas vacuoles, however, spherosomes are filled with osmiophilic lipid; hence, a clear line of demarcation between membrane and oil is difficult to ascertain after osmium fixation. However, ghosts, which contain no osmiophilic oil, show single-line membranes similar to those observed in gas vacuoles. Defatting spherosomes prior to immobilization in agar can be thought of as analogous to the degassing of gas vacuoles, since the appearance of tripartite structures is obtained. These observations, which are best ex-

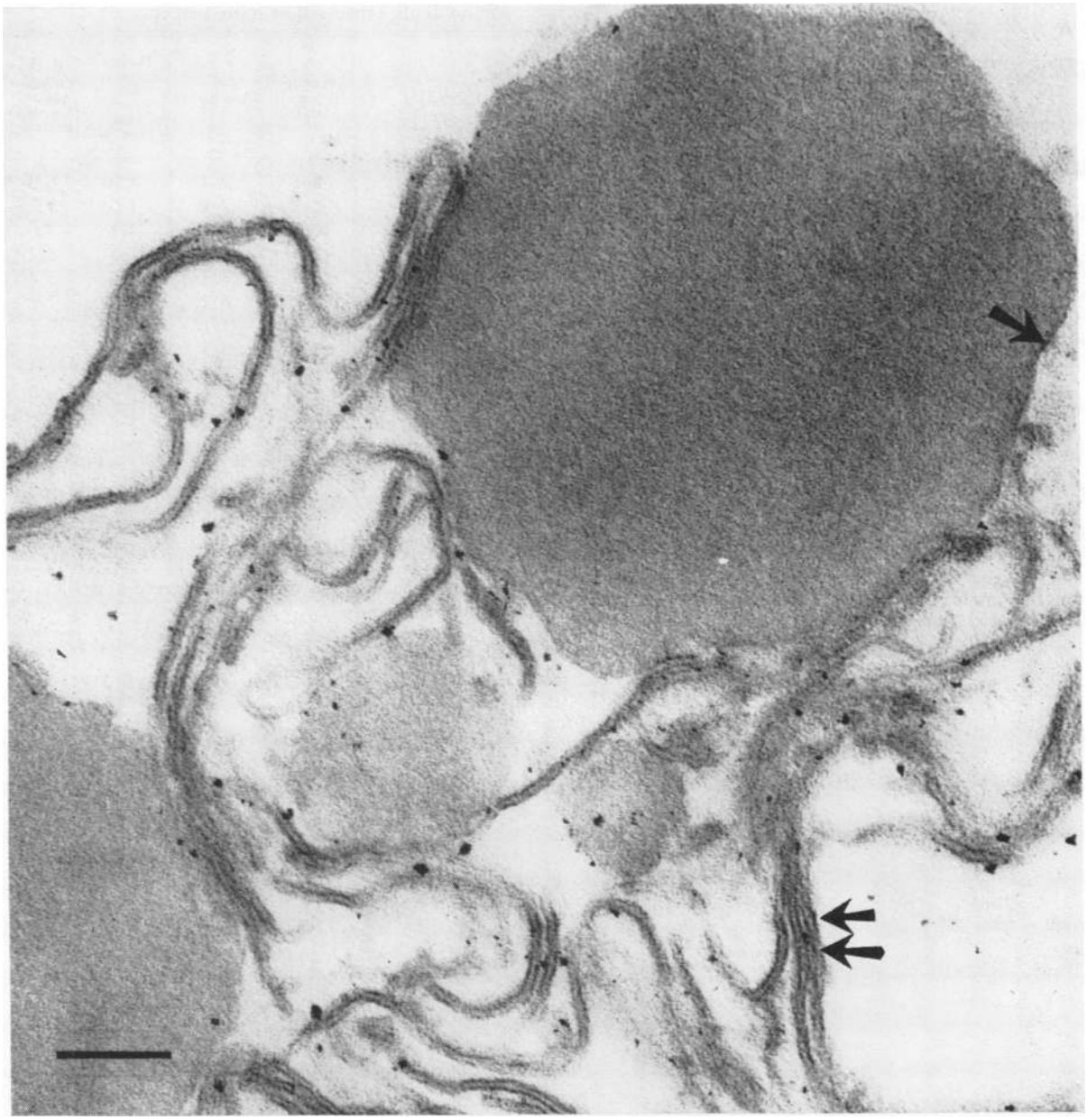

FIG. 4. Higher magnification of defatted spherosomal preparation showing the tripartite character of the membranous elements shown in Figure 3. Tripartite membranes were never observed in spherosome or ghost preparations, even when spherosomes abutted. Note also the single-line membrane (at arrow) which is barely distinguishable due to the osmiophilia of the spherosome content. Bar represents 0.1 μ.

plained by the existence of half unit-membranes that bound spherosomes, are depicted schematically in Figure 5.

Comparison of the structure of a unit-membrane to that of a half unit-membrane shows the suitability of each membrane for its respective job. A unit-membrane is comprised of a nonpolar diffusion barrier sandwiched between two polar portions that are in contact with, and "wetted" by, the polar milieu on either side of the membrane. However, the half unit-membrane presents a barrier whose two sides differ in polarity. The external, polar surface (protein) of the membrane contacts the aqueous ground plasm of the cell and the internal, nonpolar surface (lipid) contacts storage oil; thus, a barrier exists with dissimilar sides, each of which is compatible with its respective environment.

Structural Protein. If spherosomal membranes are half unit-membranes, they should contain proteins that are characteristic of unit-membranes. One protein that is characteristic of biological membranes is structural protein. Structural protein is biologically inert and apparently functions merely as physical support. It is insoluble at neutral pH and composed of amino acids with a high proportion of nonpolar side chains (5, 8). We wondered whether spherosomal membranes contained structural protein.

Zahler *et al.* (26) showed that structural protein is extracted from mitochondria by 1.4% acetic acid. Since earlier methods for the isolation of structural protein involved the use of detergents, a method which avoided the possibility of forming emulsions in our study of oil-rich spherosomes appealed to us. Extraction of spherosomes with 1.4% acetic acid produced a clear solution containing approximately 250 μg of protein per ml. Neutralization of this acidic extract caused the formation of a white flocculum as the pH approached neutrality. Struc-

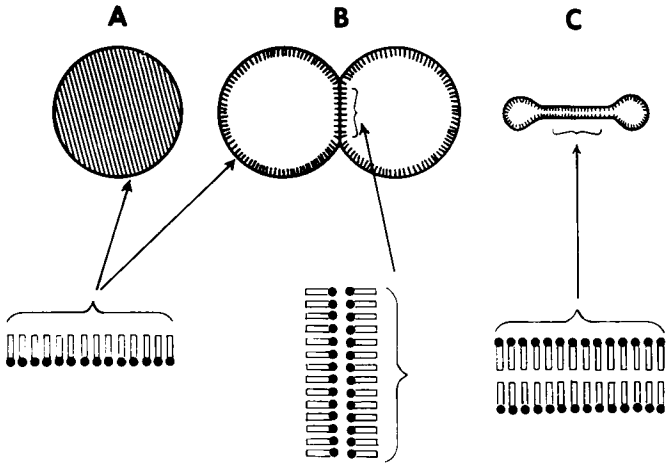

FIG. 5. Schematic presentation of results. A: Intact spherosome filled with oil. Since both the oil and membranes are osmiophilic, a distinct delimiting membrane is difficult to discern after osmium fixation. B: Juxtaposed spherosome ghosts. Membranes apposed from the external or polar (represented by black balls) sides form a thicker single line. C: Defatted spherosome. Membranes contacting from the internal, nonpolar, or lipoidal (represented by white sticks) side form a bimolecular layer of lipid.

Table I. *Amino Acid Composition of Peanut Fractions and Structural Proteins Obtained from Various Sources*

Amino acids expressed as mole percent.

Amino Acid	Peanut protein (total)[1]	Arachin[2]	Peanut Spherosome Structural Protein	Bean Chloroplast[3] Structural Protein	*Neurospora*[4] Mitochondrial Structural Protein
Lysine	3.1	2.4	4.0	6.3	6.5
Histidine	2.3	2.1	1.6	1.7	2.4
Arginine	10.0	8.6	4.1	4.0	5.5
Aspartate	12.4	14.4	7.5	8.2	9.2
Threonine	2.4	3.3	8.6	5.8	5.5
Serine	6.5	6.2	8.6	7.2	6.0
Glutamate	19.6	21.1	8.3	10.2	10.3
Proline	3.9	3.3	4.1	5.4	4.3
Glycine	11.2	7.3	14.1	11.7	8.9
Alanine	5.9	6.6	11.0	9.9	10.6
Valine	5.4	5.4	5.6	6.2	7.3
Methionine	0.9	Trace	0.4	1.1	2.1
Isoleucine	3.8	4.0	4.3	4.8	5.5
Leucine	5.9	7.4	9.2	10.2	8.9
Tyrosine	3.0	2.8	4.6	2.4	2.9
Phenylalanine	3.6	5.1	4.0	5.0	4.0

[1] Unpublished results of N. J. Neucere and E. J. Conkerton from this laboratory.

[2] Calculated from Neucere (16).

[3] Taken from Mani and Zalik (13).

[4] Calculated from Woodward and Munkres (23).

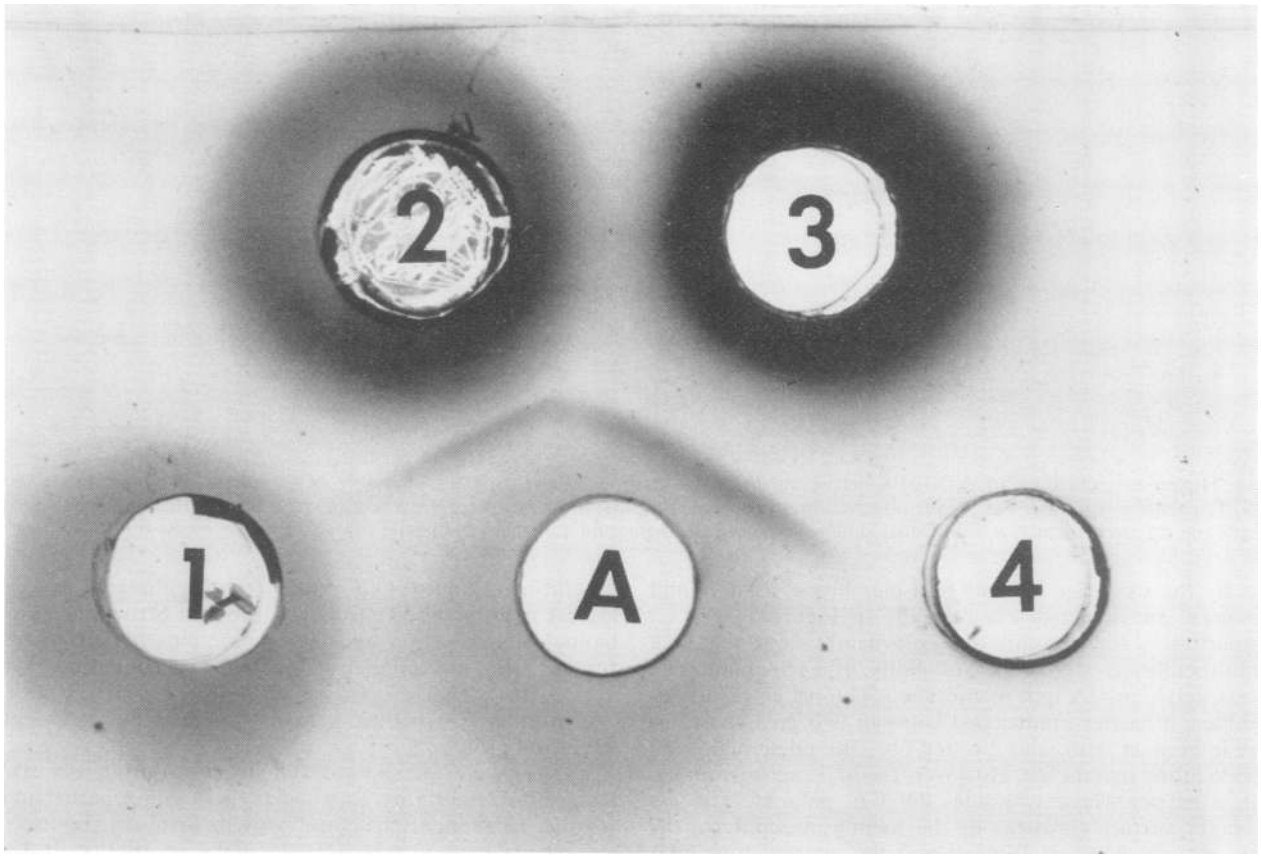

FIG. 6. Double diffusion test according to Ouchterlony. The center well (A) contains antibody to quiescent peanut seed spherosomal membranes. Wells 1 through 4 contain (1) mitochondrial protein homogenized in tris buffer, (2) mitochondrial protein homogenized in tris buffer plus detergent, (3) spherosomal protein homogenized in tris buffer plus detergent, and (4) spherosomal protein homogenized in tris buffer. Precipitin lines have formed between well A *versus* both well 2 and well 3, which coalesce, indicating an identity of proteins. The fact that the proteins were not solubilized in the absence of detergent indicates that they are membrane proteins.

tural protein taken out of solution, whether by neutralization or by lyophilization, did not redissolve in dilute acetic acid or other aqueous solutions unless detergents were present (*cf.* 4, 18).

Samples of the lyophilized spherosomal extract were hydrolyzed for 6 and 20 hr, after which they were quantitatively analyzed for amino acids. The results of the amino acid assays of the two samples were virtually identical, indicating that the molecular size was small and somewhat homogeneous. In Table I the amino acid compositions are given for peanut spherosomal structural protein, arachin (principal storage protein of peanuts), total soluble protein of peanuts, and structural protein obtained from other sources. The amino acids of total soluble protein and arachin are composed largely of acidic amino acids and arginine, which are characteristically high in oilsecd proteins. On the other hand, structural protein extracted from peanut spherosomes differs from typical oilseed proteins but is very similar in amino acid composition to structural protein extracted from other sources, *e.g., Neurospora* mitochondria and bean leaf chloroplasts (Table I).

Since the protein associated with spherosomes was derived from a membrane (albeit morphologically atypical), was obtained by a procedure devised for the extraction of structural protein from membranes, had similar solubility characteristics as membrane structural protein, and possessed an amino acid composition similar to other structural proteins, the results indicate that peanut spherosomal membranes do contain membrane structural protein.

Immunological Studies. Spherosomal protein is extremely difficult to solubilize in aqueous solutions. Dried spherosomal protein does not go into solution or even into suspension when homogenized in ordinary aqueous systems; rather, it requires the presence of a protein dispersal agent such as urea or detergents to become solubilized. To circumvent this problem, we used the medium devised by Singh and Wasserman (21) for disaggregating membrane proteins. The extreme insolubility of spherosomal protein in aqueous systems is undoubtedly a reflection of its membranous nature.

Figure 6 shows a double diffusion test according to the method of Ouchterlony (17) in which precipitin lines can be seen between antibody to spherosomal membranes and antigens of both spherosomal membranes and mitochondrial fraction membranes. Note the continuity in the precipitin lines, an indication of immunological identity. The demonstration of this identity between proteins of single-line membranes delimiting spherosomes and of unit-membranes bounding organelles of the mitochondrial fraction is interpreted to mean that, although morphologically the membranes are different, antigenically they are the same.

Thus, we conclude that the morphologically atypical single-line membrane which bounds spherosomes is in fact a biological membrane and not a nonspecific adsorption layer. It is also possible to conclude that the single dark-line bounding spherosomes is the basic unit of biological membranes; however, since the single-line membrane is the exception rather than the rule and since the tripartite structure is so widespread, it seems more reasonable to think of the single-line membrane as a half unit-membrane.

The conclusion that single-line membranes bounding spherosomes are in fact half unit-membranes raises several interesting possibilities. Since we demonstrated that the defatting of free spherosomes gives rise to tripartite structures, conversely, could not newly synthesized oil be secreted into the anhydrous, lipoidal area between the leaflets of unit-membranes to give rise to spherosomes? Also, the strong morphological resemblance of plant spherosomes to certain lipid droplets of animals, *e.g.*, adipose fat droplets and chylomicrons (2, 20), suggests that our conclusions concerning half unit-membranes bounding spherosomes could be applicable to lipid-droplet boundaries throughout the biological world.

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