MEMBRANE-TO-MEMBRANE CROSS-BRIDGES

A Means to Orientation and Interaction of Membrane Faces

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INTRODUCTION

Electron microscopy has shown various examples of membrane faces which occur in parallel, being separated by a distance of 70–350 A. This distance is constant for a defined cellular situation, although it varies from one type of association to another. Prominent instances of this include the parallel associations (stacks) of cisternae, such as the Golgi apparatus and the so-called "impaired" endoplasmic reticulum (ER) cisternae (for literature see 20, 24), as well as cases in which a cisternal (or vesicular) membrane parallels a single membrane; a particularly clear example of this being the relationship of the plasma membrane with the "subsurface cisternae" of various cells (e.g., 4, 32, 48) and with the alveolar sacs of the pellicle of many ciliates (e.g., 27, 40, 45). In a detailed electron microscope screening of such parallel membrane-membrane associations in diverse cell types, intermembranous cross-bridges were consistently observed. This suggests that the cross-bridges function as "rungs" for the stabilization of such characteristic membrane arrangements.

MATERIAL AND METHODS

Materials used in the present study were: (*a*), cultures of the ciliate *Tetrahymena pyriformis*, amicronucleate strain GL, grown under exponential conditions as indicated elsewhere (19); (*b*), secondary nuclei-containing caps of cyst-producing *Acetabularia mediterranea* cells (culture conditions as described in reference 23; (*c*), cotyledons of mustard seedlings, *Sinapis alba L.* grown in the dark for 36 hr after germination on moist filter paper, then transferred to a far-red illumination (compare 38), followed by an approximate 15 min daylight period; (*d*), testes from adult rats and Alpine newts (*Triturus alpestris Laur.*, collected from Black Forest ponds); and (*e*), Morris hepatoma, implanted in the leg musculature of Buffalo rats. The fixatives employed were glutaraldehyde (1 or 2%, 0.05 M cacodylate buffered to neutrality) and osmium tetroxide (1 or 2%, same buffer) either in sequential or in simultaneous use according to the scheme described earlier (18). Specimens were postosmicated with 2% OsO_4 for 2–12 hr in the cold (approximately 4°C). In some sequential fixations the temperature of the initial aldehyde solutions was varied in the range of 5°–10° from 4°C up to 40°C. Dehydration was performed through a series of graded ethanol solutions, and specimens were embedded in Epon 812. In some parallel experiments the material was soaked overnight with aqueous 1% uranyl acetate before the dehydration procedure. Sections were made with the Reichert ultramicrotome OmU2 (Fa. Reichert, Vienna, Austria) and were double stained with aqueous 4% uranyl acetate followed by lead citrate according to Reynolds (41), using a dilution of 1:10 of the original concentration given by Reynolds. Micrographs were taken with Siemens Elmiskopes 1A and 101.

RESULTS

In all of the systems of parallel membranes which were studied, membrane-to-membrane connections could be found. "Connection" is used here in the sense that electron contrast was continuous from one membrane face to the adjacent one, with no electron-transparent interruption being recognizable in between them larger than the grain resolution of the section (approximately 15 A) The outer face of the outer alveolar membrane of the *Tetrahymena* cortex is conspicuously parallel to the plasma membrane, with a separation distance of 150–350 A (Fig. 1). Characteristically, the inter-

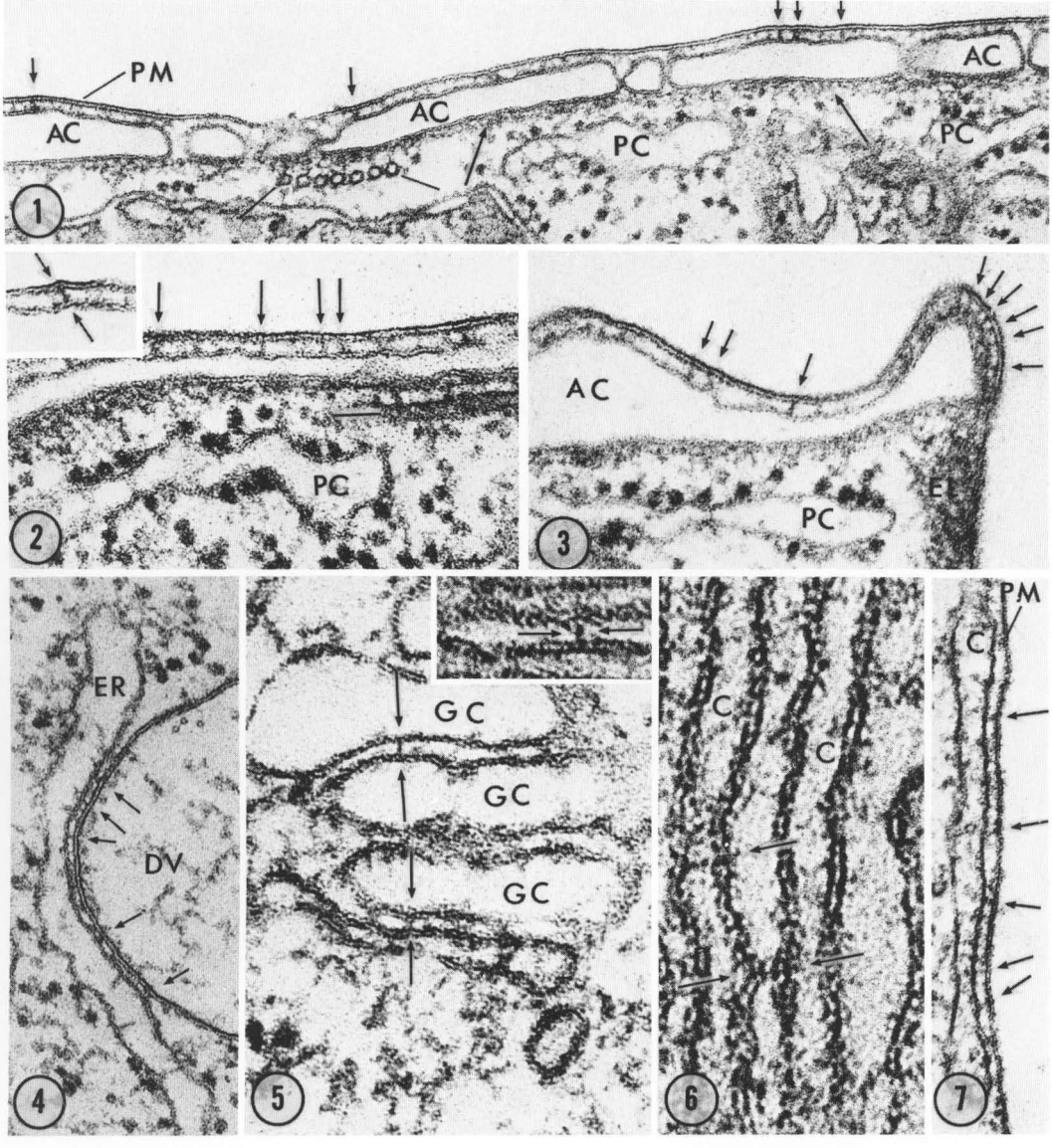
membranous space is spanned by 40–100 A thick electron-opaque bridges which apparently link the two adjacent membrane faces (Figs. 1–3), i.e. the outer face of the outer alveolar membrane with the inner face of the plasma membrane. Such intermembranous links in the ciliate pellicle are observable after different types of fixations, including those using OsO_4 alone. They were first described and discussed by Tokuyasu and Scherbaum (46) as "bridges which may be responsible for keeping constant the distance between the two pellicular membranes." Occasionally, these intermembranous cross-bridges appear regularly spaced with a minimal lateral period of approximately 110 A (Fig. 3): they seem to be slightly more frequent at positions at which the plasma membrane describes curvatures, for instance at the discharge channel of the contractile vacuole (Fig. 3; for nomenclature, see reference 29). The angle described by the membrane faces and the linker pieces apparently is not always strictly perpendicular (e.g., Figs. 2 and 3). A granular substructure of the bridges is sometimes suggested, although it is not as clearly resolved, as this is more frequently the case with the cross-bridges between the inner alveolar membrane and its associated microtubules of the "longitudinal bundles" (16; see also Fig. 2; nomenclature according to reference 1). In the cytoplasm of *Tetrahymena*, single or stacked cisternae of rough ER can often be recognized to closely parallel the membranes surrounding food or digestive vacuoles (Fig. 4; cf. 19). The region of such cisternae, which is immediately adjacent to the vacuolar membrane, is distinguished by two features: it is free from ribosomes and it is connected with the vacuolar membrane by cross-bridges about 40–80 A broad and 50–100 A long.

Smooth cisternae organized into stacks are known for numerous types of cells, the most prominent example being the dictyosomal stacks of the Golgi apparatus. Close inspection of such situations has led to the demonstration of intermembranous cross-bridges between the cytoplasmic faces of adjacent cisternae. This was observed with plants (e.g., onion and cress root tips) as well as with animal material. Figs. 5–7 provide an example of the presence of such cisternal interconnections in rat spermatogenetic cells. The only other regular elements so far known to occur in the intercisternal zone of dictyosomes are the intercisternal elements which have been found in various plant cells (7, 37, 47). These, however, are different in that they have not been shown in contact with the membrane and seem to exhibit a filamentous association parallel to the membrane surface rather than being perpendicular to it. The intercisternal cross-links can be found in dictyosomal stacks (Fig. 5) as well as in apparently nonsecretory smooth cisternal aggregates (Fig. 6). Here, again, a subarchitecture is frequently suggested in the form of linear arrays of large granules about 40–60 A. A particularly interesting case is denoted by the lower pairs of arrows in Fig. 6: here, two adjacent membranes are in local contact mediated by closely aggregated dense "granules" which themselves appear to be a morphologically constitutive part of the membrane. In addition, it should be remarked that cross-links between plasma membrane and ER "subsurface cisternae" can be observed in the very same spermatogenetic cells as well (Fig. 7).

In various cell types, especially in those which undergo degenerative processes, special associations of ER can be observed, the so-called "paired cisternae." In these associations smooth membranes, frequently identifiable as being continuous with the rough ER system or the nuclear envelope, are parallel, with a separation distance of 150–300 A, and can be integrated into large cisternal stacks.

This basic phenomenon is observed in such diverse cell types as differentiating sieve elements (11, 50), in various tumorous and normal cells (10, 13, 24, 26, 33) and also in ciliates (20). With the Morris hepatoma cells of our study, the smooth faces of such stacks (Fig. 8) are bridged by electron-opaque, stalklike, or thin filamentous material (Fig. 9). The width of the intermembranous connective elements varies from 40 to 100 A (Figs. 8 and 9). In the same cells such membrane-to-membrane cross-links are also observed between the plasma membrane and certain peripheral vesicles (Fig. 11). Such electron-opaque connections between smooth ER cisternae and the plasma membrane have recently been noted by Satir and Gilula (44) for the septate junction areas of mussel gill epithelia. In a few cases, in which two such membranes were particularly closed, single dense granules could be resolved within the membranous space, sometimes embedded in an amorphous, electron-opaque "glue" material (Fig. 10).

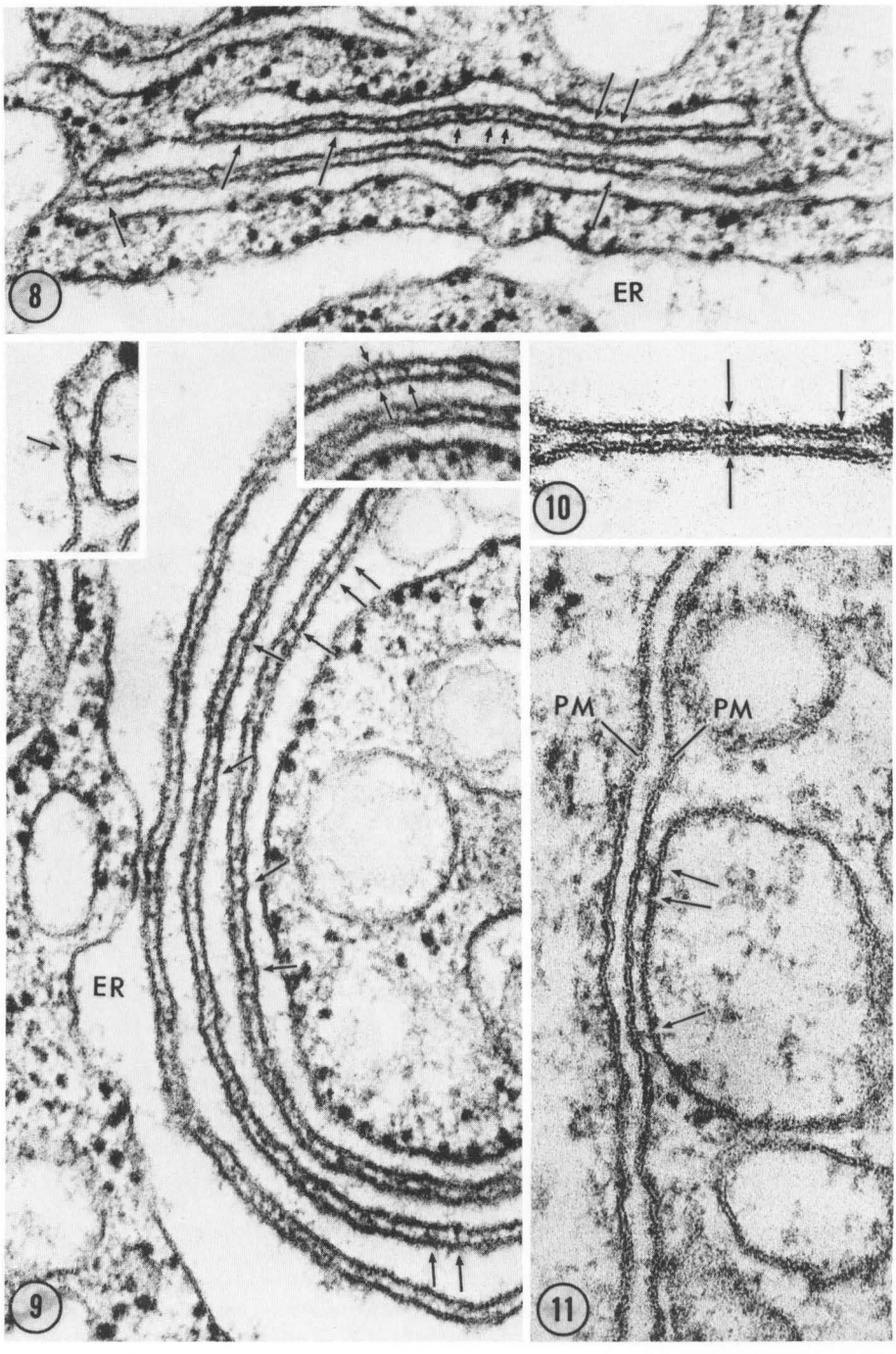
An especially tight linkage between two different membrane types apparently takes place during the plasmotomic phase of cyst formation in the



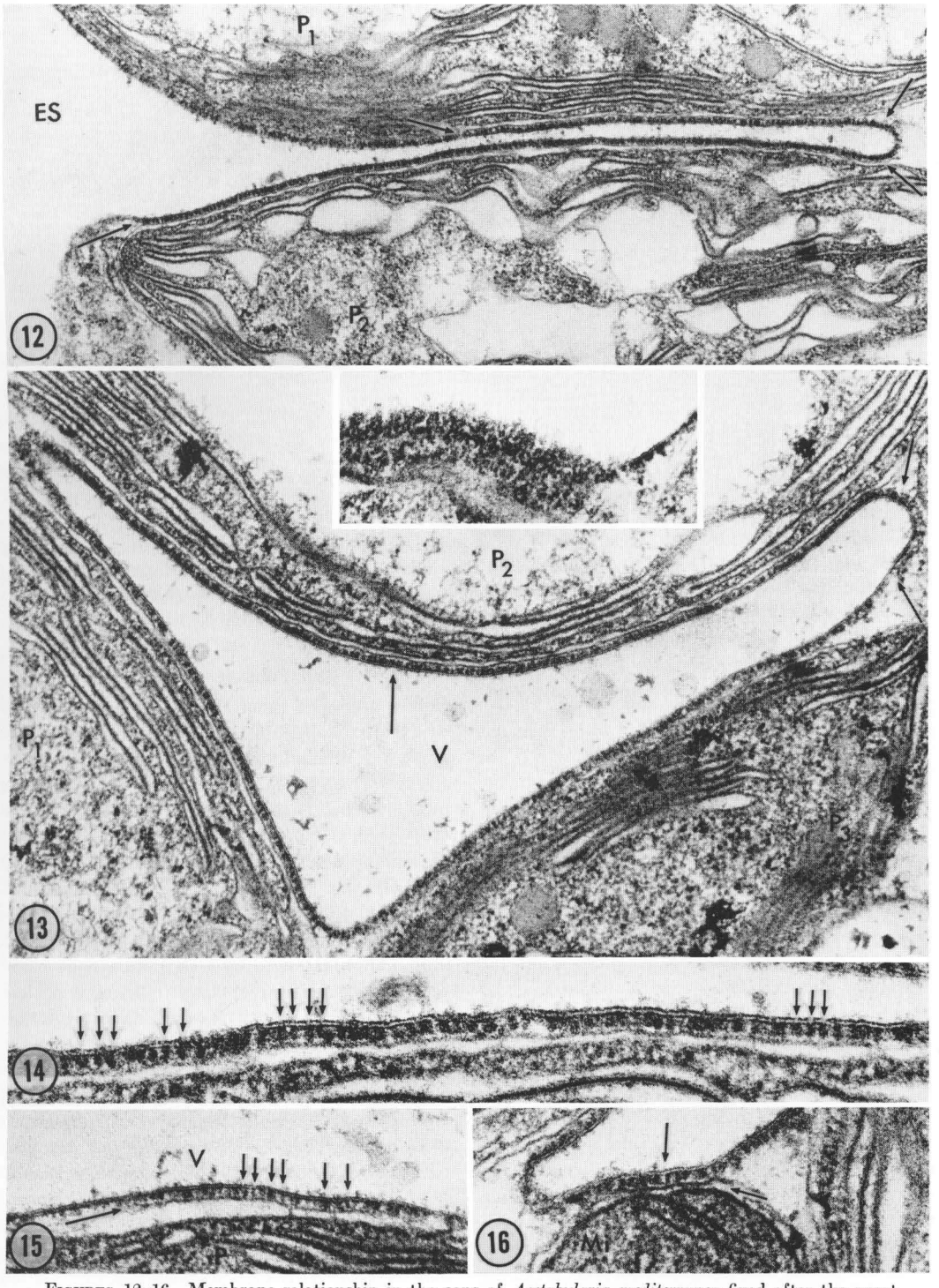
FIGURES 1–3 Structural details of the pellicular region of *Tetrahymena pyriformis*. The plasma membrane (*PM*) is underlaid by the alveolar cisterna (*AC*). The outer membrane of the alveolus shows a conspicuous parallelism with the plasma membrane and is connected to it by dense cross-bridges (e.g., at the small arrows in the upper part of the figures). The inner alveolar face is associated with the filamentous web, the "epiplasmic layer" (*EL*; denoted in Figs. 1 and 2 by the longer arrows). This epiplasmic material can be connected to the bundles of cortical microtubules (denoted by the bars). The whole pellicle is underlaid by the peripheral cisterna of rough endoplasmic reticulum (*PC*) which also maintains a certain parallelism with the alveolar cisterna. Note the somewhat regular spacing of the alveolar membrane–plasma membrane cross-bridges in Fig. 3. In Fig. 2 (at the right) one recognizes cross-bridge–like microtubule–epiplasmic web connections. Fig. 1, × 77,000; Fig. 2, × 112,000; Fig. 2 inset, × 125,000; Fig. 3, × 120,000.

FIGURE 4 Close and parallel association of rough endoplasmic reticulum (*ER*) cisterna with the membrane of a digestive vacuole (*DV*) of *Tetrahymena*. Ribosome-free areas of such ER-cisternae appear linked to the vacuolar membrane with dense cross-bridges (arrows). × 100,000.

FIGURES 5–7 Intermembranous elements in membrane-membrane associations in the spermiogenetic cells of rat testis. Fig. 5 demonstrates intercisternal cross-bridges in a dictyosomal stack (arrows). A granular substructure of such cross-bridges is sometimes suggested (e.g., at the upper pair of arrows in Fig. 5 and in the inset). Fig. 6 shows part of a stack of cisternae (*C*) in which intercisternal linkage (lower pair of arrows) is recognized besides intracisternal cross-bridges (upper arrow). Fig. 7 shows the existence of intermembranous bridges (indicated by the arrows) between a smooth ER-cisterna (*C*) and the plasma membrane (*PM*) in such a spermiogenetic cell. Fig. 5, × 150,000; Fig. 5 inset, × 260,000; Fig. 6, × 300,000; Fig. 7, × 90,000.



FIGURES 8–11 Parallel associations of membrane faces in Morris hepatoma cells. Intercisternal bridges are recognized within the stacks of paired *ER* (Fig. 8) which are a characteristic feature of such cells. Such intercisternal elements can appear either as a linear array of "dots" in the intercisternal space (e.g., at the arrowheads in the central part of Fig. 8) or as typical membrane-to-membrane cross-bridges (arrows of Figs. 8 and 9). Note that the width of such bridges is somewhat variable (compare, e.g., the two insets of Fig. 9). Fig. 10 shows two closely adjacent but separate membranes of Golgi apparatus–derived vesicles: here the plasmatic interspace is filled with the relatively indistinct material which sometimes suggests a bridging (arrows) or amorphous "gluelike" interaction between the two membranes. Fig. 11 shows the frequently seen association of special vesicles with the plasma membrane (*PM*) and the intermembranous cleft as being bridged by dense elements (arrows). Fig. 8, × 90,000; Fig. 9, × 100,000; Fig. 9 left inset, × 165,000; Fig. 9 right inset, × 110,000; Fig. 10, × 250,000; Fig. 11, × 155,000.



FIGURES 12–16 Membrane relationship in the caps of *Acetabularia mediterranea* fixed after the onset of cyst formation. Such cellular stages show characteristic membrane profiles which are set with dense particles on their cytoplasmic surface (Figs. 12–16, arrows). These particle-set membranes can be seen as surrounding cisternal, vesicular, or vacuolar spaces (*V*) and also as being continuous with the plasma membrane (Fig. 12; *ES*, extracellular space). Frequently, these membranes are closely parallel to the outer membrane of the chloroplasts (Figs. 12–15; $P_1 - P_n$, plastids) or mitochondria (*Mi*, Fig. 16), and the dense stalks seem intimately to connect both types of membranes. In cross-section, the elements bridging the intermembranous space reveal a regular spacing (e.g., Fig. 14) whereas no obvious regularity has been recognized in sections grazing such membranes (inset of Fig. 13). Frequently, these intermembranous "cross-bridges" are relatively thick (approximately 150 A), whereas in some cases they appear as thin as 50–90 A (e.g., Fig. 15). Fig. 12, × 90,000; Fig. 13, × 60,000; inset of Fig. 13, × 70,000; Fig. 14, × 150,000; Fig. 15, × 90,000; Fig. 16, × 95,000.

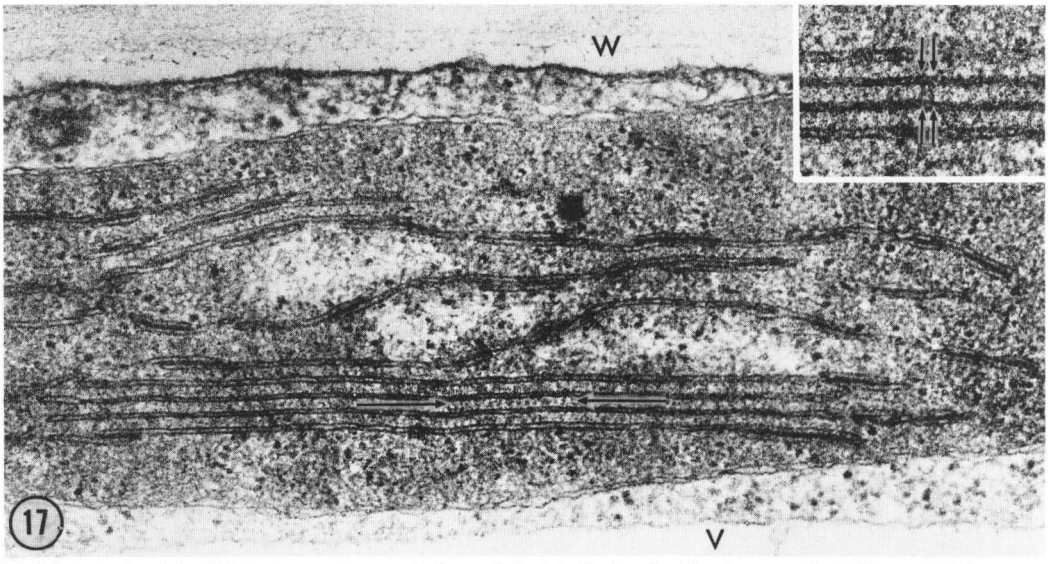


FIGURE 17 Parallel but separate associations of thylakoids in plastids of mustard seedling cotyledons. Within the interthylakoidal space electron-opaque elements are recognized (arrows) which sometimes can appear as bridges connecting the adjacent membrane faces (inset). *W*, cell wall; *V*, vacuole. × 70,000; inset, × 110,000.

caps of the green alga *Acetabularia* (Figs. 12–16). In this developmental stage cisternal or vesicular membrane profiles can be observed which, at many sites, appear to be continuous with the plasma membrane (Fig. 12). Such observed continuities are consistent with an origin of plasma membrane from the endomembrane system of the cell. These membranes are frequently closely parallel to the outer membrane of the plastids (Figs. 13–15) and mitochondria (Fig. 16), with a distance of approximately 200 A. Here, again, the plasmatic intermembrane space is bridged by dense material which most often is present in the form of relatively thick rods (approximately 150 A; Fig. 14), but sometimes can also appear with the dimensions of "normal" cross-bridges (50–90 A; Fig. 15). From the observation that this type of membrane linkage is observed exclusively after the onset of cyst formation, it may be hypothesized that it provides a means of an ordered distribution of cellular organelles during plasmotomia. Although we have made a special point to look for it, no distinct regular pattern of such cross-linking elements could be recognized in tangential views of these special membranes (Fig. 13).

The thylakoidal membranes are a system characterized by an especially pronounced tendency of membrane-membrane interaction. This includes closely appressed associations, as well as others, which are parallel but separated by a stroma interspace with dimensions ranging from approximately 100 A (12) up to 2000 A, the latter being the case in the pyrenoidal regions of the green alga *Carteria* (28). In a detailed examination of such nonappressed parallel thylakoidal associations (compare, e.g., also the findings with certain mutants of maize, [2] and *Chlamydomonas*, [21]) in the cotyledons of mustard seedlings grown under far-red illumination, followed by a 15 min daylight exposure, dense material appears in a more or less regular arrangement of particles, in between the thylakoids, which in this case are anomalously stacked (Fig. 17). Here such intermembrane material is distributed somewhat like that of the intercisternal elements of plant Golgi cisternae. Occasionally, however, these interthylakoidal elements reveal a typical cross-bridge appearance (Fig. 17, inset). Similar filamentous cross-bridges, although spanning a much longer distance, have also been shown in pyrenoidal thylakoids of *Carteria* (28). This demonstrates that the "separated" parallel thylakoids nevertheless can have morphological contact through such interthylakoidal cross-bridges.

Membrane-to-membrane cross-bridge–like continuities, however, are not definitely limited

to the intercisternal space, but are also found within the intracisternal phase (e.g., Fig. 6). The observation that such are less numerous and never have been found in an ordered array somewhat obscures their significance. However, the existence of intracisternal membrane-to-membrane cross-bridge connections could serve as a clue in answering the interesting question: "What keeps such cisternae flattened?"

DISCUSSION

Although the possibility that the cross-bridges observed are fixation artifacts, due to the coarse precipitation of proteinaceous "ground-substance," cannot be fully excluded, the regular spacing which they often display (e.g., with the *Tetrahymena* cortex) speaks against this. It is a well established concept that microtubules can be linked to each other and packaged into aggregate bundles by distinct 40–60 A broad lateral cross-bridges ("side-arms"), which are sometimes spaced along the tubular axis with a regular periodicity (e.g., 3, 22, 35, 42). It is widely hypothesized that it is this linkage which is responsible for the structural stability and the specific orientation of microtubular bundles. Additionally, such side-arms have also been proposed to take part in translocations of microtubules relative to each other in a sliding filament-like mechanism (36, 43). Recently, it has become evident that a similar cross-bridge–mediated type of linkage is also a means of microtubule-biomembrane associations: such has been demonstrated for plasma membranes (6, 9, 31), for the ER (15, 39), for both membranes of the nuclear envelope (e.g., 14, 17, 30, 49), and for the membranes of special vesicles and vacuoles (e.g., 16, 25, 45).

The observations described in the present study suggest that similar cross-bridging is also the principle by which membranes can be linked to membranes, whether they are of the same type or different, and that this is the principle of organization by which two adjacent membrane faces are kept so conspicuously parallel. As is indicated by the presence of the intracisternal bridges, such a principle is not necessarily restricted to the parallelism of adjacent cisternae but might also be extended to the parallelism of the two membranes of the single cisterna itself (see also the example of reference 5). The "tektin-hypothesis" of Mazia and Ruby (34) proposes a similarity of certain "actin-like" proteins (including the microtubules) and of membrane proteins. In this connection it is interesting to know that cross-bridge linkages can be noted in the supercomplexing of the following combinations of partners: microtubules-microtubules, microtubules-biomembranes, biomembranes-biomembranes. Although it is unknown whether the cross-bridges between microtubules are identical in chemical nature and function with those connecting the membranous faces, the present authors tend to visualize them as belonging to one class of structural elements which allow the formation of larger morphological aggregates by "through-crystallization," i.e. such cross-bridges themselves participate as constituents in a complex tubule ↔ filaments ↔ membrane meshwork. In this view, e.g., the association of Golgi cisternae or the various forms of closely stacked smooth ER might be the result of possibly exothermic self-assembly of membrane faces.

The associations of membrane faces with membrane faces can morphologically be divided into (*a*) those which are closely appressed with no interspace left between them (such as is known, e.g., for many thylakoidal and some Golgi membrane systems); and (*b*) those which are close and parallel but keep a defined distance from each other. Both types of close associations are found, e.g., with plastidal thylakoidal membranes, sometimes even in closely related plant cells like the xanthophycean algae *Botrydium* and *Tribonema* (e.g., 12). In addition, both types of morphological association can be envisaged as membrane-membrane "quasi-crystalline" aggregates, the only difference being that in the case of (*b*) the aggregation is mediated by the material of the cross-bridges.

It is not clear from which material the membrane-to-membrane cross-bridges derive: from a pool of subunits, not structured at the electron microscope level, from membranous material, or from constituents of the membrane-attached ribosomes of the rough ER. The latter origin is suggested by the appearance of the "paired ER-cisternae" (see also 33) and by the situation of the *Tetrahymena* alveoli and digestive vacuoles. Further observations are necessary to solidify the hypothetical concept of membrane-membrane cross-bridges. It is also worth examining the relationship of these intracellular membrane-to-membrane cross-bridges to the extracellular membrane linker pieces such as those of the septate desmosomes and the intersynaptic cleft (8).

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