LATERAL FUSION OF MEMBRANES IN BACTEROID-CONTAINING CELLS OF LEGUMINOUS ROOT NODULES

B. E. S. GUNNING
Department of Botany, Queen's University of Belfast, N. Ireland

SUMMARY
Bacteroid-containing cells of leguminous root nodules (Pisum and Trifolium) provide an environment in which the membrane envelope surrounding each bacteroid can exhibit unusual properties. The bacteroids are crowded, and frequently neighbouring membrane envelopes come into close contact. In such cases the two envelopes can fuse laterally to give a symmetrical tripartite membrane, probably consisting of a composite inner layer sandwiched between the outer leaflets of the two parent membranes. The expanse of fused membrane forks at its periphery, i.e. is confluent with both of the envelopes from which it is derived, and seems to be relatively stable.

INTRODUCTION
The behaviour of tripartite membranes during the process of lateral fusion that precedes liberation of the contents of a cytoplasmic vesicle to the exterior of a cell has been examined in detail recently by Palade & Bruns (1968). The plasma membrane and the membrane of the vesicle first become appressed so that their respective cytoplasmic leaflets merge back-to-back, giving a 5-layered composite double membrane. Later, the central layer is eliminated and the contents of the vesicle are then separated from the extra-cytoplasmic environment by only a single, 3-layered membrane which forks where the parent plasma membrane and vesicle membrane diverge. Eventually this remaining membrane is itself eliminated.

These transient stages are revealed when the overall process is interrupted by application of a rapidly acting fixative. It would appear, however, that in certain conditions some of the intermediate configurations are comparatively stable. Five-layered, back-to-back arrangements may be observed in suitably prepared grana of chloroplasts (Weier, Stocking & Shumway, 1967), and their occurrence in myelin is well known. The present report shows that the subsequent stage, in which the central of the 5 layers is lost, can be seen in the bacteroid-containing cells of leguminous root nodules.

MATERIALS AND METHODS
Longitudinal or transverse slices of the leg-haemoglobin containing zones of effective nodules of Pisum arvense L. and Trifolium repens L. were fixed overnight at room temperature in 2.5% glutaraldehyde in 0.025 M phosphate buffer, pH 6.9. After successive rinses in buffer the tissue
B. E. S. Gunning

was post-fixed for 3 h at 0 °C in osmium tetroxide dissolved at 2 % in the same buffer, rinsed again, dehydrated in a series of ethanol solutions, infiltrated, and embedded in Araldite-Epon (Mollenhauer, 1964). Sections cut with a diamond knife using a Cambridge Instruments Co. ultramicrotome were stained sequentially with uranyl acetate and lead citrate (Reynolds, 1963) and examined in a GEC-AEI EM6B electron microscope fitted with a cold trap, thin metal objective apertures, and the condenser-1 modification of Meek (1968).

OBSERVATIONS

The large cells in the core of a functional, nitrogen-fixing leguminous root nodule are packed with very numerous Rhizobium bacteroids. The bacteroids occupy a very substantial proportion of each cell, and the cytoplasm of the host, within which they lie, becomes largely confined to crevices between them. In sectioned material, the symbiotic partners are seen to be separated by three concentric structures (Fig. 2). These are the plasma membrane and wall of the bacteroids—both tripartite and typical of gram-negative bacteria—and a membrane contributed by the host, and known simply as the 'membrane envelope'. Some authors have considered that the membrane envelope arises de novo (Dart & Mercer, 1963a, b, 1964, 1966; Jordan & Coulter, 1965), others that it is a derivative of the endoplasmic reticulum (Jordan, Grinyer & Coulter, 1963; Mosse, 1964). In other investigations, including more recent work employing the improvements in fixation offered by the glutaraldehyde-osmium tetroxide procedure, it is interpreted as being morphologically equivalent to the plasma membrane, each Rhizobium (or group of Rhizobia) becoming enclosed by endocytosis at the time of liberation from the extracytoplasmic infection thread (Bergersen & Briggs, 1958; Dixon, 1964; Goodchild & Bergersen, 1966).

Figure 2 shows that in the Pisum and Trifolium nodules examined here the membrane envelope is clearly tripartite and about 9–10 nm in thickness. It is therefore unlikely that it is derived from the endoplasmic reticulum, in which the membrane is only 5–6 nm thick. Other features of the membrane envelope are that it is slightly asymmetric, and that granular material can often be seen (e.g. asterisks, Figs. 2 and 8) on the surface of the leaflet facing the bacteroid.

The bacteroids are so congested that there is often only a very tenuous film of host cytoplasm between neighbours. An example in which the cytoplasm is reduced to a mere 5 nm is shown in Fig. 3, and it is but a short step from this condition to complete elimination of cytoplasm over areas of actual contact between membrane envelopes. Figures 4 and 5 illustrate a zone of contact where, in places, a 5-layered, back-to-back configuration has arisen. This stage must be very short-lived, judging by the infrequency with which it can be found. Many micrographs initially thought to depict it were eventually interpreted as showing a single tripartite membrane tilted just sufficiently to superimpose one dark leaflet at the top of the section upon the opposite leaflet at the bottom of the section. This too gives rise to a central electron-dense line (e.g. Figs. 7, 8).

Junction and coalescence of two membrane envelopes, with reduction in thickness corresponding to the assumption of a composite tripartite arrangement, is shown in Figs. 6–8. The expanse of laterally fused membrane derived in this way is presumably
more or less circular, with the two parent membranes diverging at its periphery to envelop the remaining portions of their respective bacteroids.

**DISCUSSION**

Interpretation of these micrographs is beset by the customary problems of extrapolating from static images to a dynamic reality. Three possibilities exist, assuming that the images are not the result of fixation artifacts.

1. The images represent transient stages of a process of fusion, culminating in the complete removal of the membrane envelopes between two neighbouring bacteroids.

2. The images represent transient stages of a division process analogous to the development of a cleavage membrane.

3. The images represent stages in the attainment of a comparatively stable condition in which forces tending to extend the area over which the envelopes are laterally fused are balanced by counter forces.

The first possibility is feasible, for neighbouring bacteroids are bound to come into intimate contact in the crowded conditions of the infected cells. It is known that there
can be more than one bacteroid per envelope, and this could well result from fusion of originally discrete individuals. Figure 1A–F is a diagrammatic representation of stages in such a process, with B corresponding to Fig. 3, C to Fig. 4, and E to Figs. 6–8. Figure 1D shows 2 possible modes of elimination of the merged cytoplasmic leaflets, though no such stages have in fact been identified with certainty.

A cleavage, or division process as envisaged in the second possibility could in theory proceed as the reverse of fusion, i.e. could be depicted as in Fig. 1F, E,...A. However this seems unlikely, for there is no obvious reason why an extensive area of laterally fused membrane envelope need develop. The sequence F, G, H, A, is a more realistic view of a cleavage process, indeed, Dixon (1964) illustrates a stage corresponding to G and interprets it in this way. No images corresponding to G or H have been found in the present work, and it is therefore considered that, in a mature cell, division of bacteroids and their envelopes is infrequent, or may not occur at all, and that at any event it probably would not give rise to the laterally fused stage E.

The third possibility differs from the first only in that the penultimate stage (Fig. 1E) becomes stabilized. As pointed out by Palade & Bruns (1968), the frequency with which a transient stage is preserved and observed depends upon its longevity. In the present case laterally fused membrane envelopes are relatively common, whilst the subsequent stage (Fig. 1F) is very infrequent. Even when the latter is found there is an alternative interpretation: it could be a section cut near the base of the arms of a Y-shaped bacteroid, and not part of a fusion (or division) sequence. On grounds of frequency of observation it therefore seems reasonable to conclude that the third possibility is the most likely. Another argument in its favour is that complete coalescence and elimination of membrane envelopes between bacteroids would be disadvantageous, for it would reduce the surface area for exchanges between the symbiotic partners.

The forces that lead to removal of the central leaflets of the 5-layered back-to-back stage (Figs. 1C, 5) are by no means obvious. Analogy with myelin would suggest that this configuration can be stable, perhaps more so than the subsequent tripartite laterally fused condition. However, once elimination has commenced it can be conjectured that mutual attraction of the exposed hydrophobic interior portions of the parent membranes would tend to extend the fused area. Counter forces which could create an equilibrium such that the laterally fused stage is comparatively long-lived might relate to compression of the inter-bacteroid pockets of host cytoplasm, which become progressively more confined as the fused area extends, or to stresses in the surface leaflets of the membranes.

Normally, cell membranes separate unlike compartments. The fusion process described here produces a tripartite membrane that is abnormal in that it lies between two morphologically identical (peri-bacteroid) spaces. A further unusual feature of the composite membrane is its symmetry. The granular material found on the outer leaflet of a membrane envelope (Fig. 2) is present on both faces of the fused structure (Fig. 8). Unless there are unseen rearrangements at the molecular level, the fused membrane would seem to consist of two identical outer leaflets, derived from the outer leaflets of the parents. Perhaps the most striking feature, however, is that shown in
Lateral fusion of membranes

Figs. 6-8—a single tripartite membrane confluent with two membranes of similar thickness.

Palade & Bruns (1968) have described lateral fusion of vesicles of various sorts with the plasma membrane, and it may be envisaged that much the same occurs elsewhere, for example in tonoplasts of plant cells during coalescence of vacuoles. These are situations in which there is nothing to prevent completion of the overall process once it has been set in motion, and the intermediate tripartite stage is correspondingly transient and difficult to observe. It would seem that, by contrast, the bacteroid-containing cell provides an environment in which the congested conditions not only create abundant opportunities for lateral fusion, but at the same time are favourable for the formation of stable composite membranes. As a consequence, unfamiliar features of membranes such as symmetry, ability to fork, and location between like compartments become conspicuous.

Having found that membranes can behave in this way in one, admittedly atypical, cell type, it becomes pertinent to ask whether membranes in other cells might be capable of exhibiting comparable properties. Thus, while it is entirely possible that bacteroid membrane envelopes are especially prone to lateral fusion, it might also be the case that other cells simply do not provide suitable conditions, so that any potential for fusion that their membranes may possess remains latent, or else is only expressed fleetingly. The significance of this consideration lies, of course, in the implications that the process of lateral fusion holds for interpretations of the molecular architecture of cell membranes. It is much easier to envisage lateral fusion of protein-lipid-protein membranes (Branton, 1969) than that of membranes based on repeating units.

 Provision of facilities for electron microscopy by the Science Research Council is gratefully acknowledged.

REFERENCES


(Received 22 November 1969)

**ABBREVIATIONS ON PLATES**

- *b* bacteroid
- *p* bacteroid plasma membrane
- *d* dictyosome
- *tw* bacteroid wall
- *me* membrane envelope

Fig. 2. *Trifolium repens*. Part of a bacteroid (left hand side) showing its plasma membrane (*p*), wall (*tw*) and the membrane envelope (*me*) that bounds the cytoplasm of the host. Fine granular material is commonly seen on the outer leaflet of the membrane envelope (asterisks). The host cytoplasm is dense. Cisternae of rough endoplasmic reticulum with granular contents and part of a dictyosome (*d*) are seen here. Bacteroids characteristically have dispersed nucleoplasm and ribosomes; the latter are smaller than those of the host. Parts of two other bacteroids (*b*) are included. × 140,000.
Lateral fusion of membranes
Fig. 3. *Trifolium repens*. The membranes and walls between two juxtaposed bacteroids. At the arrow the gap between the two membrane envelopes is about 5 nm. Scale marker, 100 nm; × 240,000.

Fig. 4. *Pisum arvense*. The membrane envelopes of two bacteroids make lateral contact between the arrows. × 160,000.

Fig. 5. A portion enlarged from Fig. 4 to highlight the back-to-back arrangement of the envelopes. Arrows indicate the point of divergence of the envelopes, and 5 layers (3 electron-dense alternating with 2 less dense) are visible in the circled area. × 260,000.

Fig. 6. *Pisum arvense*. The membrane envelopes of adjacent bacteroids come together and fuse at the edges (arrows) of an expanse of tripartite membrane. × 160,000.
Lateral fusion of membranes
Fig. 7. *Trifolium repens.* The point of divergence (single arrow) of the parent membrane envelopes from a laterally fused composite tripartite membrane 9–10 nm in thickness. At the bottom of the picture (between the joined arrows) the membrane is tilted so that a central dense line is seen. The plasma membrane of the right-hand bacteroid is invaginated (asterisk); the invagination and nearby vesicles contain granular material (see also Dart & Mercer, 1963c). x 240,000.

Fig. 8. *Pisum arvense.* The composite tripartite membrane shown here forks at the single arrows. It, and the tripartite bacteroidal wall, are in places tilted sufficiently to generate a central dark line (between joined arrows). The granules seen on the bacteroidal face of a membrane envelope (Fig. 2) occur on both faces of the fused composite membrane (asterisks). The structure between the question marks is quite common in sections of bacteroids in *Pisum* nodules, but has not to the author’s knowledge received comment in the literature on *Rhizobium.* It consists of a parallel series of axial structures, possibly very fine cylinders, about 8 nm in diameter. Around each of these is wound a steep double helix of fine fibrils, each one making one revolution in a length of 45 nm. The fibrils approximate in thickness to that of the dispersed nucleoplasm fibrils. x 160,000.
Lateral fusion of membranes