THE MEMBRANES OF THE BASAL LABYRINTH IN KIDNEY CELLS OF THE STICKLEBACK, *GASTEROSTEUS ACULEATUS*, STUDIED IN ULTRATHIN SECTIONS AND FREEZE-ETCH REPLICAS

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SUMMARY

The structure of the basal labyrinth in kidney cells of freshwater sticklebacks was studied in ultrathin sections (after fixation with permanganate, osmium tetroxide, and combinations of glutaraldehyde with osmium tetroxide) and in freeze-etch replicas (after pretreatment with glutaraldehyde and/or glycerol, or without pretreatment). The structure of the basal labyrinth in sticklebacks, and probably in other teleost species, differs essentially from the type of labyrinth found in kidney cells of mammals like the rat. In the latter animals, the space enclosed by the membranes of the labyrinth is intercellular. In the stickleback the labyrinth consists of an intracellular system of branched membranes lining narrow saccular spaces. These spaces communicate with the exterior of the cells by means of small pores, located in the lateral and basal parts of the outer cell membranes.

All chemical fixation procedures used introduced specific structural artifacts. It is concluded that the structure of the basal labyrinth is relatively well preserved after fixation with potassium permanganate, with a mixture of glutaraldehyde and osmium tetroxide, or with osmium tetroxide when applied for 10 min only. The unit-membrane structure was, however, absent after all procedures involving osmium tetroxide.

In freeze-etch replicas determinations were made of the numbers of small particles covering the surfaces and fracture faces of the membranes of the basal labyrinth and of the outer cell membranes. The numbers per unit area of surface proved to be markedly constant and specific for each of the four faces of both types of membranes. Specific differences were found between the particle densities of the outer cell membranes and the membranes of the basal labyrinth. This finding points to functional differences between these types of membranes.

Particle densities were not influenced by pre-incubation with glycerol. After fixation with glutaraldehyde, the particles adhering to the outer and inner surfaces had decreased in number.

It is concluded from this study that membrane structure, as revealed in thin sections as well as in freeze-etch replicas, is consistent with Singer's 'fluid lipid-crystal protein' model.

INTRODUCTION

The use of freeze-etch replicas facilitates the 3-dimensional reconstruction of cell structure. Furthermore, membrane structure can be studied at high resolution with the freeze-etch technique, since surfaces and fracture faces of cell membranes are revealed in the replicas. In this study concerning the second proximal segments of the kidney of the 3-spined stickleback (*Gasterosteus aculeatus*), this technique is used for both purposes.

Attention is paid to the basal labyrinth in these cells, a system of membranes closely

associated with mitochondria. A basal labyrinth is known from many renal and some extra-renal cell types involved in the transport of ions across epithelia. The membranes of the labyrinth probably contain the enzyme complexes which account for the transport activity. The labyrinths described in many species of vertebrates and invertebrates show marked structural similarity when observed in thin sections (Ericsson & Trump, 1969; Wendelaar Bonga & Boer, 1969). However, in a former study of the kidney of the stickleback (Wendelaar Bonga, 1973), indications have been obtained that in this species the basal labyrinth of the renal cells is structurally different from that in kidney cells of, for instance, the rat (Bulger, 1965). In the latter, the basal labyrinth consists of membranes which line interdigitating cytoplasmic processes of adjoining cells. In the stickleback, the labyrinth seemed to consist of an intracellular membrane system. A reconstruction of this labyrinth, based on ultrathin sections and freeze-etch replicas, is presented in this paper. Since it is known that specific structural artifacts occur as a consequence of the standard chemical fixation techniques for electron microscopy (Rosenbluth, 1963; Riemersma, 1970), especially in cells of teleost kidneys (Bulger & Trump, 1965), the effects of several fixation procedures on cell structure are compared. Moreover, the effects of glycerol, as a cryoprotectant, and of glutaraldehyde, as a fixative prior to freeze-etching, are analysed.

Freeze-etch studies of cellular membranes have revealed that the surfaces and fracture faces of membranes are more or less densely covered with small (6·5–13·0 nm in diameter) particles. These particles have been regarded by several authors (Deamer & Branton, 1967; Tourtelette & Zupnik, 1973) as protein complexes which account, at least in part, for the functional characteristics of a membrane. This hypothesis implies that differences in membrane function may be reflected by differences in the numbers of these particles per unit area of surface. Thus, if the density of these particles should prove to be uniform and specific for a given type of membrane in cells of animals kept under the same conditions, then particle density might be a valuable parameter for membrane activity. Therefore, in this study of the kidney cells of sticklebacks, particle densities are determined of the surfaces and fracture faces of the outer cell membranes and the membranes of the basal labyrinth. It has been determined whether the particles adhering to these membranes are present in constant and characteristic densities. In addition, the effects of glycerol and glutaraldehyde on the number and distribution of the particles are analysed.

MATERIALS AND METHODS

Freshwater sticklebacks, of the *trachurus* form, were obtained from laboratory stock. They were kept in tanks with running tap water for at least 4 weeks at a temperature of about 15 °C and a daily light period of 8 h. Only adult females 68–70 mm in length and of body weight varying between 3·0 and 3·5 g were used. The animals were killed by decapitation.

For preparation of ultrathin sections for electron microscopy the tissues were fixed as follows:

(a) $KMnO_4$ (1·5 %) buffered in 0·1 M veronal-acetate (pH 7·2) for 15 min, at room temperature. Afterwards tissue was rinsed twice and stained in uranyl acetate (2 %) for 45 min. After rinsing in distilled water and dehydration in ethanol and propylene oxide, embedding followed in Epon 812.

(b) OsO_4 (0·5 %) in 0·2 M phosphate buffer (pH 7·2), for 10 min or 1 h at 4 °C; dehydration and embedding as under (a).

(c) Glutaraldehyde (3 %) in o·1 M phosphate buffer (pH 7·2) for 30 min at 4 °C; rinsing in buffer for 15 min; postfixation with OsO_4 and further processing as under (b);

(d) A mixture of glutaraldehyde (3 %) and OsO_4 (1 %) in a o·1 M phosphate buffer (pH 7·2) for 1 h at o °C; dehydration and embedding as under (a).

Ultrathin sections of material fixed after procedure b, c or d were stained with Reynold's lead citrate.

Freeze-etch replicas were made of small tissue blocks after: (1) infiltration with 25 % glycerol in o·1 M phosphate buffer (pH: 7·2) for 15 min; (2) no pretreatment; or (3) prefixation with 3 % glutaraldehyde in o·1 M phosphate buffer (pH 7·2) for 25 min, rinsing in the same buffer for 10 min, and infiltration with glycerol as under (1).

The tissue blocks were subsequently frozen in liquid freon, for 1 s, and stored in liquid nitrogen. Replicas were made using Balzers BA360M Freeze Etch equipment. Freshly cleaved specimens were etched for 3 min at − 100 °C and shadowed with platinum and carbon. The replicas were cleaned in a saturated solution of $K_2Cr_2O_7$ in 70 % H_2SO_4, for some hours, and subsequently in 40 % NaOH solution for 1 h. They were then rinsed in distilled water and examined on unsupported 300-mesh grids. For quantitative purposes membrane areas of 1 μm^2 per animal were sampled in micrographs at a final magnification of about 80 000 times.

The results were analysed statistically using Student's t-test.

Thin sections and replicas were examined in a Philips EM 300 or in a JEOL JEM 100B electron microscope.

OBSERVATIONS

Ultrathin sectioning of the second proximal tubules

The nephronic tubule of the stickleback is differentiated into a first and a second proximal segment and a collecting tubule. The cells of the second proximal segment are studied, since they are the most numerous and easy to distinguish in the electron microscope. During the reproductive period, most of the tubular cells of males, including the cells of the second proximal segment, are engaged in the production of mucus for nestbuilding. As in adult males ultrastructural indications of mucus production are occasionally found outside the breeding season, only female kidneys are used, to avoid interference by phenomena related to mucus production with those of ion transport.

The cells of the second proximal segments are prismatic. The lateral cell boundaries are straight. Laterally projecting cytoplasmic processes were never observed. The apical cell membrane is differentiated as a brush border. The cells are laterally connected by intercellular junctions. Near the top of the cells, there are band-like differentiations forming a complex of tight-junction, intermediate junction, and a desmosome. More basally, some additional desmosomes are usually present. Gap junctions (Goodenough & Revel, 1970) forming patch-like areas of contact, with a specific 2·0-nm-wide spacing between adjacent lateral membranes, are common. The apical cytoplasm contains a great number of vesicular structures involved in the uptake or the release of still unknown substances. Some strands of granular endoplasmic reticulum, an occasional Golgi zone and some lysosomes are present around the apical part of the nucleus. The basal labyrinth occupies the central and lateral parts of the cytoplasm. The paired membranes of the labyrinth enclose more than 90 % of the mitochondria. The latter organelles are long and slender and their long axis is usually oriented perpendicularly to the cell base (Wendelaar Bonga, 1973).

The effects of several preparatory procedures on cell structure, especially the structure of the basal labyrinth, are dealt with below.

Potassium permanganate. In tissue sections fixed with $KMnO_4$ the preservation of the cytoplasmic matrix, of the nucleoplasm and of the mitochondrial matrix is very poor. The cellular membranes, however, are clearly delineated and intact (Fig. 3), showing the triple-layered unit-membrane structure (Robertson, 1960). At high magnification (Figs. 8, 9) the electron-dense layers of the membranes show electron-transparent gaps. Contrast of the membranes proved to be increased when propylene oxide was replaced by acetone during the dehydration procedure.

The membranes of the basal labyrinth are paired, enclosing a narrow space, and they are continuous with the outer cell membrane. Most of the membrane pairs make contact with the basal part of the outer cell membrane, a few with the lateral part of this membrane. The basal labyrinth has a complicated structure, since the membrane pairs branch and anastomose in many places. In the narrow space enclosed within the membranes of the labyrinth small vesicular structures are occasionally present (Fig. 3).

Osmium tetroxide. When osmium tetroxide is applied for 1 h or longer, as is usually recommended, the cells are badly preserved (Fig. 4). The membranes have a wavy appearance and they are interrupted at many places. Most of the membrane pairs forming the basal labyrinth have lost contact with the outer cell membranes. Fracturing of the membranes and loss of membranous material probably take place during fixation. A marked phenomenon is the absence of the triple-layered structure of the membranes. At high magnification the membranes have a finely granular appearance, without notable stratification. The mitochondria are shrunken.

When, however, fixation is for a very short time (10 min), preservation of cell structure is much better and more in line with the picture of permanganate-fixed tissue (Fig. 5). The membranes are straight and uninterrupted, while most membrane pairs of the basal labyrinth are in contact with the outer cell membrane. As after prolonged fixation, however, the membranes do not have a triple-layered appearance, although they are less granular than after fixation for 1 h. Vesicular structures confined between the membranes of the labyrinth are absent from material fixed with osmium tetroxide, in contrast to tissue fixed with permanganate. But as after use of the latter fixative, membrane contact is improved when propylene oxide is replaced by acetone during dehydration.

Osmium tetroxide preceded by glutaraldehyde. The cytoplasmic, mitochondrial and nuclear matrices are well preserved and are more electron-dense than after the foregoing procedures. Membrane contrast, on the other hand, is very poor (Fig. 6), although it is slightly improved when acetone replaces propylene oxide as a dehydrant. The membranes lack the triple-layered structure characteristic of the unit membrane, and they are very granular (Figs. 10, 11). Most of the membranes of the basal labyrinth have lost contact with the outer cell membranes, as after prolonged fixation with osmium tetroxide. But in contrast to the results of the latter procedure, the membranes are intact, while the distal ends of the membrane pairs of the labyrinth have fused with each other. The loss of contact may, therefore, have an origin which is different from that found after fixation with osmium tetroxide. This conclusion is

corroborated by the results of an additional experiment. After permanganate fixation the majority of the membrane pairs of the labyrinths are in contact with the outer cell membranes. But loss of contact and fusion of the membrane pairs were observed when freshly dissected tissue was immersed for at least 30 min in phosphate buffer, prior to fixation in permanganate. Thus, this phenomenon probably represents a post-mortem change and is not induced by glutaraldehyde.

Mixture of osmium tetroxide and glutaraldehyde. This procedure gives good preservation of cell structure (Fig. 7). The membranes are intact and straight, although the triple-layered structure is absent. Compared to the result of fixing in glutaraldehyde followed by osmium textroxide, membrane contrast has improved, while most of the membrane pairs of the basal labyrinth are in contact with the outer cell membranes. Thus, neither membrane rupture and loss of membrane material, as after prolonged fixation with osmium tetroxide, nor loss of contact between the labyrinth and the outer cell membranes, as after osmium tetroxide following glutaraldehyde, occur when tissue is fixed with a mixture of osmium tetroxide and glutaraldehyde.

Freeze-fractured and etched replicas of the second proximal tubules

Pretreatment with glycerol. A general description of cell structure after freeze-etching will be based on material pretreated with glycerol. After fracturing of the frozen material, large membrane faces are present in the replicas. These faces are
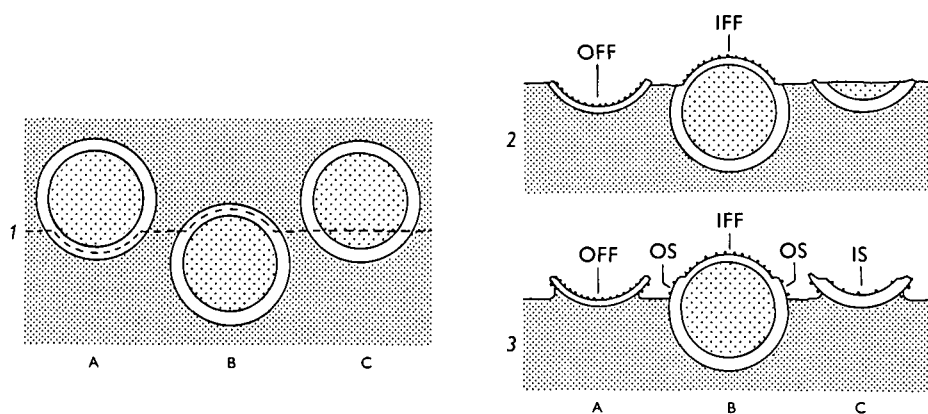

Fig. 1. The process of fracturing and etching. When frozen cells or organelles are broken, the fracture plane (*1*, broken line) may follow the limiting membrane and split it (*1* A, B), or the structure may be cleft (*1* C). Fracturing results in exposure of the outer fracture face (OFF, *2* A), or the inner fracture face (IFF, *2* B). After etching (*3*), the outer (OS, *3* B) and inner (IS, *3* C) surfaces are revealed.

densely covered by particles of $8 \cdot 0 - 12 \cdot 0$ nm diameter. When the objects are etched in addition (i.e. subjected to sublimation of superficially located ice from the fracture plane) small membrane zones are revealed which are less densely studded with particles. Our observations are consistent with the interpretation of Branton (1966) that membranes are normally split during fracturing, in a plane through the centre of the membrane, which gives rise to fracture faces; subsequent etching reveals the true

membrane surfaces. Thus, each membrane shows 4 faces in the replicas: an inner and an outer fracture face (IFF and OFF), and an inner and an outer surface (IS and OS). There is still no uniformity in the literature in the nomenclature of the faces. The conventions used in this study are illustrated in Fig. 1.

The apical vesicles are prominent in the replicas (Fig. 12). Invaginations, of the same diameter as the vesicles, are found at the bases of the microvilli. This points to uptake or extrusion of the contents of the vesicles. Some paired membranes, which are located laterally or apically from the nucleus, show numerous small projections into the cytoplasm (Fig. 13). Since the diameters of the projections match those of the vesicles, the latter probably fuse with or arise by constriction from the membranes.

Table 1. *Number of particles per surface unit* ($1 \mu m^2$) *of inner surface* (*IS*), *inner fracture face* (*IFF*), *outer fracture face* (*OFF*) *and outer surface* (*OS*) *of the membranes of the basal labyrinth* (*a*) *and of the outer cell membranes* (*b, c and d*) *of cells of the second proximal tubules*

	Basal labyrinth	Outer cell membranes		
	a	*b*	*c*	*d*
IS	596 ± 50	595 ± 45	520	170
IFF	3398 ± 153	1844 ± 135	1980	2360
OFF	3789 ± 124	2670 ± 73	2450	2970
OS	1282 ± 51	1212 ± 82	1160	230

a and *b*: pretreatment with glycerol; means ± S.E. of 5 animals are given.
c: no pretreatment; means of 2 animals.
d: pretreatment with glutaraldehyde and glycerol; means of 2 animals.

Occasionally these membranes are seen to be continuous with the lateral cell membranes (Fig. 13). Thus, the lumen enclosed by the indented membranes communicates with the intercellular space. It could not be established whether the membranes are part of the basal labyrinth or of the endoplasmic reticulum. Similarly indented membranes are not present in the cells of the first proximal tubules, while the small apical vesicles are likewise absent. The mitochondria are often globular or oval, in contrast to the long and slender mitochondria usually found in tissue sections.

An unexpected observation was made concerning the way the membranes of the labyrinth and the outer cell membranes are connected. In cross-sections of kidney cells the picture is similar to that in thin sections. The membrane pairs of the basal labyrinth are in contact with the outer cell membranes (Fig. 14). But when surfaces of the basal and lateral parts of the cell membranes are revealed (Fig. 15), pore-like invaginations are seen. These slit-like pores are frequently arranged in rows. They apparently represent the area of contact between the labyrinth and the outer cell membranes, as illustrated in Figs. 16 and 17. It appears, therefore, that the narrow space enclosed by the membranes of the basal labyrinth is continuous with the extra-cellular space. At their apical ends the membranes of the labyrinth form saccular processes, which are branching and anastomosing (Figs. 16, 18) and which end blindly. The membranes of the labyrinth often closely enwrap the mitochondria (Figs. 17, 18).

When infiltration with glycerol was prolonged to more than 30 min, most membrane pairs of the labyrinth lost contact with the outer cell membranes.

The number of membrane-bound particles per unit area of surface was determined for the 4 faces of the outer cell membranes and the membranes of the basal labyrinth (Fig. 19). Since preliminary counts failed to show any difference in particle density between the lateral and basal parts of the outer cell membranes, these parts were sampled without discrimination. The apical areas, forming the microvilli, were excluded. The results, based on 5 animals, are presented in Table 1, a, b. The data show that the number of particles per unit area is very characteristic for each of the membrane faces, since standard errors are relatively small (3-10 %). It appears furthermore that the particle density of the fracture faces is considerably higher than that of the surfaces. There are more particles found at the OFF than at the IFF, while those covering the
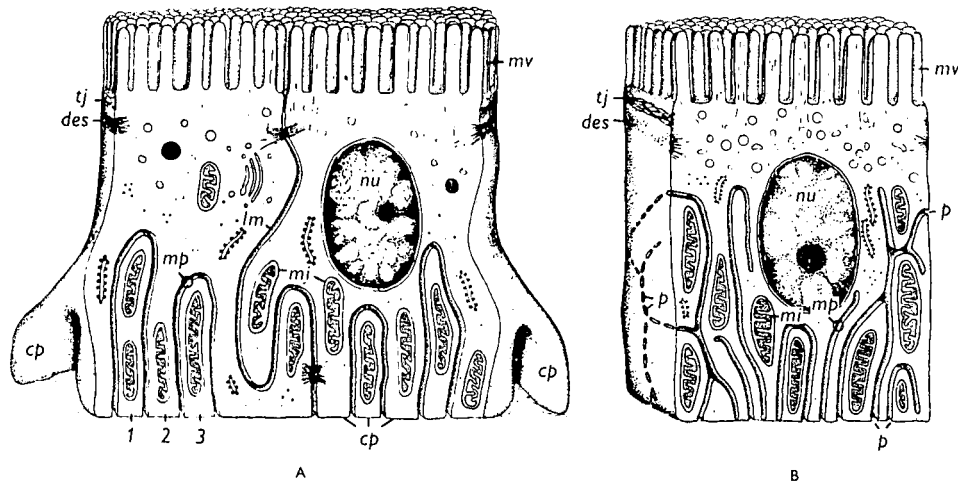
Fig. 2. Schematic drawing of the structure of kidney cells. *des*, desmosome; *lm*, lateral cell membrane; *mi*, mitochondrion; *mv*, microvilli; *nu*, nucleus; *tj*, tight junction.

A, cells of the proximal convoluted tubules of the rat (modified after Bulger, 1965). The basal labyrinth consists of outer cell membranes which line cytoplasmic processes (*cp*). The processes *1* and *3* belong to a different cell from that of process *2*. Thus, the membranes forming a membrane pair (*mp*) originate from adjacent cells.

B, reconstruction of a cell of the second proximal tubules of the stickleback. The lumen enclosed by the membranes of the intracellular labyrinth is in contact with the extracellular space, by slit-like pores (*p*). The cell is prismatic and cytoplasmic processes are lacking.

OS outnumber those associated with the IS. These conclusions hold for both the outer cell membranes and the membranes of the basal labyrinth. Comparison of both types of membranes shows that the number of particles adhering to the IS of the cell membranes is similar to that of the IS of the labyrinth ($P = 0.5$). The same holds for the numbers found at the OS of these membranes ($P > 0.05$). Marked differences appear when the particle densities of the fracture faces of these membrane types are compared: the numbers of the IFF of the labyrinth exceed those of the IFF of the

outer cell membranes by 80 % (P < 0·001). For the OFF this difference amounts to 40 % (P < 0·001).

Freeze-etching without cryoprotectant. When pretreatment with glycerol is omitted and the tissue is frozen immediately after dissection, the replicas show considerable distortion phenomena due to the formation of ice crystals (Fig. 20).

The way the membrane pairs of the labyrinth make contact with the outer cell membranes is similar to that described under pretreatment with glycerol. The mitochondria, on the other hand, are not oval, but long and slender, as after chemical fixation. Additional experiments showed that the degree of the rounding of the mitochondria observed after pretreatment with glycerol is positively related to the duration of the pretreatment, whether this involved immersion in buffered glycerol or in buffer only.

Most cellular membranes have a rough appearance, and the particles can often hardly be distinguished against this background. Particle counts were nevertheless possible in some parts, although sufficiently large sampling areas were only obtained for the outer cell membranes. The data (Table 1c) show that the particle densities are similar to those found after pretreatment with glycerol.

Freeze-etching after pretreatment with glutaraldehyde and glycerol. Fixation with phosphate-buffered glutaraldehyde prior to infiltration with glycerol and freezing induces profound structural changes in the kidney cells. Most membranes of the basal labyrinth have lost contact with the outer cell membranes. The latter show only an occasional pore-like invagination. The mitochondria retain their long and slender shape. Marked changes were found in the density of the particles. Determinations were made of the numbers of particles of the outer cell membranes. The inner and outer surfaces are relatively smooth when compared to non-fixed membranes (Table 1d): the numbers are reduced by about 70 % for the IS, and by about 80 % for the OS. The number of particulate structures present on the fracture faces is, on the other hand, slightly higher than that found in unfixed material. It was, however, often hard to determine, since the background appeared rougher, while the particles were less clearly delineated than in unfixed material. These differences may account for the relatively high numbers found on the fracture faces.

The structure of the basal labyrinth

The study of thin sections shows that there are differences in the structure of the basal labyrinth which are related to the method of fixation. However, all preparatory procedures have in common that the basal labyrinth appears as an intracellular system of paired membranes. The membranes of each pair run closely parallel to each other, and more-or-less perpendicularly to the base of the cell. In many places these membranes make contact with the lateral or basal parts of the outer cell membranes. The slit-like space enclosed by the membranes is apparently in open connexion with the exterior.

The results of the examination of freeze-etch replicas are consistent with, and complementary to, those obtained from the sections. The replicas especially show that the

membranes forming the labyrinth are arranged as compressed saccular and tubular elements, which branch and anastomose frequently, justifying the term labyrinth. The finding that the lumen of the labyrinth communicates with the extracellular space by means of rows of pores could not be deduced from the sections and demonstrates the value of the freeze-etch technique. This technique furthermore reveals differences between the membranes of the basal labyrinth and the outer cell membranes as far as the density of the membrane-bound particles is concerned.

The structure of the basal labyrinth, as deduced from our observations, is presented in Fig. 2B.

DISCUSSION

Chemically fixed and sectioned material

Our observations on thin sections, fixed by different methods, show that each fixative introduces specific changes in cell structure. After permanganate fixation vesicular structures were found between the membranes of the basal labyrinth. Since such structures were absent after application of other fixatives, most or all of these structures may represent artifacts. Rosenbluth (1963) has described similar vesicles in the spinal ganglia of toads, and considered them to be artifacts due to rearrangement of membrane material during permanganate fixation.

Membrane artifacts introduced by prolonged fixation in osmium tetroxide are well known (e.g. Low, 1954). In our material the destructive effect of this fixative was very marked after application for 1 h. Our finding that fixation for 10 min gives much better results is surprising since fixation for at least 1 or 2 h is generally recommended (Riemersma, 1970).

In sections fixed for 10 to 15 min with permanganate or osmium tetroxide, or in freeze-etch replicas of unfixed material, most membrane pairs of the basal labyrinth are continuous with the outer cell membranes. When glutaraldehyde is used as a fixative, prior to osmium tetroxide or permanganate or prior to freeze-etching, lack of contact is common. Loss of contact has been observed before by Bulger & Trump (1965) in thin sections of the kidney of the midshipman, *Porichthys notatus*, after fixation with glutaraldehyde and osmium tetroxide. According to these authors, this phenomenon occurs during fixation. Our results show that it also takes place in tissue immersed in buffer. Thus, this phenomenon probably represents a post-mortem change. This conclusion implies that glutaraldehyde is not an adequate fixative for the kidney cells of the stickleback. If glutaraldehyde and osmium tetroxide are used as a mixture, the negative effects occurring when both fixatives are applied separately are diminished to a great extent. Our results show that in tissue fixed with the mixture, loss of contact between the labyrinth and the outer cell membranes, as well as serious membrane damage, are absent.

After all fixational procedures involving osmium tetroxide – but not after permanganate fixation – the trilaminar unit-membrane structure is absent. Although this phenomenon has been mentioned before (Korn, 1966; Robertson, 1967), osmium tetroxide fixation leads to the unit-membrane appearance in most tissues (Robertson,

1972). Its absence in our material indicates that disturbance of membrane architecture takes place during fixation.

Membrane contrast proved to be higher after dehydration with acetone than with propylene oxide. Both dehydrants are lipid solvents and are known to remove lipids even from fixed membranes. Larger amounts are dissolved by propylene oxide than by acetone (McGee-Russell & De Bruyn, 1968). Therefore, the extensive loss of membranous material may be due to removal of lipid components. This interpretation implies that the electron-dense layers of the unit-membrane represent, at least for the main part, lipid material. In the original concept of the unit-membrane (Robertson, 1960), these dense layers have been interpreted as being mainly protein layers, covering a lipid bilayer at both sides. This location of the membrane-bound proteins, based on the Danielli–Davson model, has been criticized on biochemical and biophysical (Green & Perdue, 1966; Korn, 1966, 1969; Lenard & Singer, 1966) and ultrastructural evidence (Sjöstrand & Barajas, 1968; Kopp, 1972). In the alternative model of Lenard & Singer (1966), the fluid lipid-crystal protein model, proteins and lipoproteins are represented as particulate units, more-or-less evenly distributed in a lipid bilayer. Sjöstrand & Barajas (1968) have suggested a comparable structure for mitochondrial membranes. Kopp (1972) has obtained evidence, in line with our inferences, that the electron-dense outer membrane layers are exclusively formed by lipids and not by proteins. He has found that the osmiophilia of the yeast plasmalemma almost completely disappears when the lipids are saturated. His results show that the location of the proteins within membranes cannot be established with osmium tetroxide. Robertson (1967, 1972) has shown that, in artificial bilayers, the outer polar ends of the lipids have affinity for osmium tetroxide. Thus, the unit-membrane structure is probably not due to the staining of proteins. It can, on the other hand, satisfactorily be interpreted as a lipid bilayer. Our high-power electron micrograph of permanganate-fixed membranes (Fig. 9) shows that the electron-dense layers are interrupted by many electron-transparent gaps. These gaps, as well as the electron-transparent space enclosed by the electron-dense layers, may contain proteins, embedded in or penetrating through the lipid matrix, as supposed in the fluid lipid-crystal protein model. This interpretation is in line with freeze-etch results (see below).

Freeze-etched material

All chemical fixation methods used introduce specific structural artifacts. Freeze-etch replicas are supposed to give more reliable information, since chemical fixation can be avoided. The results of Plattner (1970) have shown, however, that artifacts inherent in chemical fixation may occur in freeze-etch replicas. He found volume changes of cellular organelles and rupture of membranes after use of glycerol as a cryoprotectant. However, the changes observed in our material after prolonged treatment with this chemical, like the rounding of mitochondria or the loss of contact between the labyrinth and the outer cell membranes, do also occur in cells immersed in buffer. Moreover, glycerol did not noticeably influence the density of the membrane-bound particles. This is in contrast to the results of Kirk & Tosteson (1973), who observed a reversible decrease of particle numbers of the OFF of erythrocyte mem-

branes. We found, on the other hand, that glutaraldehyde leads to a marked decrease of the numbers adhering to both membrane surfaces of the kidney cells. This may be due to conformational changes induced in the particles, which make them less conspicuous. The absence of a similar reduction of the particles of the fracture faces may be due to their location: these particles are confined within the membrane interior during fixation.

In the present study 4 different faces are distinguished for each membrane. This observation is consistent with that of Branton (1966), and Deamer & Branton (1967). These authors have concluded that during fracture membranes are split through their central plane. This process is assumed to result in separation of the lipid monolayers constituting the membrane matrix. The faces exposed after fracturing represent, therefore, inner adjoining faces. The outer surfaces are revealed after etching. This interpretation has been supported by the demonstration that macromolecules, like ferritin (Pinto da Silva & Branton, 1970) or fibrous actin (Tillack & Marchesi, 1970), when bound *in vitro* to erythrocyte membranes, are not found after freeze-fracturing, but exclusively after etching. The same holds for membrane-bound ribosomes (Wartiowaara & Branton, 1966).

The results of our particle counts show that the number of particles per unit of surface area is very characteristic, and different for each of the 4 faces of the membrane studied. This may hold for membranes in general, since it has recently also been demonstrated in thylakoid membranes of *Euglena gracilis* (Miller & Staehelin, 1973). The particles are supposed to represent proteins since artificial lipid bilayers have smooth faces, while artificial membranes prepared from protein-lipid complexes show faces covered with many particles (Moor, 1966; Moor & Mühlethaler, 1963). Furthermore, exposure of erythrocyte membranes to pronase results in an almost complete loss of particles (Branton, 1971). Thus, the freeze-etch results indicate that the membrane-bound proteins are particulate and more-or-less evenly distributed in a lipid matrix. Consequently, these results are in line with the fluid lipid-crystal protein model of Lenard & Singer (1966).

The IFF and OFF are joined tightly together before fracturing. But although the IFF and OFF are densely covered with particles, indentations corresponding to the particles have not been found. The IFF and OFF are, therefore, not fully complementary.

Only inferences can be made concerning the function of the particles. Proteins have been regarded as essential for maintenance of the structural integrity of cellular membranes. There are firm indications, however, that most and possibly all membrane-bound proteins are involved in physiological membrane functions. Korn (1966, 1969) demonstrated that the protein content of myelin, a membrane type which is metabolically highly inactive, amounts to only 20 % of the dry weight of these membranes. In most other, metabolically more active, types of membranes the protein content varies between 50 and 70 %. Dreyer, Papermaster & Kühn (1972), using gel electrophoresis, compared the composition of the protein fraction in 4 types of membranes. They were unable to detect a single protein common to all of these membranes and, furthermore, they demonstrated that in the membranes of the outer rod segment of the bovine

retina up to 90 % of the protein content was accounted for by rhodopsin. The data of Dreyer *et al.* indicate that the widespread occurrence of a specific structural protein is improbable and suggest that most proteins are involved in membrane-bound physiological functions. This conclusion implies that particle density should reflect the functional state of a membrane. This hypothesis is corroborated by the results of Tourtelette & Zupnik (1973). These authors noted that treatment of cells of *Acholeplasma laidlawii* with puromycin, which impairs membrane functions, led to a reduction in the number of particles on the cell membranes. Thus, the differences in particle density as found in our study between the outer cell membranes and the membranes of the basal labyrinth point to functional differences between these types of membranes. Recently we found that induction of functional changes in the kidney cells of the stickleback, by exposure to seawater, is accompanied by specific changes in the numbers of particles on both the outer cell membranes and the membranes of the basal labyrinth (S. E. Wendelaar Bonga & M. Veenhuis, in preparation).

Structure of the basal labyrinth

The presence of a basal labyrinth is widespread in the animal kingdom. This structure has been found in epithelia involved in the transport of water and ions in vertebrates and in invertebrates (Ericsson & Trump, 1969; Wendelaar Bonga & Boer, 1969).

Three-dimensional reconstruction of the basal labyrinth in kidney cells of rats (Bulger, 1965) has revealed that it is constituted by the outer cell membranes, which line cytoplasmic processes. These processes are long and extensively interdigitating and contain numerous mitochondria. The study of freeze-etch replicas has confirmed this observation in rats (Leak, 1968). The space confined by the membranes of the labyrinth is therefore extracellular. This type of labyrinth has been presented as being common to all vertebrates, including teleosts (Ericsson & Trump, 1969). The structure of the basal labyrinth in sticklebacks is, however, essentially different. It is clear from the present observations, especially from those made on freeze-etch replicas, that this labyrinth is made up of a system of intracellular membranes forming a branching network of tubular and saccular elements. The spaces confined by this labyrinth are in contact with the extracellular space by short slits and pores. In this respect the kidney cells of the sticklebacks are more closely related to teleostean chloride cells than to mammalian kidney cells. Chloride cells have been described in the gills of many teleosts, including sticklebacks (Bierther, 1970). In these cells a complicated intracellular network of narrow branching and anastomosing tubules, with a circular profile, probably acts as a guiding system for ion transport. The tubules are in contact with the outer cell membranes and their lumen communicates with the exterior of the cells (Conte, 1969).

The type of basal labyrinth found in sticklebacks is not limited to this species. It also occurs in the kidney cells of the ten-spined stickleback *Pungitius pungitius* and the plaice *Pleuronectes platessa* (authors' personal observations). It may be common to teleosts in general.

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Figs. 3–11. Basal labyrinth after different fixation procedures. *bl*, basal lamina; *bm*, basal part of the outer cell membrane; *lm*, lateral part of the outer cell membrane; *mi*, mitochondrion; *mp*, membrane pair of the basal labyrinth.

Fig. 3. Potassium permanganate. Most of the membrane pairs of the labyrinth are continuous with the outer cell membrane (arrows); *ves*, vesicular membrane structure confined within the membrane pairs. × 60 000.

Fig. 4. Osmium tetroxide, 1 h. The membranes of the labyrinth are wavy and broken in many places. Most membrane pairs are not in contact with the outer cell membrane. The mitochondria are somewhat shrunken. × 35 000.

Fig. 5. Osmium tetroxide, 10 min. The membranes are straight and uninterrupted. The membrane pairs of the labyrinth make contact with the outer cell membrane (arrows). × 35 000.
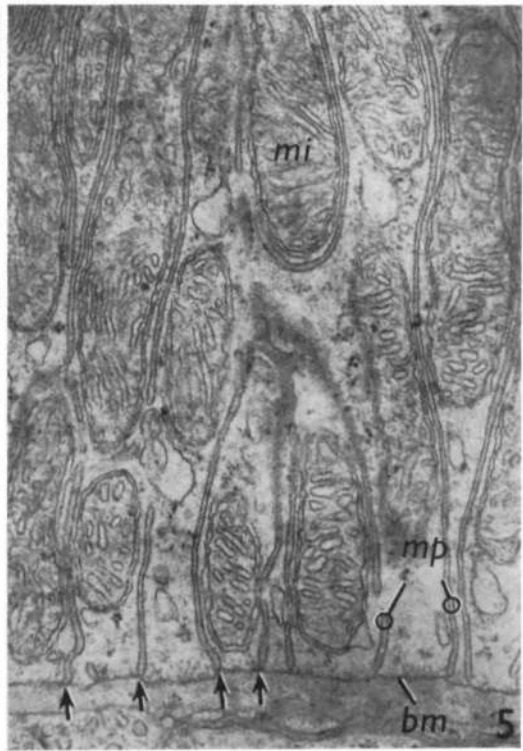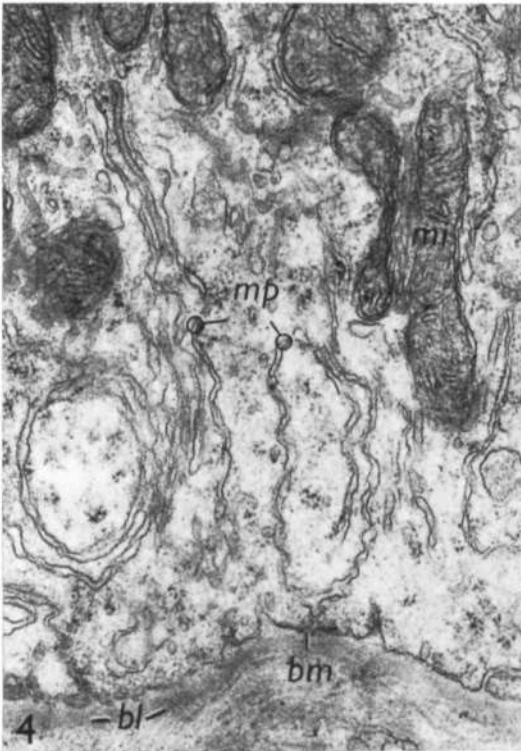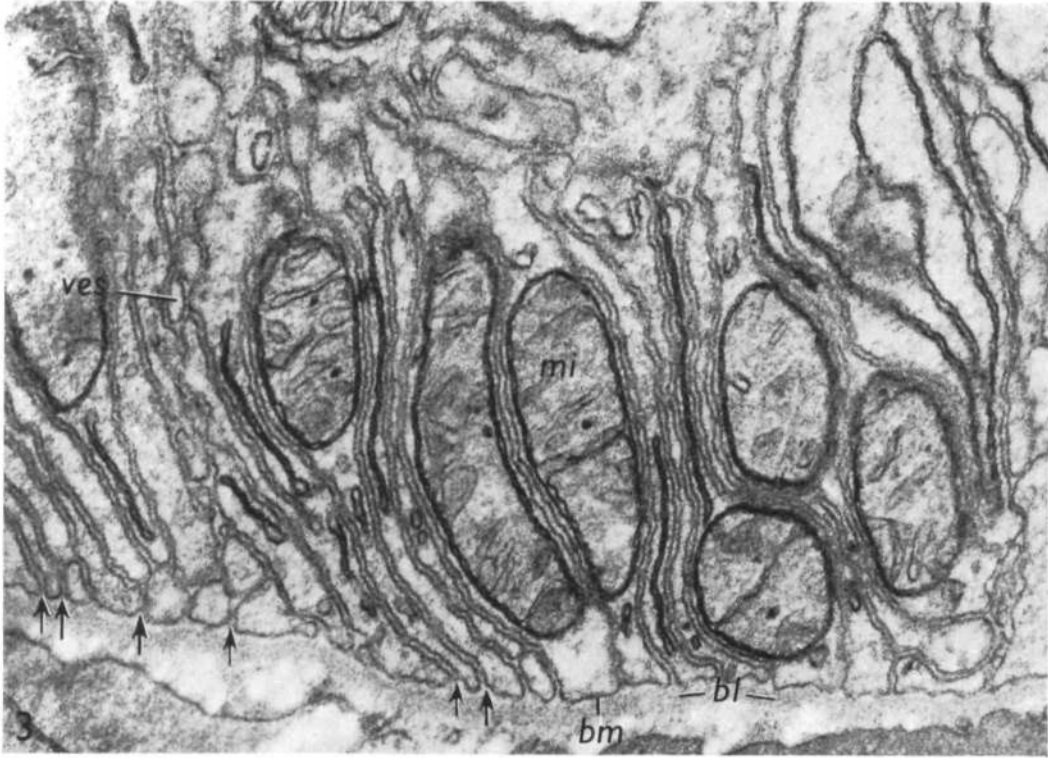
Fig. 6. Osmium tetroxide preceded by glutaraldehyde. Most membrane pairs are not in contact with the outer cell membranes. The membranes are uninterrupted. Membrane contrast is poor. × 50 000.

Fig. 7. Mixture of osmium tetroxide and glutaraldehyde. Most membrane pairs of the labyrinth are in contact with the outer cell membrane (arrows). Membrane contrast is better than in Fig. 6. × 50 000.

Fig. 8. Potassium permanganate. The membranes show the triple-layered unit membrane structure. × 90 000.

Fig. 9. Detail from area outlined in Fig. 8, showing 4 membranes. × 650 000.

Fig. 10. Osmium tetroxide preceded by glutaraldehyde. A membrane pair has lost contact with the outer cell membrane (arrow). The membranes are granular and lack a trilaminar structure. × 100 000.

Fig. 11. Detail of area outlined in Fig. 10, showing 2 membranes × 650 000.
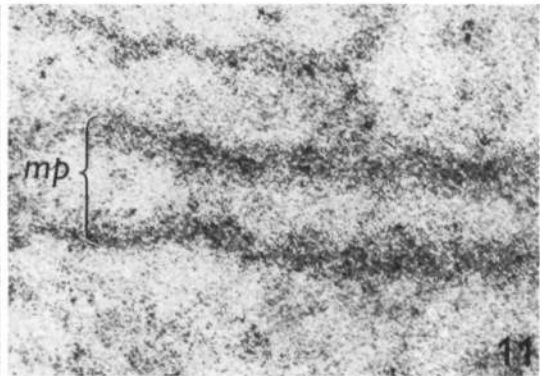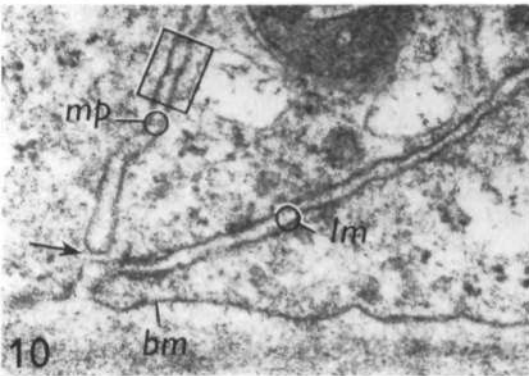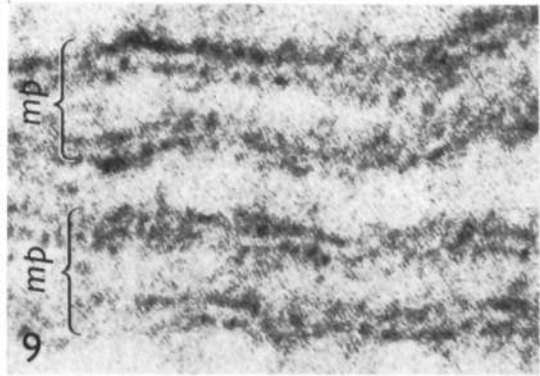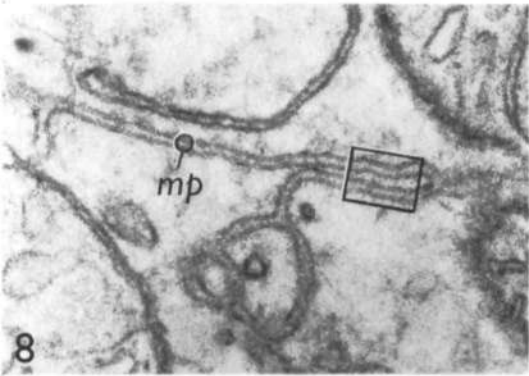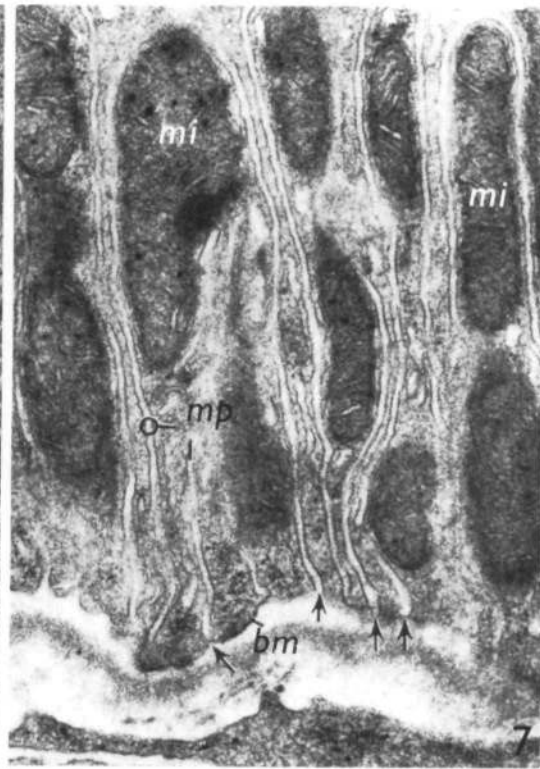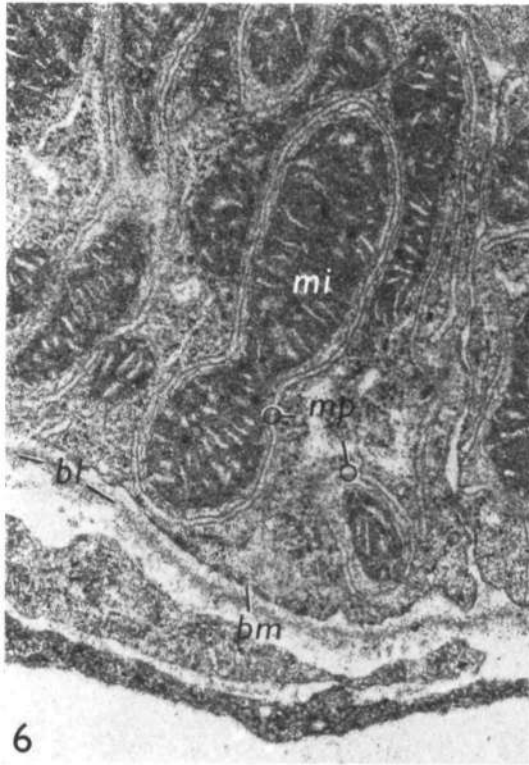
Figs. 12–19. Freeze-etch micrographs of cells pretreated with glycerol. *bl*, basal lamina; *bm*, basal part of the outer cell membrane; *iff*, inner fracture face; *is*, inner surface; *lm*, lateral part of the outer cell membrane; *mi*, mitochondrion; *mp*, membrane pair of the basal labyrinth; *off*, outer fracture face; *os*, outer surface. The encircled arrows in the left upper corners indicate the direction of metal shadowing.

Fig. 12. Apical area of a kidney cell showing microvilli (*mv*) and many small (*sv*) and some large (*lv*) vesicles. × 30 000.

Fig. 13. Central part of a cell. Some small vesicles (*sv*) are present in the cytoplasm. Membranes (*m*) representing endoplasmic reticulum or basal labyrinth make contact with the lateral cell membrane. The vesicles may arise by budding from the membranes, as is suggested by vesicle-like indentations in the membranes, which project towards the cytoplasm (arrows). The pores (*p*) probably mark the places where similar indentations have been broken off during fracturing. × 62 000.

Fig. 14. Basal part of the cells. Most membrane pairs of the basal labyrinth make contact with the basal cell membrane (arrows). × 53 000.
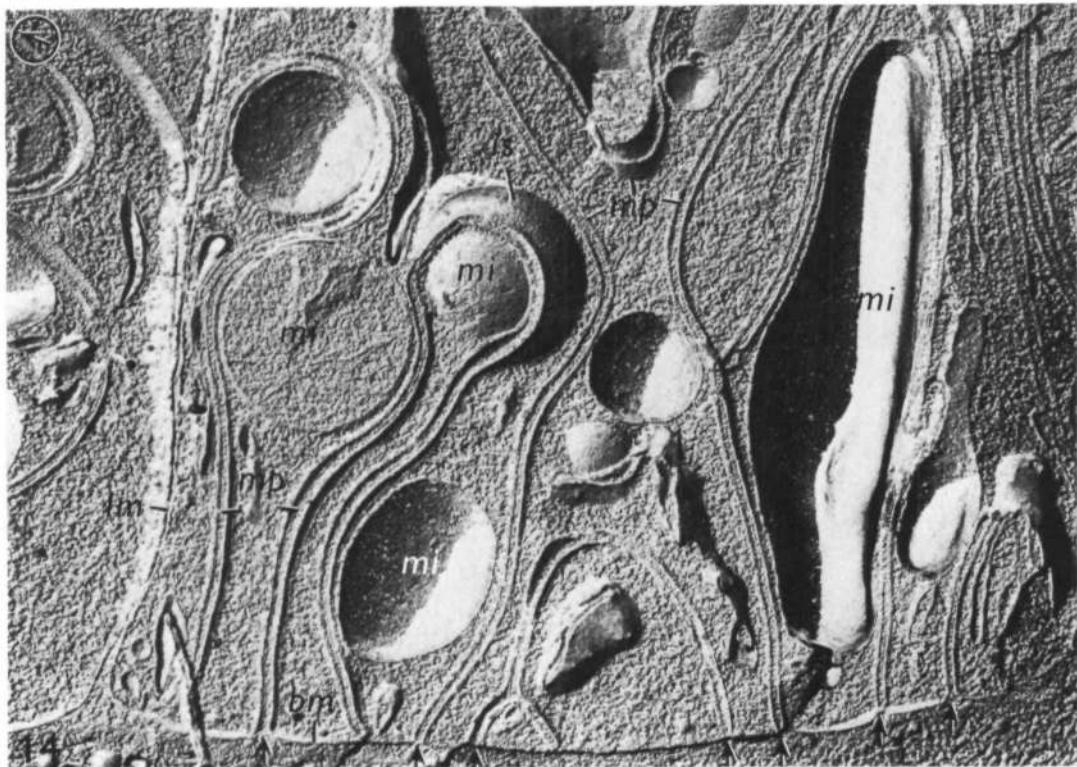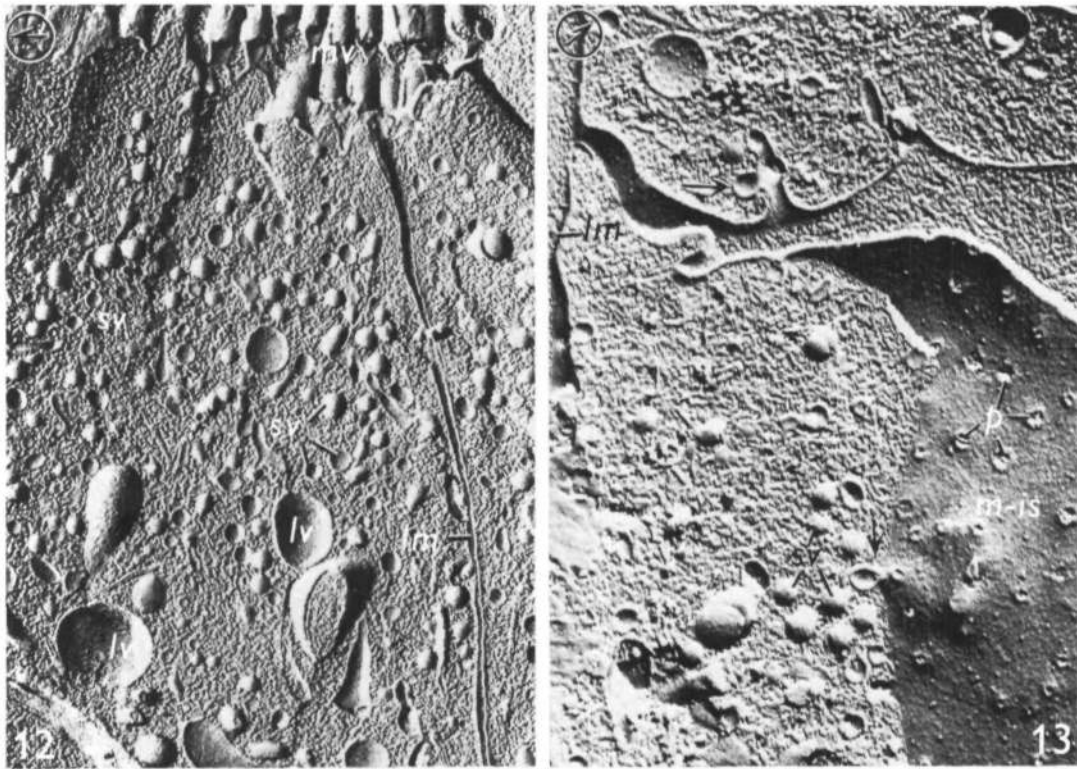
Figs. 15–18. Show details of the basal labyrinth.

Fig. 15. Inner surface of a lateral cell membrane showing rows of slit-like pores (*p*), marking the areas of contact with the basal labyrinth. × 50000.

Fig. 16. Contact between membranes of the basal labyrinth and the basal cell membrane. × 75000.

Fig. 17. The mitochondria are enwrapped by the membrane pairs of the labyrinth. These membranes are continuous with the lateral cell membranes (arrow). × 55000.

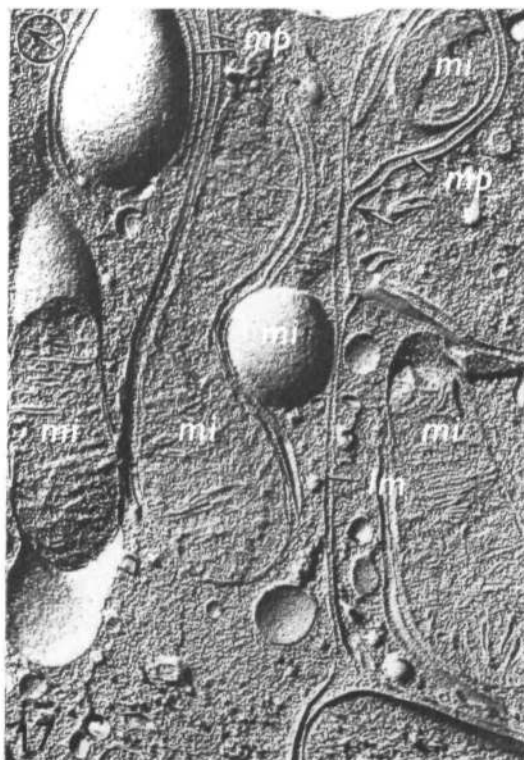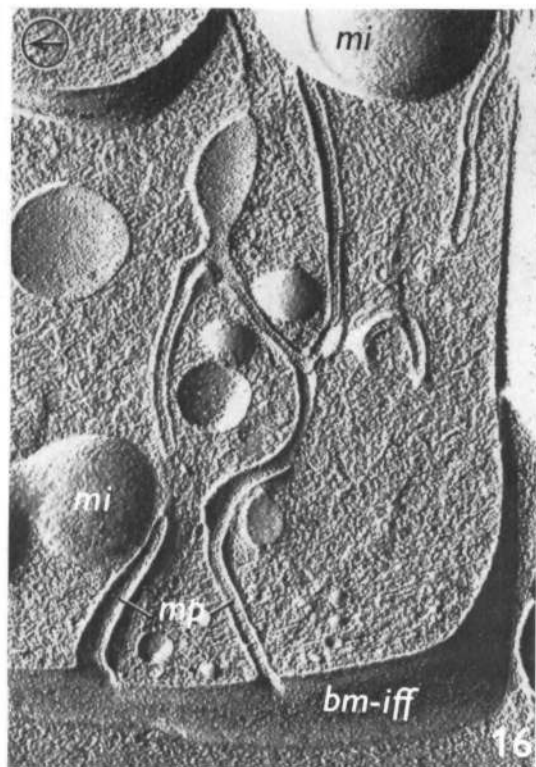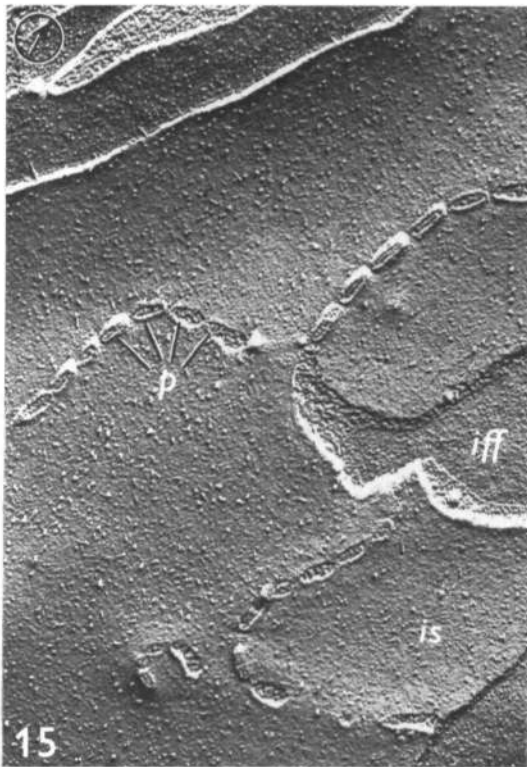Fig. 18. Membranes of the basal labyrinth, closely associated with mitochondria. × 50000.
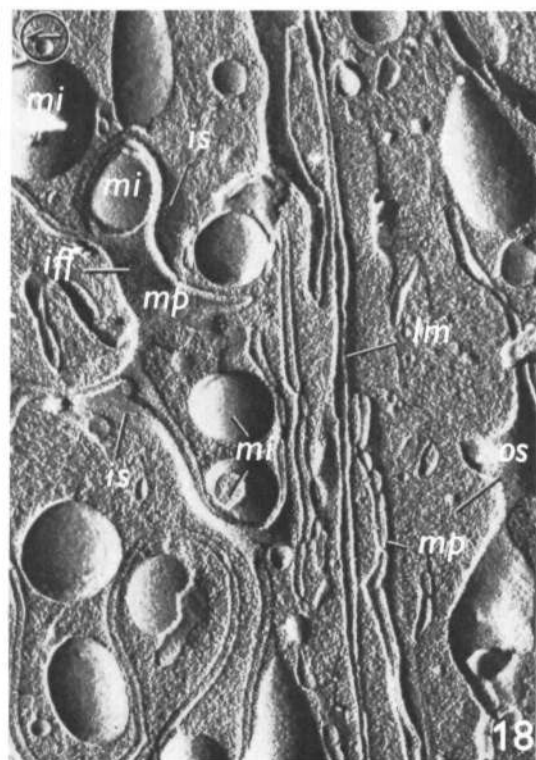
Fig. 19. Surfaces and fracture faces of the membranes of the basal labyrinth and of the outer cell membranes of freshwater sticklebacks, showing the membrane-bound particles. A, basal labyrinth; B, outer cell membrane; *1*, inner surface; *2*, inner fracture face; *3*, outer fracture face; *4*, outer surface. × 50 000.

Fig. 20. Freeze-etched cells without pretreatment with glycerol. Ice formation is seen between the cells; *nu*, nucleus; *p*, nuclear pores. × 10 000.