Short papers submitted expressly for this section, reporting original and significant findings of immediate interest and judged to be acceptable without major revision, will be published within approximately three months. See inside back cover for details.

HEXAGONAL ARRAY OF SUBUNITS IN INTERCELLULAR JUNCTIONS OF THE MOUSE HEART AND LIVER

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Hexagonally packed subunits have previously been observed on the surface of plasma membranes in at least two different systems. They were first described by Robertson (1) in electrical synapses of the Mauthner cells after permanganate and osmium-tetroxide fixation. Benedetti and Emmelot (2) have illustrated almost identical patterns in isolated liver plasma membrane fractions which had been negatively stained. We report here a new technique, based on the observations of Doggenweiler and Frenks (3), which has allowed us to demonstrate the presence of similar structures in sections of mouse heart and of mouse liver. When tissue blocks are treated before dehydration and embedding with a preparation of lanthanum salts, the extracellular space becomes filled with an essentially amorphous electron-opaque material. After such treatment hexagonally packed structures are clearly delineated in specialized intercellular junctions of the heart and liver (4, 5).

MATERIALS AND METHODS

Tissues are fixed in a formaldehyde–glutaraldehyde mixture (6), are washed in buffer, and then are post-fixed for 2 hr in OsO_4–collidine at pH 7.2–7.4. A 2–4% solution of lanthanum nitrate is brought to pH 7.6–7.8 with 0.01 N NaOH, with vigorous stirring. The addition of base must be made slowly to avoid premature precipitation of lanthanum hydroxide. When the titration is done properly, a faint opalescence or traces of flocculent material appear at pH 7.8, the pH at which lanthanum hydroxide becomes insoluble. An aliquot of the lanthanum solution is added to the fixatives so that the final lanthanum concentration is 0.5–1%. The final pH of the lanthanum–osmium tetroxide–collidine solution is about 7.2 and is faintly cloudy. It seems likely that, as lanthanum solutions are brought to a high pH, a colloidal compound is formed which, as described below, permeates the extracellular space as a tracer. While we deem it best to add lanthanum to all the solutions up to the alcohols, this is not necessary and lanthanum can be added during the OsO_4 fixation alone. Adding the lanthanum only to the aldehyde fixing fluid or to the buffer rinse solution is less satisfactory, since the heavy metal seems to be washed out from the blocks again during subsequent exposure to aqueous solutions. The exposure to lanthanum solutions should be done at room temperature.

RESULTS

Sections cut from blocks treated with lanthanum as described above contain a material of high electron opacity which fills the extracellular space to a variable degree. In most cases the lanthanum has not penetrated the central portion of the block, but the intermediate zone between the center and the surface of the block is well impregnated. At the periphery of the blocks one finds electron-opaque material consistently only in those regions where the extracellular space is very

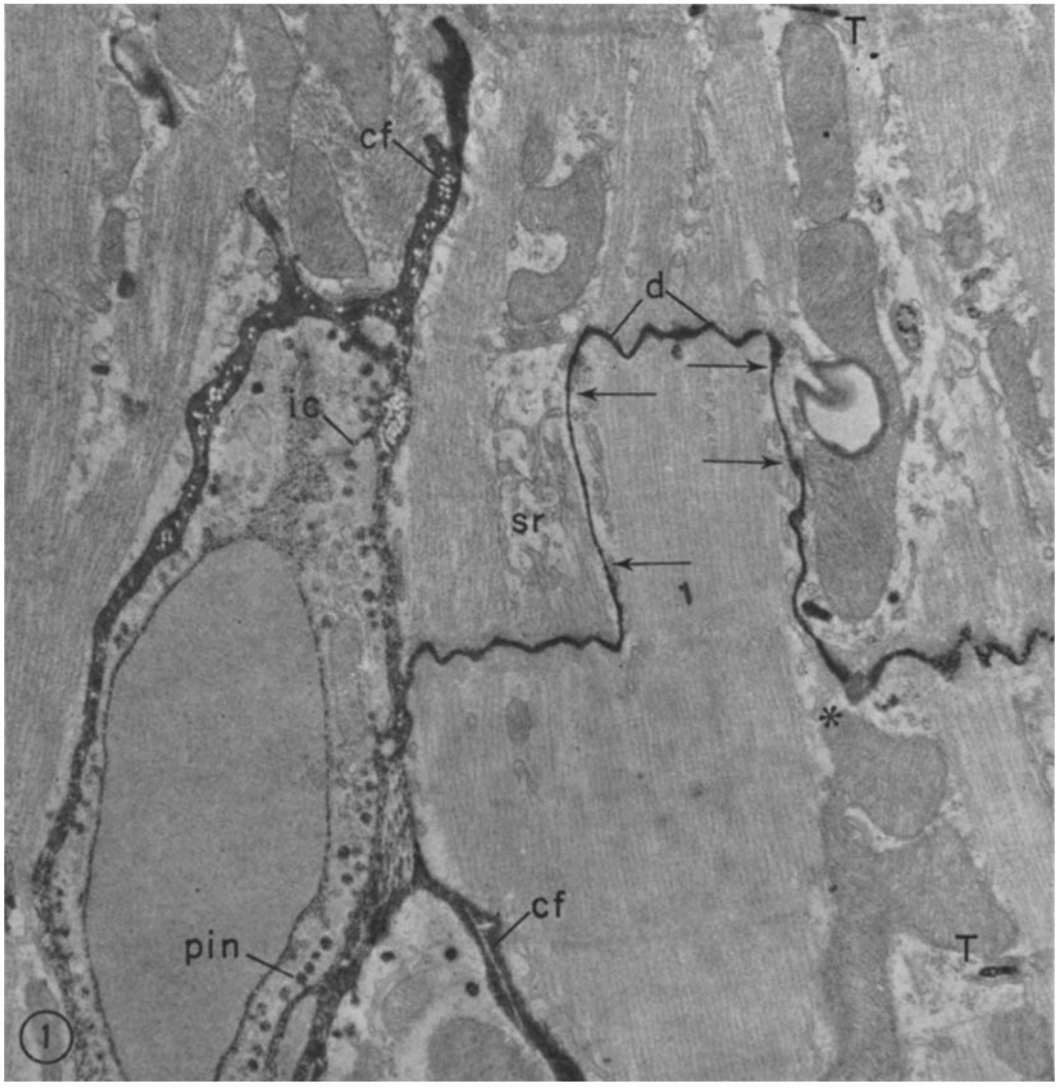

FIGURE 1 Longitudinal section from a block of mouse heart treated with lanthanum solutions. The extracellular space is filled with electron-opaque material which outlines collagen fibrils (*cf*). Pinocytosis vesicles (*pin*) which open to the basal face of the capillary are filled, and some electron-opaque material has leaked into the lumen of the capillary, presumably via the intercellular cleft (*ic*). The gray punctate appearance of the plasma surrounding the red blood cell is due to the presence of a low concentration of lanthanum tracer in the capillary lumen. The T system of the muscle (*T*), because it communicates with the extracellular space, is also delineated by lanthanum. The tubules of the sarcoplasmic reticulum (*sr*) however are not connected with the sarcolemma and do not stain. The extracellular space in the intercalated disc has also been permeated and lanthanum delineates the adhering junctions (*d*) as well as areas of close membrane apposition such as are seen between the pairs of horizontal arrows. At higher powers these would have the appearance illustrated in Fig. 2. At the star a tangential section through a similar junctional area, which at a higher power would have the appearance illustrated in Figs. 5 and 6 is shown. × 45,000.

narrow. The pattern of deposition of electron-opaque material reflects the fact that it can easily be washed out of the tissue again. The lanthanum preparations seem to act as a tracer of the extracellular space rather than as a stain for specific molecular components. Attempts at staining the extracellular space in very small blocks or thin slices obtained on a tissue chopper are usually completely negative, as the lanthanum leaches out again too rapidly for any to be retained. Although a priori one would expect that lanthanum salts would bind strongly to various components of the extracellular space, we have found little, if any, evidence to support this hypothesis. While it is likely that some binding occurs (3), we believe this to be of minor importance in our results, and find it more consistent with the data to interpret regions rendered electron opaque by lanthanum preparations as areas that are accessible to "small" colloidal molecules diffusing from the medium through the intercellular space. The lanthanum preparation does not penetrate into cells except in rare cases where there is a rupture of the plasma membranes.

In the mouse heart (Fig. 1) dense material is seen throughout the extracellular space, where structures such as collagen fibrils are outlined, and have the same appearance as that observed in negatively stained specimens. The basement lamina surrounding the muscle cells is permeated and has the same high electron opacity as the connective tissue space. Pinocytosis vesicles opening onto the surface of the muscle or in capillaries, and the large transverse tubules characteristic of heart muscle, are also filled with electron-opaque material. The regions of the intercalated discs stand out in bold relief as the intercellular space is permeated by lanthanum, and in cross-sections

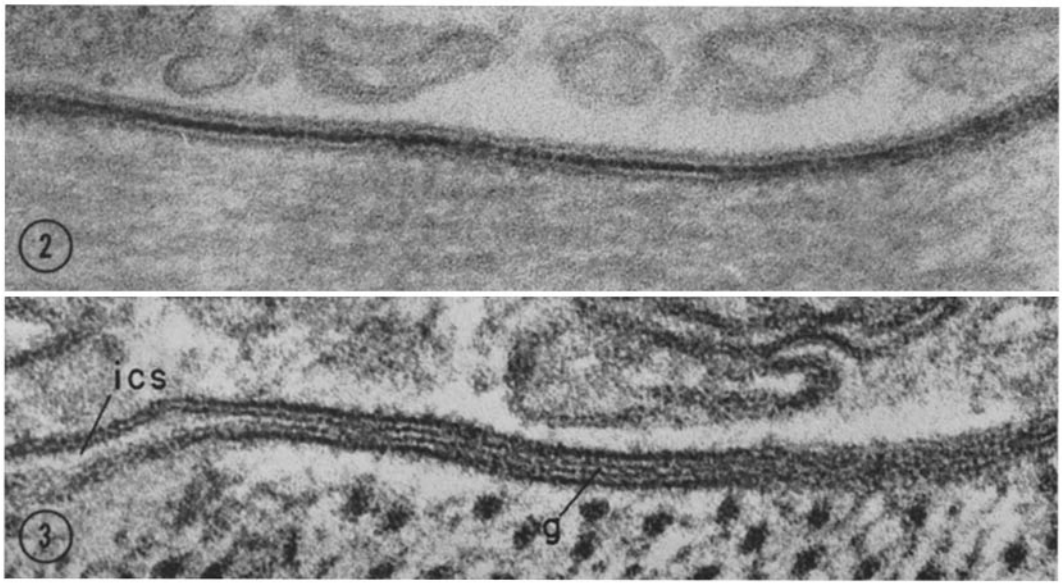

FIGURE 2 A high magnification view of a cross-section through a junctional area similar to those illustrated between horizontal arrows in Fig. 1. The junction appears to be pentalaminar, but differs from the usual occluding junctions because the central region is broad and has a higher electron opacity than the inner leaflets. The width of the intermediate line measured by densitometry and optical micrometry corresponds closely to the width of the gap and outer leaflets of the junctional area, which are illustrated in Fig. 3 as seen in uranium-stained specimens. × 200,000.

FIGURE 3 A high magnification view of a region similar to that shown in Fig. 2 but in a block stained with uranium. The normal intercellular space (*ics*) is reduced to a gap about 20 A wide (*g*) in the junctional area. The width of the gap is constant throughout the junctional area. It is therefore unlikely that images such as this one represent sections through the edge of a tight area. Both the "gap" and the adjacent outer leaflets of the plasma membrane are probably permeable to lanthanum. × 250,000.

of desmosomes (maculae adherentes) the usually dense "intermediate" line appears in negative contrast, outlined by electron-opaque material. Cell membranes are visualized as a lightly stained inner leaflet and a clear zone which represents the central element of the usual "unit" structure. No definite outer leaflet can be distinguished as its electron opacity matches that of the neighboring extracellular space.

The regions of close membrane apposition, the so-called tight junctions of cardiac musculature (8, 9),[1] are of special interest in blocks treated with lanthanum. In cross-section they appear as pentalaminar structures, but the intermediate line which is usually thin, and of low electron opacity in a typical occluding junction, is broad and extremely dense; this suggests that lanthanum salts can permeate these junctions (Fig. 2). Preparations which were treated en bloc with uranyl acetate (18), but not with lanthanum (Fig. 3), show that these junctions are not, in fact, typical occluding junctions. Instead of a fusion of the outer leaflets there is actually a gap of about 18 A which separates the outer leaflets of apposed plasma membranes, a configuration similar to that described by Rosenbluth in *Ascaris* muscle (7). The width of the lanthanum containing intermediate line is about 55 A as determined with optical micrometers, and from densitometric tracings. This is equal to the width of the intermembrane gap seen in specimens stained with uranium en bloc plus that of the outer leaflets of the apposed cell membranes. One can conclude therefore that both the gap and the outer leaflets of the plasma membranes are permeable to the

[1] Fawcett, D. W., and S. McNutt. Data in preparation.

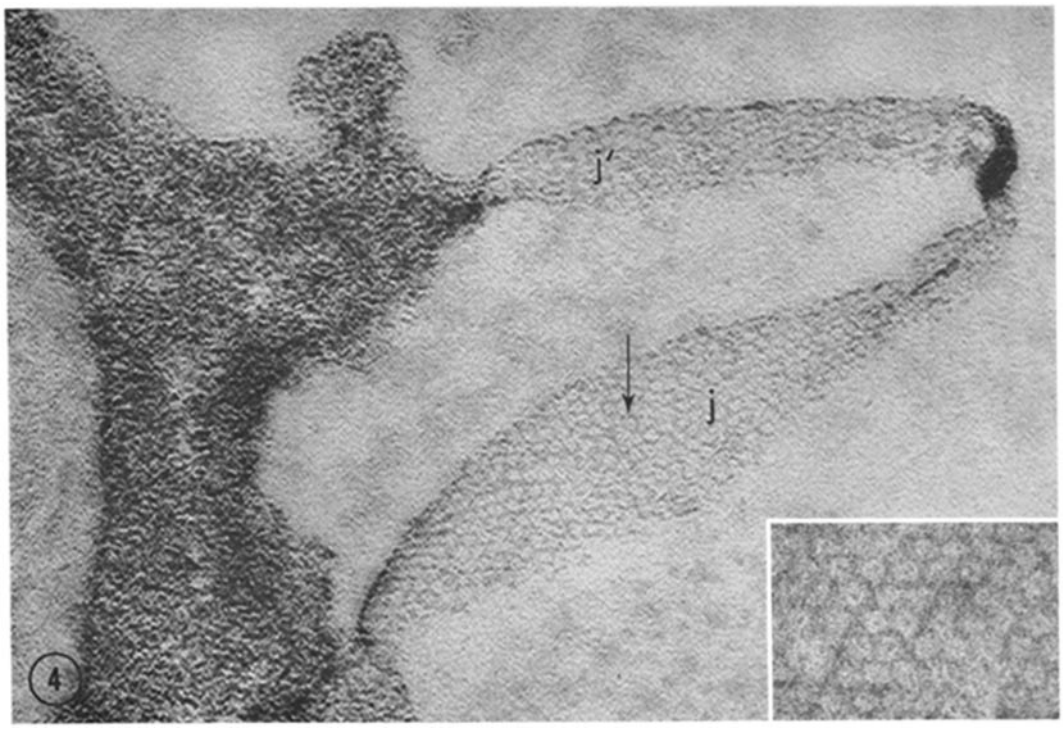

FIGURE 4 A high magnification view of a tangential (*j*) and a near tangential (*j'*) section through a junctional area in the mouse heart. In tangential sections the lanthanum clearly delineates hexagonally packed structures (at *j*, and inset). Each of the subunits has an electron-opaque core (arrow), and an electron-transparent wall some 30–40 A thick (inset). The center-to-center spacing of the subunits is of the order of 90 A. In the less perfectly tangentially oriented parts of the junctional area (*j'*), the membrane subunits appear distorted, although they are still recognizable. × 210,000; inset, × 420,000.

lanthanum tracer in a junction which had been presumed tight.

If one examines sections tangential to such a junction, one finds that electron-opaque material delineates hexagonally packed structures (Figs. 4 and 6). The hexagonal nature of the packing of the subunits has been confirmed both by the print rotation technique of Markham (11) and by optical diffraction (12). Each of the subunits in the array has an angular outline, but their exact shape is difficult to determine. The center-to-center distance between the subunits is 90–95 A, and the diameter of each is about 70–75 A, so that the space permeable to lanthanum surrounding each particle is about 10–20 A, wide. In the center of each particle there appears to be an electron-opaque core 10 A or less in diameter (Fig. 4); it would appear therefore that each subunit is a hollow prism some 50 A tall (two external leaflets each some 15 A wide, and a gap of 20 A), with a wall 30–35 A thick and a small core. It is not clear how the lanthanum tracer penetrates into the core of the prism. These prisms and their packing are essentially identical to the "hexagons" observed by Robertson in $KMnO_4$-fixed electrical synapses (1). A striated pattern with a spacing of 90 A or 45 A is seen in oblique sections through the specialized close junctions of the heart (Fig. 5). Tilting and stereomicroscopy have demonstrated that this pattern is an optical effect resulting from oblique views along the faces of the prisms as was already deduced by Robertson (13).

A study of the mouse liver yielded similar observations to those just described in heart. Besides the junctional area at the bile canaliculus there is also an extensive junctional area between the space of Disse and the bile capillary, as described recently in the mouse liver by Heath and Wissig (14). Examination of blocks stained with uranium have shown that in these junctions just as in the heart there is a 20 A gap between the apposed cell membranes. Here again use of lanthanum impregnation demonstrates a hexagonal packing

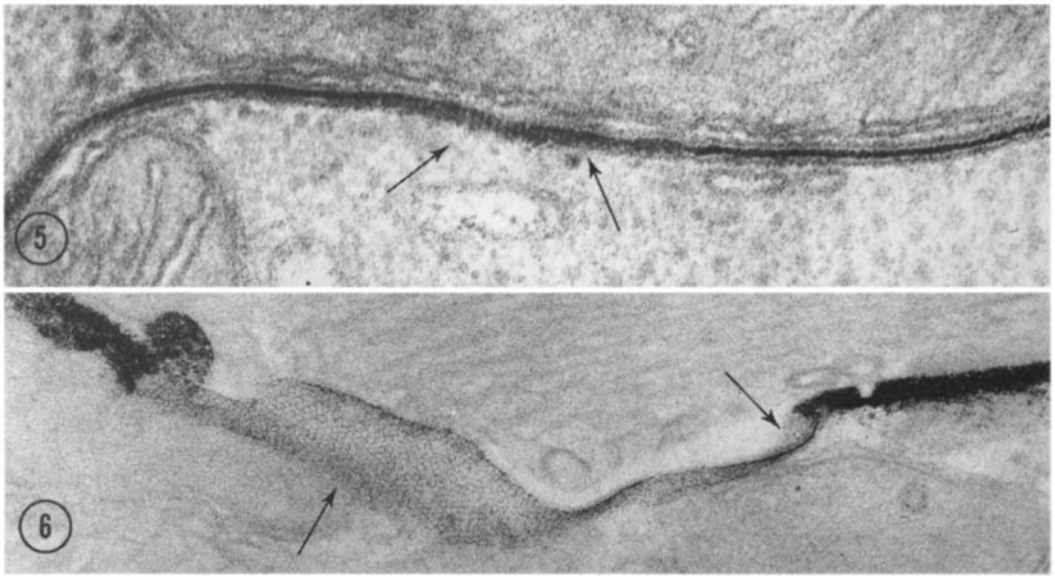

FIGURE 5 When cut normal to the plane of the membranes the junctions appear pentalaminar. In oblique sections, however, as seen between the arrows, a striated pattern appears similar to that described by Robertson in the Mauthner cell electrical synapse. This pattern represents oblique views along the faces of the hexagonally packed structures found in the junctional area. × 150,000.

FIGURE 6 A low power electron micrograph showing the appearance of a junctional area as seen from different angles. At the upper arrow the junction is cut almost normally and one can see the dense intermediate line also illustrated in Figs. 2 and 5. In tangential view (lower arrow) a regular pattern appears as the lanthanum outlines hexagonally packed structures in the junctional area. × 100,000.

of particles in tangential views of the junctional areas. The appearance of the pattern is so similar to that reported by Benedetti and Emmelot (2) in negatively stained, isolated cell membranes of rat liver that it is tempting to suggest that the membrane areas containing hexagonal structures observed by these authors were probably isolated junctional areas, rather than unspecialized plasma membrane fragments.

In conclusion one may say that impregnation of tissue blocks with lanthanum seems to be useful for tracing out the extracellular space in electron microscope specimens. Although the size of the tracing molecules is not known at the present time, the tracer can permeate spaces 20 A wide or less. By a combination of lanthanum tracing and uranium staining one can distinguish the following. (*a*) There are truly tight junctions (zonulae occludentes), not discussed here in detail, in which there is a complete obliteration of the extracellular space, no permeation of lanthanum and no demonstrable hexagons. Such junctions have been described previously (10) and we have confirmed their existence in distal and proximal kidney tubules, and in intestinal epithelium. (*b*) There are also cell junctions in which there is a minute gap between the external leaflets, permeation by lanthanum, and in which hexagons can now be detected as described here in mouse liver and heart. Since measurements of cross-sections of mouse heart intercellular junctions stained with lanthanum show that the electron-opaque tracer permeates both the outer leaflets and the intercellular gap, we may conclude that the substructures characteristic of these junctions must be part of either or both of these layers. Robertson (13) has suggested that the hexagons are part of the outer leaflets in the case of the synapses in the Mauthner cell. It is not possible, with the techniques used, to decide whether the nonpolar layers of the plasma membrane are also involved. It is of interest to note that those sites at which hexagons can be demonstrated in the junctional area by lanthanum, i.e. liver and heart junctions, are regions in which cells are known to be in electrical interconnection (15, 16). A similar correlation obtains in the Mauthner cell (17). A hexagonal pattern such as described here may prove to be characteristic of some, but not necessarily all, junctions involved in electrical interconnection between cells.

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