

GAP JUNCTION STRUCTURES

VI. Variation and Conservation in Connexon Conformation and Packing

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ABSTRACT Correlation of structural changes in isolated gap junctions with the mechanism of channel gating is complicated by the effects of isolation procedures and the lack of a direct functional assay. The effect of variations in the isolation procedure are examined by comparison of the structures of gap junctions isolated by different protocols. X-ray diffraction data from over two hundred specimens are compared to provide a basis for identification of invariant aspects of the connexon structure and variable properties related either to functional switching or experimental modifications. We discuss the relationship between subunit tilt, lattice symmetry and packing, and membrane curvature and demonstrate that membrane curvature may be a natural consequence of the structure of the connexons and the patterns of interactions between them.

INTRODUCTION

Gap junctions are membrane junctions consisting of an array of morphological units that span the plasma membranes of two adjoining cells and the ~30-Å wide extracellular gap between the membranes (Goodenough and Revel, 1970; Caspar et al., 1977; Makowski et al., 1977). Each of the morphological units is composed of twelve copies of the connexin molecule arranged as a pair of hexamers called connexons, one connexon associated with each membrane. A gated aqueous channel extends along the center of the morphological units, connecting the cytoplasm of the two cells and providing a pathway for the transfer of ions and other small molecules between the cells (Bennett, 1973; Bennett and Goodenough, 1978). Changes in connexon structure possibly associated with channel gating have been observed by x-ray diffraction (Makowski et al., 1977; 1982) and three-dimensional image reconstruction from electron micrographs (Unwin and Zampighi, 1980).

Detailed structural studies have concentrated on gap junctions from mouse or rat liver which can be isolated in the form of large two-dimensional crystalline lattices suitable for analysis by x-ray diffraction and Fourier microscopy. Electron micrographs of gap junctions in tissues frozen rapidly to 4°K from the living state (Raviola et al., 1980) show that in gap junctions from mouse liver, the connexons are closely packed but not in crystalline arrays.

This indicates that the gap junction lattices studied by electron microscopy and x-ray diffraction are formed as a consequence of the preparative procedure. Treatments known to uncouple cells connected by gap junctions may result in increases in the packing density of membrane particles and curvature of the junction membranes (Peracchia, 1977; Baldwin, 1977; 1979; Raviola and Raviola, 1978; Raviola et al., 1980). Thus the formation of junction lattices suitable for detailed structural studies may be affected by the physiological state of the junction. It is possible that a correlation exists between changes in connexon structure associated with channel gating and changes in lattice packing and membrane curvature. Changes in the lattice packing associated with structural changes have been observed (Makowski et al., 1977; Unwin and Zampighi, 1980). In this paper we demonstrate that membrane curvature may be a natural consequence of gap junction structure when the connexons are closely packed in the junction lattice.

It is difficult to assess the significance of all the modifications and variations in the isolated junctions induced by the preparative procedures. During isolation, the junction protein may be subject to proteolysis by endogenous enzymes; the connexon conformation may be changed by the action of physiological effectors; and the lipid composition and lattice constant of the junction plaques may be altered by the action of detergents. Identification of the

factors responsible for structural changes requires controlled pairs of experiments in which only a single variable is changed. However, structural variability due to unidentified factors may obscure the effect of some variables and changes in some important experimental variables may result in a disordered junction lattice difficult to characterize structurally. Comparison of x-ray diffraction data accumulated during the past decade from specimens prepared by a variety of methods provides a basis for identifying invariant aspects of the junction structure and the accessible range of structural variation related either to functional switching or to experimental procedures.

Gap Junction Structure

The drawing in Fig. 1 summarizes much of the information available about gap junction structure. The morphological units of the gap junction lattice consist of 12 copies of the connexin molecule arranged as a pair of hexamers (Ma-

kowski et al., 1977). The molecular weight of connexin from mouse liver appears to be ~26,000 (Henderson et al., 1979) and from rat liver, ~28,000 (Hertzberg and Gilula, 1979; Nicholson et al., 1981). As illustrated in Fig. 1, the connexin molecules in each connexon are related to one another by a sixfold rotation axis and the two connexons making up a morphological unit are related by twofold axes perpendicular to the sixfold axis and in the center of the gap. Thus, each morphological unit has point group symmetry 622.

The intercellular channel has an average radius of ~10 Å along most of its length. At the cytoplasmic surfaces of the membranes the channel is broader. It appears to have a funnel-shaped entrance that narrows from an average radius of 25 Å at the level of the cytoplasmic surface of the bilayer to ~10 Å radius at a point 15 Å below the bilayer surface (Makowski et al., 1983).

The gap junction protein appears to be divided into two domains (Makowski et al., 1983). One domain, with a

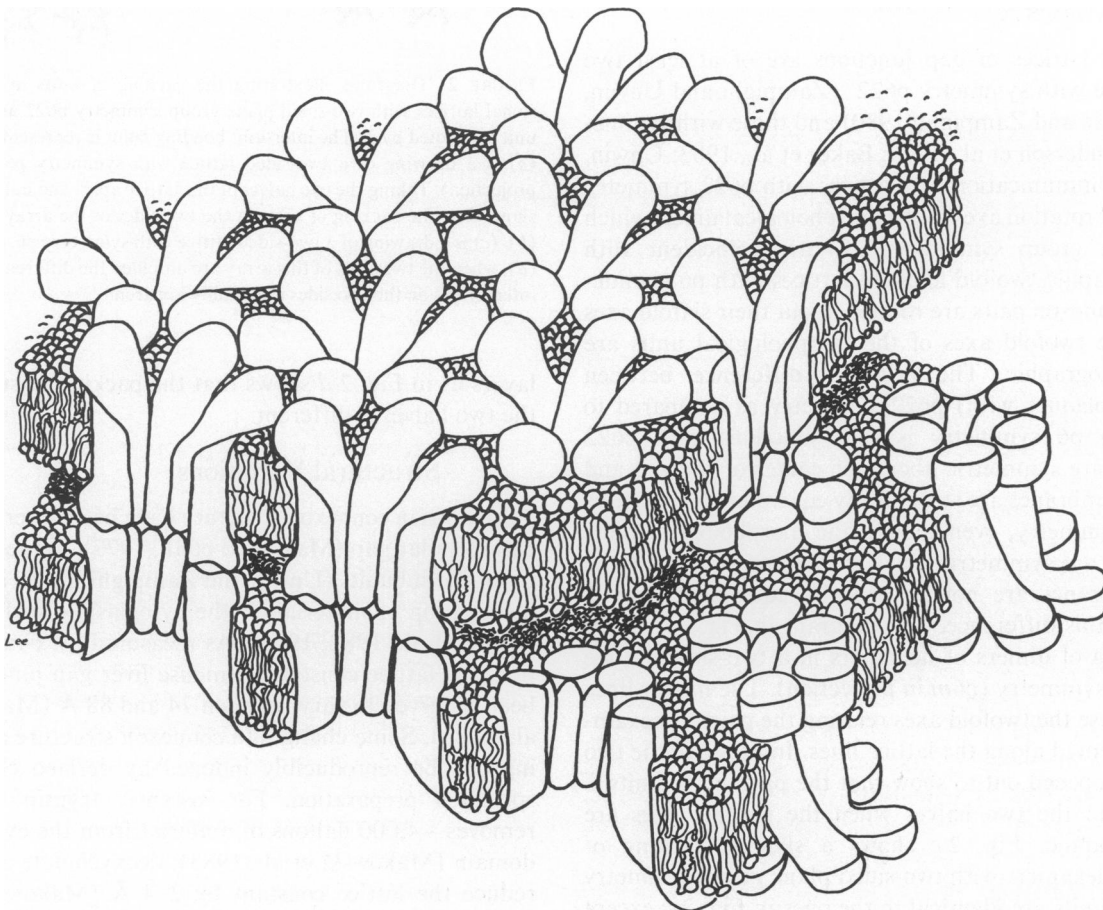


FIGURE 1 Drawing of the structure of gap junctions isolated from mouse liver as deduced by x-ray diffraction. The gap junction units are made up of twelve copies of the connexin molecule arranged into two hexamers (connexons), one associated with each membrane. Each connexin molecule is divided into two domains. The trans-membrane domain spans one bilayer and half of the extracellular gap. The cytoplasmic domain is tightly associated with the lipid polar head groups on the cytoplasmic surface of the membrane. An aqueous channel extends along the center of the connexons. At the cytoplasmic surface of the membrane the channel is ~25 Å in radius, narrowing to ~10 Å radius ~15 Å inside the membrane. One of the gating structures responsible for the closing of the channel is apparently located at the base of the funnel-shaped opening, ~50 Å from the center of the gap.

molecular weight of ~15,000, spans the bilayer and half of the gap and is contained largely within a radius of 30 Å from the sixfold axis. Three-dimensional reconstructions of negatively stained junctions (Unwin and Zampighi, 1980) and three-dimensional electron-density maps calculated from x-ray diffraction data¹ indicate that this domain is tilted 5–20° relative to the membrane perpendicular. Within the bilayer, this transmembrane domain appears to have a high proportion of β -sheet conformation with the strands of the β -sheet running approximately parallel to the membrane surfaces (Makowski et al., 1982). The second domain is somewhat smaller and occupies the cytoplasmic surface of the gap junction membrane. It appears to be relatively labile, being sensitive to digestion by trypsin (Makowski et al., 1983) and perhaps other proteases. This cytoplasmic domain is closely associated with lipid polar head groups and extends from distances of >20 Å from the sixfold axis, forming a funnel-shaped entrance to the narrow transmembrane channel.

Symmetry

Hexagonal lattices of gap junctions are of at least two types; those with symmetry $p622$ ¹ (Zampighi and Unwin, 1979; Unwin and Zampighi, 1980) and those with symmetry $p6$ (Henderson et al., 1979; Baker et al., 1983; Unwin, personal communication). In lattices with $p622$ symmetry the twofold rotation axes of the morphological units (which have point group symmetry 622) are coincident with crystallographic twofold axes. In lattices with $p6$ symmetry, the connexon pairs are rotated about their sixfold axes so that the twofold axes of the morphological units are noncrystallographic. The principal difference between (junction plaques with) $p622$ symmetry as compared to those with $p6$ symmetry is that junctions with $p622$ symmetry are symmetric about the center of the gap and the two membranes are structurally equivalent. In a lattice with $p6$ symmetry, even though the morphological units themselves are symmetric about the center of the gap, the two membranes are not structurally equivalent. Fig. 2 illustrates this difference. The diagram in Fig. 2 *a* shows the packing of dimers of hexamers in a two-sided lattice with $p622$ symmetry ($p6m$ in projection). The mirror lines arise because the twofold axes relating the pairs of hexamers are oriented along the lattice lines. In Fig. 2 *b*, the two layers are opened out to show that the packing of units is the same in the two halves when the twofold axes are crystallographic. Fig. 2 *c* shows a skewed packing of dimers of hexamers with two-sided plane group symmetry $p6$. These units are identical to the ones in Fig. 2 *a* except that they have been rotated in the lattice so that their twofold axes are noncrystallographic. Opening out the two

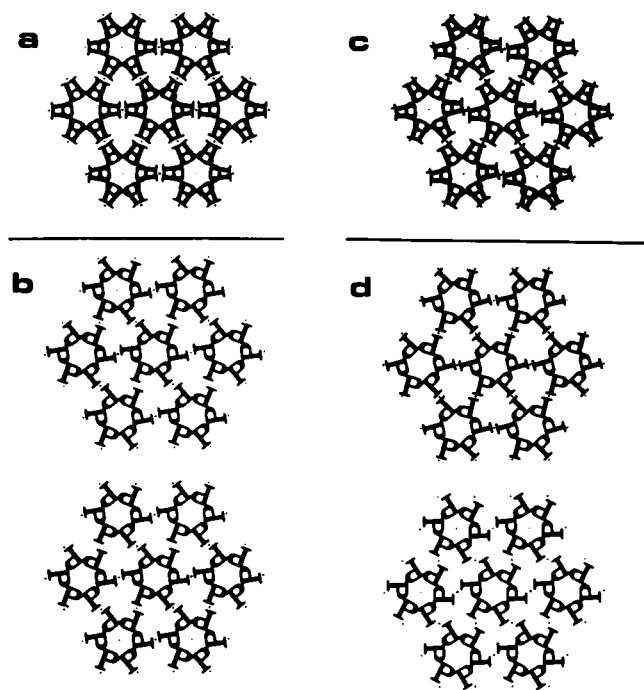


FIGURE 2 Diagrams illustrating the packing of units in two-dimensional lattices with two-sided plane group symmetry $p622$ and $p6$. Each unit is denoted by P . The inter-unit bonding point is represented by a dot. (a) is a drawing of a two-sided lattice with symmetry $p622$ ($p6m$ in projection). Taking the two halves of this lattice apart and unfolding them shows that the packing of units in the two sides of the array is the same (b). (c) is a drawing of a two-sided lattice with symmetry $p6$. As shown in (d), when the two sides of this array are unfolded the different patterns of interactions on the two sides are readily apparent.

layers as in Fig. 2 *d* shows that the packing of subunits in the two halves is different.

Structural Variations

Variations in connexon structure have been observed in the extracellular gap (Makowski et al., 1977), in the degree of tilt of the subunits (Unwin and Zampighi, 1980) and in the distribution of material on the cytoplasmic surface (Makowski et al., 1982, 1983). As measured by x-ray diffraction, the lattice constant of mouse liver gap junctions has been observed to vary between 74 and 88 Å (Makowski et al., 1982). Some changes in connexon structure and packing can be reproducibly induced by defined changes in specimen preparation. For instance, trypsin treatment removes ~4,000 daltons of material from the cytoplasmic domain (Makowski et al., 1983); deoxycholate appears to reduce the lattice constant by 2–4 Å (Makowski et al., 1982); and dialysis in distilled water for several days alters the tilt of the connexon subunits (Unwin and Zampighi, 1980). However, structural differences are also seen among specimens prepared by identical protocols (Makowski et al., 1977). Lattice constant differences of 2–4 Å are commonly observed among specimens prepared according to the same procedure. This is comparable to the

¹Makowski, L., D. L. D. Caspar, D. A. Goodenough, and W. C. Phillips. 1983. Three-dimensional structure of gap junctions. Manuscript in preparation.

consistent differences seen, for instance, between controlled pairs of specimens isolated with and without deoxycholate. When the magnitude of structural variation is comparable to the structural change induced by a change in protocol, the positive identification of a structural effector requires the repeated observation of consistent structural changes between controlled pairs of specimens.

SPECIMEN PREPARATION

Comparison of Isolation Procedures

Details of the isolation procedure influence the extent of proteolytic cleavage of the junction protein, the regularity of the hexagonal junction lattice, and the curvature of the junction membranes. Because there is no functional activity that can be assayed in isolated junctions, the criteria for satisfactory purification have been chemical homogeneity and morphological regularity. Isolation of gap junctions from mouse or rat liver involves initial isolation of a plasma membrane fraction in low-ionic-strength carbonate buffer followed by detergent treatment to solubilize nonjunctional membranes (Goodenough, 1974; Henderson et al., 1979; Hertzberg and Gilula, 1979; Fallon and Goodenough, 1981; Nicholason et al., 1981). Most x-ray diffraction experiments have used junctions lightly fixed with glutaraldehyde. No structural differences between fixed and unfixed specimens have been identified by x-ray diffraction. However, because use of glutaraldehyde may result in a closing of the membrane channel (Spray et al., 1981), it is possible that most of the junctions we have studied by x-ray diffraction have been in the closed, high resistance state.

The initial x-ray diffraction studies (Caspar et al., 1977; Makowski et al., 1977) used mouse liver gap junctions isolated by a procedure (referred to below as protocol I) requiring exogenous collagenase and hyaluronidase to remove contaminants (Goodenough et al., 1974). In these preparations, the apparent molecular weight of connexin in SDS was ~20,000 daltons. Interpretation of electron density profiles calculated from x-ray diffraction patterns from these junctions (Makowski et al., 1977) indicated that there were 23,000–28,000 daltons of protein per connexin molecule present in these junctions. Comparison of these results with the 26,000–28,000 molecular weight of unproteolyzed connexin suggests that although the junction protein was nicked by the action of proteases, most of the junction protein remained associated with the isolated junction plaques. Lattice constants of gap junctions isolated by this procedure varied from 80–88 Å. Treatment of these isolated junctions with trypsin led to the formation of junction vesicles with sharply curved surfaces (Goodenough, 1976) and very highly ordered crystalline lattices (Makowski et al., 1977).

A second protocol (protocol II) was developed (Fallon and Goodenough, 1981) that utilized the detergent Brij 58 and required no exogenous proteases. The apparent molec-

ular weight of connexin in junctions isolated by this procedure remained 21,000 daltons. Interpretation of electron density profiles calculated from x-ray diffraction patterns from these specimens (Makowski et al., 1983) indicates a total protein mass of ~24,000 daltons per connexin molecule. Lattice constants of junctions isolated by variations of this protocol varied from 74 to 88 Å. Treatment with trypsin removed ~4,000 daltons of protein mass per connexin molecule (Makowski et al., 1983) but no increase in lattice order or membrane curvature resulted from this treatment.

Fig. 3 shows electron-density profiles of gap junctions isolated by protocol I and protocol II. The two profiles are very similar in most features. The electron density profile of the specimen isolated by protocol I (---) has a slightly broader bilayer with slightly less density projecting into the cytoplasm. These features may correspond to differences in lipid composition and degree of proteolysis in the two specimens. Recent x-ray diffraction studies of unproteolyzed gap junctions isolated from rat liver have demonstrated that except for differences near the cytoplasmic surface the membrane profile is not substantially altered by the proteolysis.

RESULTS

Morphological Regularity

Among junction plaques in a single specimen a high degree of morphological regularity and homogeneity is usually observed. Fig. 4 is an x-ray diffraction pattern from a specimen of gap junctions isolated by protocol II. The lattice constant of this specimen is 78 Å and the lattice is very highly ordered, with equatorial lattice sampling being observable to at least 9 Å spacing. This indicates that most if not all of the junction lattices in the specimen are highly ordered with almost identical lattice constants. Along the meridian it is possible to see continuous diffraction to at least 10 Å spacing. This indicates that the pair of membranes is well-ordered in the direction perpendicular to

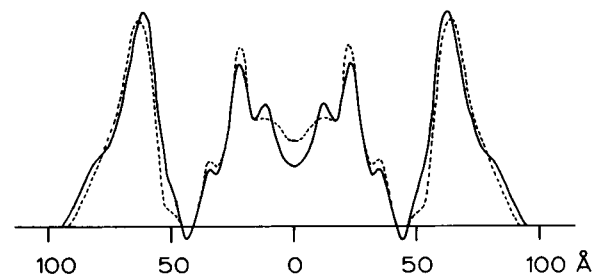


FIGURE 3 Comparison of the electron density profiles of specimens isolated by protocol I (---) (specimen E153 [cf. Makowski et al., 1977]) and by protocol II (—) (specimen I150 [cf. Makowski et al., 1983]). The two electron density profiles are very similar except for small differences in the width of the bilayers and the distribution of density on the cytoplasmic surfaces of the membranes. Both profiles were calculated using meridional data extending to 11 Å spacing.

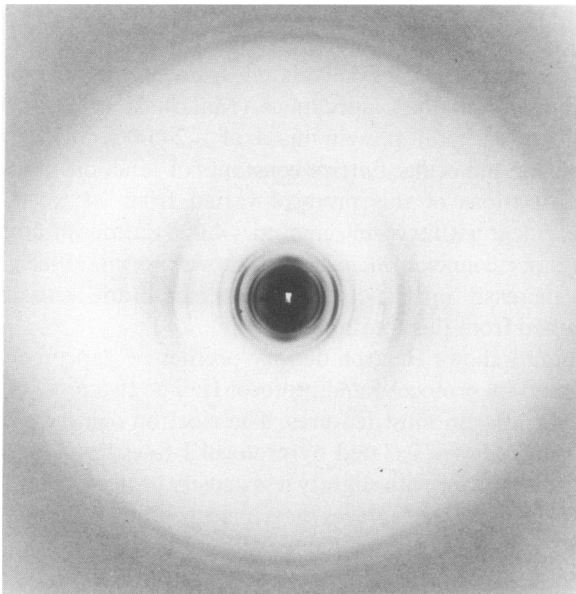


FIGURE 4 X-ray diffraction pattern from isolated mouse liver gap junctions (specimen L68). The meridian extends vertically above and below the center of the pattern and the equator to the right and left of the center. Sharp reflections along the equator index on a hexagonal lattice with lattice constant of 78 Å. Continuous diffraction along the meridian arises from the electron density contrast in the direction perpendicular to the membrane surfaces and can be used for the calculation of an electron density profile similar to those in Fig. 3. Strong diffraction from the β -sheet conformation can be seen at ~ 4.7 Å spacing along the meridian at the top and bottom of this diffraction pattern.

their surfaces with the pair separation varying by not more than a few angstroms throughout the specimen. Centered at about 4.7 Å spacing on the meridian there are three to five sharp diffraction fringes. This has been interpreted as arising from β -sheet conformation in the connexin protein subunits (Makowski et al., 1982). A spacing of about 1/60 Å between fringes is consistently observed. This modulation arises from the regularity on spacing and extent of the β -sheet domains. The sharpness of these fringes indicates that the β -sheet domains in the two connexons making up a morphological unit are within about an angstrom or less of being the same distance apart in all the junctions of the specimen.

Correlation of Lattice Constant with Membrane Pair Separation

Changes in the conformation of the gap junction protein were observed within the extracellular gap using x-ray diffraction from gap junctions isolated by protocol I (Makowski et al., 1977). Changes in the lattice constant of the hexagonal junction lattice were correlated to changes in the membrane pair separation as measured by the position of the third zero of intensity of meridional diffraction in these specimens. Although potentially affected by other variations in membrane structure, the position of this zero of intensity is a sensitive measure of the center-to-center

separation of the two membranes making up the junctional plaque. The center-to-center separation between membranes is roughly $3/2 D$, where D is the spacing of the zero (in Å⁻¹) (see Kirschner and Caspar, 1972). As the lattice constant decreased from 87 to 82 Å, the apparent membrane pair separation decreased from ~ 86 to 82 Å. This decrease in pair separation appears to be a result of a decrease in the width of the extracellular gap while the widths of the bilayers remained constant. As gap width decreased the cross-sectional area of the protein in the gap increased, so that the volume of protein in the gap remained unchanged. Although variations in protocol I were attempted, no experimental variable was identified as responsible for the observed structural variations.

The position of the third zero of meridional intensity is plotted in Fig. 5 as a function of lattice constant for 33 specimens isolated by variations of protocol I (●) and 198 specimens isolated by variations of protocol II (○). The mean zero position for specimens isolated by protocol I was 0.01794 ± 0.00018 Å⁻¹ (95% confidence limits) with a standard deviation of 0.00050 Å⁻¹. The samples appear to exhibit a decrease in zero position with increasing lattice constant. The mean zero position of specimens with lattice constant < 84.0 Å is 0.01284 ± 0.00024 Å⁻¹. The mean for specimens with a lattice constant of 85.0 Å or greater is 0.01764 ± 0.00031 Å⁻¹. For this relatively small sample of specimens the data indicate a significant correlation of meridional zero position with lattice constant.

For specimens isolated by variations of protocol II no such correlation is observed. No significant variation in the mean position of the third meridional node is observed for any lattice constant between 74 and 88 Å. For 198 specimens, the mean is 0.01768 ± 0.00006 Å⁻¹. Comparison of these figures with those calculated from protocol I would indicate that the two samples are representative of different populations. However, examination of Fig. 5 suggests that the specimens isolated by protocol I may be a subpopulation of the population represented by the sample of specimens isolated by protocol II. Inevitably, small samples may occasionally exhibit a statistically significant correlation that does not, in fact, exist in the sampled

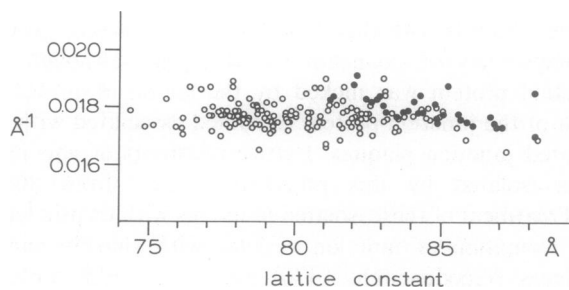


FIGURE 5 A plot of the position of the third zero of meridional intensity as a function of lattice constant. ● indicate specimens isolated by variations in protocol I and ○ indicate specimens isolated by variations in protocol II.

population (e.g., Diaconis and Efron, 1983). It is possible that the apparent correlation of lattice constant and membrane pair separation evident in the positions of solid circles (●) in Fig. 5 is a sampling artifact. However, the differences in isolation protocol suggest that the two populations are different and a statistical analysis of the data demonstrates that it is unlikely that the populations are the same. Both samples represent subpopulations of the structural states accessible to isolated mouse liver gap junctions. Distribution of membrane pair separation (measured from third meridional node position) is approximately Gaussian with a standard deviation of ~2.8% independent of the lattice constant for the 19% range of variation from 74 to 84 Å.

Structural Variability

The distribution of lattice constants and apparent membrane pair separations represented by the data from 231 specimens in Fig. 5 are indicative of real structural variations. Errors in measurement of the position of the third meridional node and lattice constant are ~1%. The standard deviation in the position of the meridional node for the 231 specimens is 2.8%, which is significantly greater than the experimental errors. The variation in these measurements, therefore, indicates significant differences in membrane pair separation. The data in Fig. 5 indicate a variation in membrane pair separation from about 80 Å to over 90 Å. These variations may be due not only to movement of the lipid bilayers but also to changes in connexon structure or lipid composition. Some of the structural variability apparent in this data arose from controlled changes in specimen preparation made in attempts to improve specimen orientation or designed to test the effect of particular treatments on gap junction structure. However, differences between specimens prepared by repetition of the same procedures were often as large or larger than the differences between pairs of specimens from the same isolation that had been treated differently.

It is difficult to identify the causes of structural variations among specimens prepared by nominally identical procedures. The quality of x-ray diffraction patterns such as that in Fig. 4 demonstrates the structural homogeneity of single specimens and indicates that different specimens prepared following as closely as possible the same procedures may not have the same structures. Differences in lattice constant are indicative of differences in lipid content, which may be induced by slight variations in detergent treatment. Differences in the position of the third meridional node most likely indicate some change in connexon structure leading to change in membrane pair separation. The data plotted in Fig. 5 represent the range of structures accessible to gap junctions isolated from mouse liver. They provide a context in which to assess the statistical significance of small structural variations

observed between controlled pairs of specimens isolated with a difference in one experimental variable.

Treatments that gave rise to statistically significant structural changes consistently in several experiments included digestion with trypsin; the addition of tannic acid; and the addition of thorazine. Trypsin caused a change in the electron density distribution at the cytoplasmic surface, but no consistent change in the lattice constant (Makowski et al., 1983). Both tannic acid and thorazine appeared to increase the lattice constant slightly and to alter the membrane profile. Addition of either deoxycholate or Brij 58 to the isolation protocol caused a decrease in lattice constant of 2–4 Å, presumably by removal of lipid. The presence of EGTA in the initial homogenization caused a rearrangement of mass on the cytoplasmic surface. This may have been caused by a conformational change in the protein or by a change in the degree of proteolysis. In single experiments addition of bromelain and of imidazole was correlated to structural changes large compared with variations among specimens prepared by the standard procedures. No consistent significant structural changes were observed on addition of BaCl₂, NaCl, Ca⁺⁺, LaNO₃, HgNO₃ or acetone.

Membrane Curvature is a Natural Consequence of Connexon Structure

Gap junction plaques that are curved, annular, or vesicular have been observed under many conditions. They appear to be a common morphological feature of some tissues (see e.g., Bennett and Goodenough, 1978). It has been suggested (Peracchia, 1977; Raviola and Raviola, 1978; Raviola et al., 1980) that membrane curvature may be a consequence of uncoupling (closing of the junction channels) during preparation of tissues for ultrastructural studies. Usually curvature of the junction appears to be accompanied by a decrease in the center-to-center packing of connexons in the lattice (Bennett and Goodenough, 1978).

The symmetric nature of the gap junction structure and physiology appeared to be inconsistent with the generation of membrane curvature. Junction curvature implies nonequivalence of the two membranes making up the junction. Inasmuch as the junction morphological units are made up of two equivalent halves—two hexamers related by twofold axes in the center of the gap—it seemed unlikely that their structure could lead directly to membrane curvature. However, a structural asymmetry between the two junction membranes is generated when the 622-point group symmetry of the morphological units is not reflected in the symmetry of the gap junction lattice. As shown in Fig. 2, when the gap junction lattice symmetry is p6, the packing of connexons in the two membranes is nonequivalent.

Consider the photographs of the model in Fig. 6. These show planar lattices of morphological units with lattice symmetries p622 and p6. When there are no strong forces tending to push the units closer together the lattice will

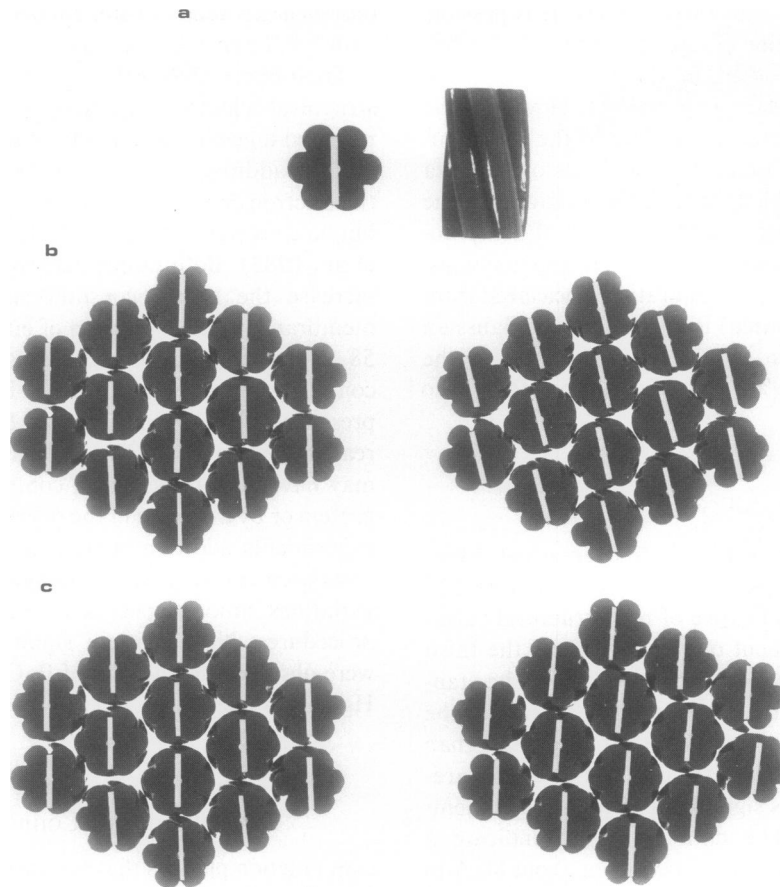


FIGURE 6 Lattices with symmetry $p6$ and $p622$. The model morphological units *a* were chosen to have proportions and tilt of subunits similar to the gap junction units. White lines correspond to the direction of one of the twofold axes of the units present at the center of the units (corresponding to the center of the gap). The lattices in *b* are arranged with $p622$ symmetry (*left*) and $p6$ symmetry (*right*). Turning these lattices over *c* shows that the packing of subunits is the same on the two surfaces of the lattice with $p622$ symmetry but different on the two surfaces of the lattice with $p6$ symmetry (cf. Fig. 2).

remain approximately planar even when its symmetry is $p6$. When a force acts to push the morphological units closer together it is possible to decrease the surface area of both membranes only so far. After that, the area of one membrane can be decreased only at the expense of some increase in the area of the other membrane. The result is a bistable system in which the gap junction surface is curved in one of two possible directions, either one being more stable than a flat membrane. This is demonstrated in the photographs of the model in Fig. 7.

In Fig. 7, the model morphological units are being pushed together by an external force (applied by elastic bands). The twisting of the subunits around their sixfold axes prevents close packing of both surfaces. One surface packs very tightly as seen in Fig. 7 *a*; the other (shown in Fig. 7 *b*) is much more open. This differential packing results in a curvature of the model as seen in the edge-on view in Fig. 7 *c*.

The nature of the force leading to the close packing of connexons and causing membrane curvature is not obvious. Uncoupling agents may induce membrane curvature by a

direct effect on connexon conformation leading to both the closing of the connexon channel and an increase in the affinity of connexons for one another. However, close packing of the units giving rise to membrane curvature could also be caused by indirect effects mediated through other cellular systems and leading to a decrease of the gap junction plaque area.

SUMMARY

Gating of the gap junction channel responsible for control of intercellular communication is accomplished by changes of connexon structure presumably triggered by the interaction of physiological effectors with the connexons. Correlating structural changes in isolated junctions with the gating mechanism is complicated by the effects of preparative procedures and the lack of a direct functional assay. Comparison of the x-ray diffraction and electron microscope data we have accumulated over the past decade from specimens prepared by a variety of methods provides a basis for identifying invariant aspects of the gap junction

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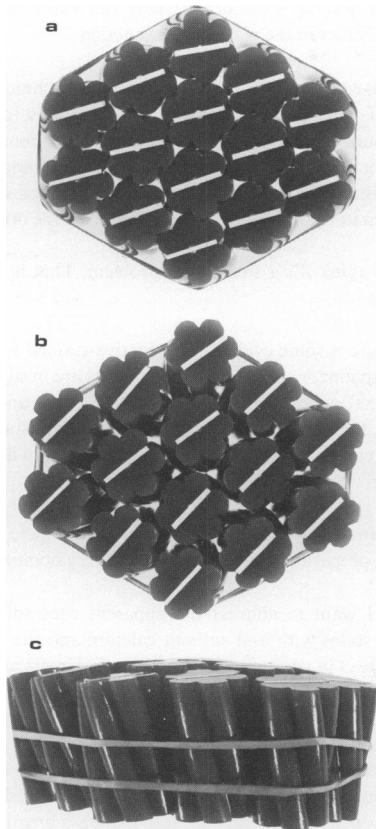


FIGURE 7 Curved lattices produced by close packing of symmetric morphological units. The area of the lattice is minimized by the pressure of two elastic bands. The top *a* and bottom *b* surfaces of the curved lattice have very different packing densities. The side view *c* shows the curvature of the lattice induced by the close packing of model units.

structure and variable properties related either to functional switching or experimental modifications.

Changes in connexon structure appear to affect lattice packing and symmetry and membrane curvature. Curvature of membrane pairs connected by morphological units that are symmetric about the center of the gap implies different packing of the connexons in the two membranes of the junction. This occurs in flat junctional membranes with $p6$ symmetry. In a closely packed membrane lattice the tilting of protein subunits in the connexons may give rise to membrane curvature.

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DISCUSSION

Session Chairman: Peter Rand *Scribes:* Donna L. Mielke, Stewart Jaslove, and Don Mackay

MONTAL: You mention the possibility of a calcium effect. Have you tested pH? Do you get any consistency with pH?

WRIGLEY: Yes, we have tested pH. One of the weaknesses of this work is that even though you can establish the pH before you look at it, when doing electron microscopy the concentration of any salts present goes through the ceiling as the sample dries down. I don't know of any way to monitor the pH continuously right down to dryness.

MONTAL: You mention that you treat your preparations with detergent. Could it be that you are losing your control or obtaining a different result because of the nature of your preparation, particularly the detergent you use?

WRIGLEY: That is also entirely possible. Since we were not looking for structure as such, merely changes thereof, we have not played around with different detergents. We have tried Lubrol, but most of the time we work with deoxycholate and try to do exactly the same procedure each time unless we're planning to do a particularly different experiment. It is bad enough that we are using detergent at all. It must be doing horrendous things to the native junction, and that may account for some of the funny things that happen.

We have, of course, tried to minutely control calcium levels. We have buffered the calcium. We have also done preps in the presence of calcium and in the presence of overkill amounts of EGTA to do the opposite thing. Every time we do that, we appear to have locked the conformation in one or another form and cannot subsequently change it again. Obviously, we have knocked out something in the junction. It is only when we do it as specified in our paper, which is essentially the Zampighi method (Zampighi and Uwin, 1979), that the stuff remains susceptible to change in our hands.

MAKOWSKI: We see a difference in preparations in which we isolate with and without deoxycholate. Deoxycholate seems to lower the lattice constant by as much as 2–4 Å, presumably by extraction of lipids from the bilayer.

BENNETT: In freeze-fracture followed by deep-etch there is no evidence that proteins stick into the cytoplasm and some evidence against it. Do you have any comment on that?

MAKOWSKI: I can't explain that. I know of no electron microscope evidence for the material we see projecting from the cytoplasmic surface. If you look at the three-dimensional reconstructions of both the negatively stained junctions and of the frozen, hydrated junctions, you don't see protein projecting into the cytoplasm. If you look at the data, in the x-ray case you find that there is unambiguous evidence for this material on the meridian, which is the direction perpendicular to the membrane plane. That is the only data that is inaccessible by electron microscopy. With the freeze-etch experiments, if these projecting portions of the proteins are not more than 10–15 Å in diameter, then you might well not see them simply because of the resolution of the deposited metal, or they may have collapsed before the replicas were made.

WRIGLEY: That is probably the answer. You do not see nearly so much detail with metal shadow. In the case of proteins sticking sideways, that is, in the plane of the membrane, I think that lattice I showed in the negatively stained picture is very probably protein, but I can't prove it. The reason I think it's protein is that, notwithstanding the inwards/outwards radial movement of the connexon protein during the structural change, the lattice constant remains the same. That suggests that there is

part of the main protein structure sticking out sideways and making protein-protein contact in the plane of the junction.

BENNETT: I have a question on the morphological techniques where you are averaging. If you have control and test conditions (and there is a tremendous amount of overlap between the two conditions looked at by these fine-structural techniques), how are you going to compare that great overlap or noise in the data with the electrical data which show that they are either all open in the one condition, or all shut in the other?

MAKOWSKI: I think it's a substantial problem. That is something we don't know how to do.

WRIGLEY: There is some evidence in negative-stained EM that in any given patch of gap junctions, individual connexons are in slightly different states of "openness" or "closedness," and that there is a range even within one patch. In the techniques we have been using with unstained material, by definition we cannot see that because we are forced to do averaging to see anything at all.

MAKOWSKI: In x-ray diffraction patterns we do have evidence that within any given preparation the structures are very homogenous.

MANNELLA: I want to address the apparent contradiction between open and closed states with and without calcium and the appearance of the junctional arrays in your unstained images. In Unwin and Zampighi's model, there is a skewing of the cylinders defining the central pore, resulting in more of an opening on the extracellular side while the state of the channel on the cytoplasmic side might be functionally closed. Your projection images could, then, reflect a different averaging out of the total mass in the central region. Thus the state which is expected to look closed might look more open in projection because there is a broader distribution of the mass in the channel region.

Also, the channel might collapse when air-dried in the absence of supporting material. Have you tried glucose-embedding, which might better preserve the structure of the interior of the channel?

WRIGLEY: That is certainly a worry, and we tried glucose many times. It had no effect whatsoever. We also tried with all kinds of variations of the auro-thio-glucose but it made no difference. It is the lack of contrast without stain that I think is precluding recovery of high-resolution information. We are using exactly the same low-dose routines and otherwise the same procedures. The reflections are just as sharp as far as they go, but yet we cannot recover anything further than 18 Å. This must be for lack of a sufficient number of units to average.

With regard to the redistribution making an appearance of open or closed, I buy that entirely. I keep saying that I don't like to use these words, "open" and "closed," and neither does Nigel Unwin. The fact that my observation seems to be opposite from other observations with calcium could easily be explained in that way.

An alternative explanation for this conflict is that the communicating channel is not down the pore through the middle, but is around the outside, which is what opens up when the pore closes. We don't know anything in detail about the distribution of lipid in that space. I'd guess Lee Makowski doesn't know too much about the detailed distribution of lipid in that space, either. I presume there is not any left in mine because we took most of it out. It is difficult to postulate that a channel exists outside of that connexon.

MAKOWSKI: In our preparations there is quite a lot of lipid; we can see that in three-dimensional maps. With regard to the work of Unwin and Zampighi, the state that they implied was closed did have more stain in the channel than the state that they implied was open. That would be consistent with Nick Wrigley's results suggesting the channel is open in the presence of calcium and closed without calcium. There certainly appeared to be more material along the sixfold axis in the absence of

calcium than in its presence. However, Nick's results were with unstained material. When you take an electron micrograph of unstained material, you tend to get a projection of the protein through the entire thickness of the structure. He sees essentially the same optical density on the axis as in the area of the protein wall of the channel; and this would imply that there is a great deal of protein moving into that channel under his conditions.

MANNELLA: That is at the resolution cut-off, though, ~20–25 Å.

WRIGLEY: In the unstained preparation it is a little better than that; 18 Å. Without stain, what little electron scattering you get is from the protein itself, so what was white in those pictures is supposed to represent protein. Nevertheless, the total levels of electron scattering are lowered so much in the unstained situation that even tiny differentials of contrast could get lost. But I think those pictures genuinely reflect a presence of protein right there on the axis. If the channel were constricted, but not completely closed under those conditions, you would need only the slightest tilt off direct normal viewing to make it seem closed because you are integrating the protein scattering through the double layer.

MANNELLA: What worries me are the studies showing that air drying can be very denaturing. Maybe by air drying unsupported crystalline arrays of the gap junction you are preserving the outside structure, but losing the structure in the channel. The only way to test this is by imaging with something in the channel to preserve the structure.

WRIGLEY: That's right, but when we put things into the gaps, it made no difference.

MAKOWSKI: We found from the x-ray results that dehydration tends to cause the gap to collapse.

WRIGLEY: Remember that in our results, there was no reason why one of them should have collapsed any more or less than the other. These unstained preparations are identical, apart from the amounts of calcium and EGTA.

ROSE: Dr. Wrigley, have you tried EDTA or EGTA on stained preparations?

WRIGLEY: Yes we have. But remember that with the stained preparations we get the "closed" appearance associated with 1,0 crystallographic reflections. That is the same appearance that is induced by the addition of EGTA, so it is difficult to say whether we have done anything to it.

ROSE: Concerning the "extraconnexon matrix," why do you say the lattice constant cannot be maintained without it?

WRIGLEY: If there is part of the lipid ocean remaining in there, that could support the protein connexons floating in it and they could maintain the same spacing, I quite agree.

ROSE: Do you mean that you maintain lattice constancy during changes in calcium or EGTA?

WRIGLEY: That is exactly what I mean. I did not measure any lattice constant prior to DOC extraction as Lee Makowski did, but I am talking specifically about the lattice constant as a "nonfunction" of calcium/EGTA. It remains constant, within ~2%, during those transitions.

ROSE: I still don't see why the matrix is necessary. Couldn't forces be acting between the subunits?

WRIGLEY: The rest of the protein is migrating radially. That ring of six objects forming the hexamer changes its diameter. In the "open" case, it is a large ring, and the rings all seem to be almost touching each other with a

large hole in the middle. In the "closed" case, when the hole disappears, the rings contract and there is a large space around them. I doubt whether forces would act across the 20 Å of open space between connexons in the contracted condition. There's got to be something there and we have shown that there is. I hesitate yet to suggest what it is.

ROSE: Dr. Makowski, could you give some rationale for using thiazine, and what were the conditions — was calcium present?

MAKOWSKI: No, calcium was not present. Thiazine was used at very, very low concentration, calculated to be close to physiological micromolar levels. The structural changes that we saw with the x-ray diffraction indicated that there was some change in both the structure and lattice constant.

DORSET: The objective lens transfer function of an electron microscope is woefully nonlinear, so you are compensating a spherical aberration term with a defocus. As is well known, Scherzer focus allows the highest resolution transfer of image information possible with all spatial frequencies combined at the same contrast sign. However, the lowest angle information is somewhat suppressed in favor of higher resolution detail at this defocus. Thus, I am wondering about your imaging conditions, whether you are near Scherzer focus, or maybe 2,000-Å defocus so that you are maximizing the transfer function to emphasize the low angle detail.

WRIGLEY: We do not routinely do any electron diffraction, but we do routinely diffract all our pictures optically and make absolutely sure that any picture we plan to use has the first zero of the transfer function comfortably outside of the highest reflections we are getting. Anything else is thrown out.

DORSET: Because the transfer function does not enter into the electron diffraction pattern, this will be an independent check of the resolution of the sample itself.

WRIGLEY: It could be an independent check. Are you suggesting that we work below Scherzer so as to gain a little more contrast and that there is no sense, at 10 Å, in pushing our first zero out to three? Yes, we do bring the first zero in by going below Scherzer and get some more contrast. When we have accidentally hit Scherzer, and therefore lost some contrast, we still tend to see the same number of reflections. That begs the question of whether we might have seen more had we had more contrast. Maybe we should try that.

DORSET: One problem we have had is that our matrix porin lattices have crystalline disorder. The best resolution we have seen so far has been in electron diffraction patterns, which are often arced. Unfortunately, in our case, the resolution possible is presently somewhat less than that afforded by the transfer function or permitted by low-dose techniques.

Another question: do you also plan to get a three-dimensional structure?

WRIGLEY: It would be nice to do so. I feel a higher priority is to correlate this supposed structural change with physiological function. This is the one burning thing that is totally lacking with gap junction. We do not even know that this hexagonal structure, which has hexameric objects some 80 Å apart, is the structure responsible for transmitting ions and small molecules between cells. This is an assumption.

WALLACE: Dr. Wrigley, you are concerned about variation between specimens, but you did not mention the radiation dose you are giving the sample. Based on the fact that you are not using glucose or low temperature, this can be a substantial source of change in structure. Are you doing temperature factor (B) corrections on your data?

WRIGLEY: You cannot correct for radiation damage. We do what we can to eliminate it by going to extremely low doses. I am not prepared to quote how low because the ways of measuring these low doses are subject to so many variations. But the point of the whole procedure we use is that it is designed to achieve low dosage on the one hand and the necessary precision of defocus on the other. It is not easy to do both of those things together. You can do it on a random basis, and I have a procedure that gets a success rate of ~80% because you can hit it right the first time. We do not know how much radiation damage there was, but repeating information extended in the stained material to nearly 10 Å.

WALLACE: Regarding Lee Makowski's work in which he sees a 4.7 Å band attributable to β sheet-type structure, given the proliferation of models that we have seen at this meeting, which are based on α -helical structures in membranes, I think it is refreshing to see that there is structural evidence that β sheet can also exist in membranes.

WOJTCZAK: Dr. Makowski, in your sucrose solution, did you have EGTA buffer? So-called ultra-pure sucrose can have up to 10^{-6} M calcium in it.

MAKOWSKI: No. Up to now the sucrose experiments have been done without EGTA. I am quite sure there was calcium present.

WOJTCZAK: Why, then, have you hypothesized that the channel in physiological condition is closed? You do not have physiological conditions.

MAKOWSKI: No, they are not physiological conditions. The channel in our preparation is closed to the penetration of sucrose. Now that we know that, we are trying to do experiments in low calcium to see if we can open the channel.

WOJTCZAK: Dr. Wrigley, you had 10^{-4} M calcium all the time?

WRIGLEY: No, that was the minimum that made it work. Sometimes, it has been 10-fold higher than that in order to achieve the transition.

WOJTCZAK: If you have added some EGTA, considering it is a calcium buffer, you still have a lot of calcium, which may be above the physiological level. According to what is known about the cardiac gap junction, there are two calcium binding sites: a high affinity site saturating at $\sim 10^{-6}$ M calcium and a low affinity site saturating at 10^{-4} M calcium. It is not clear which site is related to uncoupling. Your data probably have nothing to do with the coupling-uncoupling transition, because the gap junctions are already uncoupled. What you show is probably a delayed effect of high calcium on already uncoupled junctions. The fact that you find connexons condensed may explain discrepancies that exist in the literature concerning the connexon diameter changes.

WRIGLEY: I agree with this suggestion absolutely. It is entirely possible that the structural transition that we see may have nothing to do with coupling and uncoupling. Other workers suggest that the coupling-uncoupling transition is one of formation or dispersal of the lattice itself.

MAKOWSKI: Detailed structural studies can only be done on gap junctions in a hexagonal lattice, and the danger always exists that the connexons in the open state never form a hexagonal lattice.

ADELMAN: I tend to think that what x-ray diffraction tells you is closer to the truth than what you see with electron microscopy. If you can go to tomographic electron microscopy, I think you might be able to resolve questions about whether that protein projects beyond the lipid membrane as well as several other questions for which you are now dependent on the diffraction pattern. If you can get at either of the inner surfaces, then

going back to the old Cecil Hall shadow casting might do this, particularly if you could control the angle at which you are shadowing. This is simply a trigonometric problem of figuring out the height if the cast is made correctly. What we really see is a heavy metal replica of a surface. Whether this is done by making a cast or a shadow cast, or staining or negative staining, or whatever, that is a heavy metal replica.

WRIGLEY: I too believe the x-ray diffraction results, but there's one unfortunate thing about it. It lacks phase information.

MAKOWSKI: Because of the phase problem in x-ray diffraction, without the electron microscopy we would never have been able to get so far. We have learned a great deal about the gap junction structure from electron microscopy.

ADELMAN: I agree. Both are needed.

DONOVAN: Is there any way your methods can tell us what open and closed channels look like? You have an isolated membrane with no control of voltage across it and presumably no potential at all. Because the junction itself is potential dependent, is it even possible for you to manipulate the system, in addition to adding calcium, that you might get it in a state where you would expect it to be open or closed? Or is there a way in which you could make your preparations so that you could control potential?

WRIGLEY: The kind of situation one has to set up is, as you suggest, the situation where you can control the ionic environment of the gap junction and then measure the consequences of that in an electrophysiological sense. You would then put the same material on your electron microscope grids and look at the corresponding structure.

SPRAY: A general comment on the structure-function studies should be added. For liver gap junctions, gating mechanisms are thus far unknown. This is not really a criticism of the structural studies so far. But it points out a need to correlate an unknown functional state with what is becoming a known structural one.

WRIGLEY: It is unfortunate that we do not even know that it is that structure which conveys material back and forth.

SPRAY: An example may illustrate my point. We have also been interested in the question of whether there is a structural correlation with functional closure of gap junctions. Bob Hanna and Tom Reese have looked with ultra-low temperature freeze-fracture techniques at the structure of gap junction particles in tunicate heart (Hanna et al., 1981; see Spray et al., this meeting). Unfixed, rapidly frozen material was fractured at very low temperature and rotary shadowed. Both the control and tissues uncoupled by 5 min of CO₂ exposure produced the same sort of images. After the tissue had been in an uncoupled state for ~1 h, the particle arrangement was much more compact and regular. Our message for people doing structural studies is please, consider the functional change. As illustrated in this study, the short-term uncoupling effect is not correlated with a noticeable structural change, but the long-term exposure, which does not produce any additional physiological effect, is correlated with a gross structural change in the particle distribution.

MAKOWSKI: Is it possible that what is going on here is that at the onset of your application of the decoupler, you had a structural change in the connexons which broke off communication between the coupled cells and, at the same time, changed the structure of the connexons so that they were in a state in which they could form crystals? The formation of protein crystals in soluble proteins is a very slow process. It is possible that over a period of 1 h they could form a hexagonal lattice which might be inaccessible to connexons in the open state.