Gap Junction Structures after Experimental Alteration of Junctional Channel Conductance

THOMAS M. MILLER and DANIEL A. GOODENOUGH
Department of Anatomy, Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT Gap junctions are known to present a variety of different morphologies in electron micrographs and x-ray diffraction patterns. This variation in structure is not only seen between gap junctions in different tissues and organisms, but also within a given tissue. In an attempt to understand the physiological meaning of some aspects of this variability, gap junction structure was studied following experimental manipulation of junctional channel conductance. Both physiological and morphological experiments were performed on gap junctions joining stage 20–23 chick embryo lens epithelial cells. Channel conductance was experimentally altered by using five different experimental manipulations, and assayed for conductance changes by observing the intercellular diffusion of Lucifer Yellow CH. All structural measurements were made on electron micrographs of freeze-fracture replicas after quick-freezing of specimens from the living state; for comparison, aldehyde-fixed specimens were measured as well. Analysis of the data generated as a result of this study revealed no common statistically significant changes in the intrajunctional packing of connexons in the membrane plane as a result of experimental alteration of junctional channel conductance, although some of the experimental manipulations used to alter junctional conductance did produce significant structural changes. Aldehyde fixation caused a dramatic condensation of connexon packing, a result not observed with any of the five experimental uncoupling conditions over the 40-min time course of the experiments.

Gap junctions are thought to be the structures through which cells exchange small molecules (15, 49). Although this view has not been rigorously proven, available evidence, especially intracellular antiserum injections, provides strong support (3, 17, 25, 51, 61). Examination of gap junctions *en face* reveals the presence of subunits (connexons) that can be hexagonally packed (6, 50, 52), and freeze-fracture observations demonstrate the presence of connexons as intramembrane particles (12, 19, 34).

Physiological experiments indicate that the low-resistance pathways between cells can be switched to a high-resistance state by experimental manipulation (1, 5, 33, 37, 58). These observations raise the question of whether gap junctions between cells joined by high-resistance channels are structurally different from their counterparts in the low-resistance state (1). Thin-section studies of high-resistance junctions show them to be either normal in appearance (40) or split apart (18, 39).

Observations of negatively stained and freeze-fractured junctions revealed that connexons can be packed into more than one configuration, and it was proposed that a condensed arrangement of connexons is associated with a specific state of junctional conductance (41). To test this hypothesis, the correlation of gap junction structure and function has been measured by estimating the degree to which connexons are in a condensed configuration. Studies using chemical fixation to preserve both control and experimental gap junctions have been performed on a variety of tissues (2, 4, 10, 13, 14, 23, 27, 30, 32, 35, 38, 42–46, 55). Only a few have determined the physiological state in the same system studied anatomically (13, 14, 23, 24, 32, 40, 44).

Most studies conclude that uncoupled gap junctions have their connexons ordered in a more condensed or crystalline configuration than do control junctions. In many of the structural studies, however, the physiological state after exposure to uncoupling conditions is inferred from data gathered in other laboratories, sometimes in different tissues and organisms. Chemical fixation may take from seconds to minutes to preserve the structure of the gap junction actually visualized in the electron microscope, and it has been shown

that, in some cases, fixation alters connexon packing (57) and induces a switch from low to high resistance (8, 28).

To avoid the problems associated with chemical fixation of the tissues, it is necessary to freeze the specimens directly. Quick-freezing or fresh-freezing techniques have been applied to the study of gap junction structure (4, 21, 24, 27, 36, 38, 46, 48, 56, 57). Only some of these studies have examined the structure of gap junctions that were exposed to uncoupling conditions (21, 24, 27, 46, 48).

The purpose of the present investigation was to directly correlate the structure of the gap junction, preserved by quick-freezing, with the physiological state of the low-resistance pathways. The epithelium of the embryonic chick lens was chosen as the experimental preparation because the lens can be removed intact and easily maintained in vitro. The lens epithelium is accessible with microelectrodes, and it is thin enough that the entire thickness of the cells can be quick-frozen with satisfactory preservation.

At the present time, there is no known method to specifically switch all the intercellular channels from low to high resistance such that they can be studied after the quick-freeze technique. Experimental manipulations that do uncouple cells may cause a variety of other cellular responses as well. Therefore, it is not possible to unequivocally relate any structural change in gap junctions directly with physiological response, since one could be looking only at second- or third-order effects. In an attempt to circumvent this problem, lens epithelial cells were uncoupled by exposure to a number of different experimental conditions. We reasoned that if a specific packing arrangement of connexons was associated with the uncoupled state, then this arrangement should be common to all the experimental manipulations.[1]

A panel of reagents was identified that reproducibly altered junctional conductance, and the freeze-fracture morphology of quick-frozen gap junctions from uncoupled lens epithelial cells was compared with that from coupled cells. A number of structural parameters were quantitated, which were designed to measure the degree to which connexons are laterally associated with each other in the plane of the membrane. Measurement of the chosen structural parameters involved either the scoring of all-or-nothing events or the measurements of areas; parameters such as particle size and center-to-center spacing, which require precise measurement of very small linear distances on the replicas, were avoided.

MATERIALS AND METHODS

Fertilized leghorn chicken eggs (Spafas, Inc., Norwich, CT) were incubated at 37°C for 4 d, in order to obtain embryos from developmental stages 20–23 (22). Lenses were removed from the embryos by first cutting away the corneas and then dissecting the lenses from the surrounding optic cups. All dissections were done in Eagle's minimal essential medium with Earle's salts (control medium) (Gibco Laboratories, Grand Island, NY) equilibrated with 95% O_2/5% CO_2 at 37°C. The lenses were then used either for dye transfer or ultrastructural experiments.

Dye Transfer Experiments

Lenses were mounted in lucite chambers fitted with a No. 2 coverglass bottom. A fragment of a glass microscope slide was attached to the bottom of the chamber with vacuum grease, and the edge of the slide and adjacent coverglass charged by soaking the glass surfaces with a drop of 0.1% L-polylysine (Sigma Chemical Co., St. Louis, MO) for 2–3 min. A dissected lens was then positioned on edge in the chamber so that the posterior surface of the lens lay against the edge of the slide while the equator of the lens touched the bottom of the chamber (Fig. 1). The chamber was mounted in a custom-built stage for the Zeiss IM-35 inverted microscope (Carl Zeiss, Inc., Thornwood, NY) and held at 37°C by a peltier thermionic stage heater (Midland-Ross Corp., Cambridge, MA). The orientation of the lens permitted an edge-on view of optical sections of the epithelium and underlying lens fibers (Fig. 1). The lens capsule overlying the epithelium was digested by a localized manual pressure-injection of 400 μl of 1% collagenase and hyaluronidase (Sigma Chemical Co.) over 2 min through a hand-drawn glass capillary, followed immediately by three complete medium changes in the chamber.

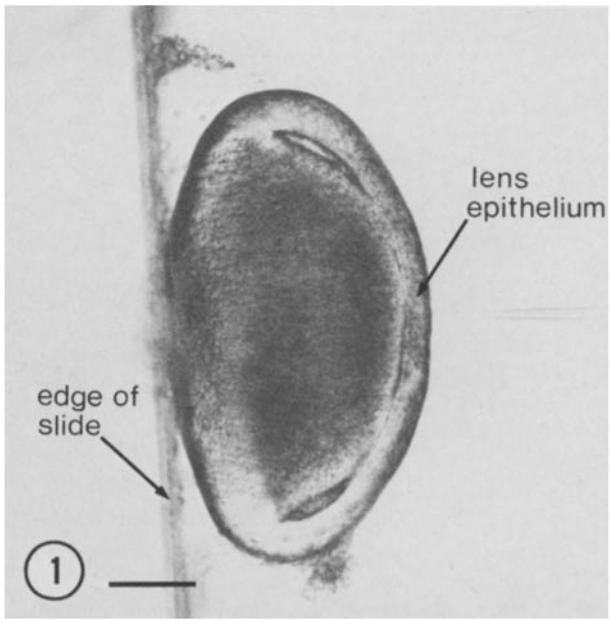

FIGURE 1 A bright-field micrograph of a living lens mounted for a physiology experiment. The lens is attached to a polylysine-coated fragment of a glass slide, such that the lens epithelium can be directly approached with a fluorescent dye-filled microelectrode. Bar, 0.1 mm. × 115.

The state of cell–cell communication between developing lens epithelial cells was assessed by impaling single epithelial cells with a glass microelectrode, followed by iontophoretic injection of the fluorescent dye Lucifer Yellow CH, as previously described (54). Dye was injected by passing 50-ms, 1-nA pulses at 10 Hz for 2–3 min. After a control injection, each lens was placed in one of several experimental media (see below). Several injections were made in the experimental medium, followed by returning to the control medium for recovery and a final injection to assay for reversibility of any observed block in dye transfer. Epifluorescence and bright-field photographs of the lenses were recorded at all stages of the experiments on 35-mm Tri-X film (Eastman Kodak Co., Rochester, NY) exposed at ASA 800.

Ultrastructural Experiments

Lenses were dissected from embryos and incubated in control medium or in experimental medium under identical conditions to those described above. The lenses were then quick-frozen against a liquid helium–cooled copper block (26) with the lenses oriented so that the lens epithelium contacted the copper block. This produced satisfactory freezing for the entire thickness of the lens epithelium. The specimens were then superficially fractured in a Balzers BAF301 freeze-etch machine (Balzers Corp., Nashua, NH) according to standard procedures. Replicas were examined in a JEOL 100 CX electron microscope (JEOL USA, Peabody, MA). Fracture faces of epithelial cell membranes were identified by ensuring that epithelial nuclei were present in the fracture plane of each replica, thus assuring that lens fiber membranes were not photographed. Gap junctions on these surfaces were photographed at a magnification of 33,000 for later analysis.

Other lenses were taken directly from control medium and placed in a fixative solution containing 1% paraformaldehyde, 2% glutaraldehyde, 90 mM

[1] Since, at the molecular level, there could be different changes in connexons all of which result in channel closure (58), there could also be different connexon packing patterns that result from uncoupling, if structural changes associated with uncoupling are detectable.

NaCl, 30 mM HEPES (Sigma Chemical Co.), and 5 mM $CaCl_2$. After fixing overnight, the lenses were equilibrated for 1 h with 30% glycerol in the same buffered salts used for fixation, but without aldehydes. The fixed lenses were frozen in Freon 22, and freeze-fractured. For scanning electron microscopy, lenses were fixed overnight as above, bisected with a razor blade, and postfixed in 2% OsO_4 in water for 1 h. The lenses were then critical point dried and examined in a JEOL JSM-35 scanning electron microscope as described previously (54).

Experimental Media

Five sets of uncoupling conditions were used in the dye transfer and ultrastructural experiments. All media were at 37°C, and all media were equilibrated with 95% O_2/5% CO_2, except the high-CO_2 medium. The media and incubation times were: (*a*) control medium equilibrated with 10% O_2/90% CO_2, for 10 min (60); (*b*) control medium plus 10^{-6} M A23187 (Calbiochem-Behring Corp., La Jolla, CA) (diluted from a stock of 1 mM A23187 in dimethylsulfoxide), for 40 min (49, 53); (*c*) control medium plus 3.5 mM *n*-heptanol, for 30 min (29); (*d*) modified Earle's salts solution with the 116 mM NaCl replaced by 90 mM Na acetate, buffered to pH 6.0 with 25 mM 2, (*N*-morpholino)ethane sulfonic acid (Sigma Chemical Co.) for 40 min (9); (*e*) control medium plus 2 mM each of NaCN and iodoacetate, for 10 min (16, 47).

Quantitative Analysis of Gap Junction Structure

The image of each freeze-fractured gap junction was printed to a final magnification of 100,000. A group of 15 consecutive photographs of gap junctions was selected at random from the 25–50 photographs taken from each lens. The first 10 of these were analyzed, with the extra photographs being used to replace any of the first 10 that could not be analyzed for technical reasons. To gather data on the in-plane packing of the connexons, each gap junction was analyzed in the following manner: (*a*) every connexon was counted and scored as to how many other connexons it touched; (*b*) the junctional area was measured with a Zeiss MOP-3 image analyzer (Carl Zeiss, Inc.); and (*c*) the area, if any, of condensed, crystallized connexons within a gap junction was measured. Junctional area is the area circumscribed by a perimeter drawn as closely as possible around all the connexons actually analyzed within a junctional plaque. The touching of connexons is a subjective parameter judged by eye. Ideally, this would represent connexons whose centers were separated by diameter of a connexon. In practice, however, given the large numbers of connexons counted, variations in connexon size due to amount of platinum, shadow angles, degree of distortion during fracturing, etc., it was not feasible to make this judgment with this degree of accuracy. The score given to each connexon was from 0 to 6, 0 for a connexon standing alone, and 6 for a connexon participating in a tight hexagonally packed array with 6 neighbors. The junctional area was the analyzed area of a junction; this was often different from the total area of the junction since some of the junction might have been hidden by debris, shadowed poorly, or had left the plane of fracture. The condensed area was that part of the junction where the connexons were arranged along hexagonal lattice lines; it is important to note that, due to short-range disorder, the connexons in condensed areas did not necessarily touch all of their nearest neighbors (11).

The data gathered from the micrographs were used to calculate five structural parameters, which were then used to compare the structure of experimentally uncoupled gap junctions with the structure of control gap junctions. The first parameter is the concentration of connexons in the junctional area; parameters 2 and 3 deal with the amount of condensed area; and parameters 4 and 5 are measures of the associations of individual connexons with nearest neighbors. The structural parameters are formally defined as follows: parameter 1 is the number of connexons per unit junctional area; parameter 2 is the amount of condensed area per unit junctional area, considering only those gap junctions that have some condensed area; parameter 3 is the ratio of the number of gap junctions that have some condensed area to the total number of gap junctions in that experimental group; parameter 4 is the ratio of the number of connexons that touch at least one other connexon to the number of connexons that stand alone; parameter 5 is the ratio of the number of connexons that touch three or more other connexons to the number of connexons that touch two or less other connexons.[2] Parameters 1, 2, 4, and 5 were calculated for each gap junction, which yielded four sets of numbers for each control or experimental group. It is these numbers, and therefore the individual gap junctions, that served as data points for comparison of the groups (even though the data in each group was gathered from more than one lens; see Table II).

Two control groups were used in these experiments, a 0-min control (lenses quick-frozen as soon as possible after dissection) and a 40-min control (lenses quick-frozen after a 40-min incubation in control medium at 37°C). The two control groups spanned the total incubation times for the experimental groups and established a range of structural variation that could take place on this time scale without there being any discernible physiological changes. Each experimental group was compared with both control groups. The controls differ from each other, representing a range of structural variability which could be neither experimentally controlled nor interpreted. To detect a meaningful structural charge as a result of cell uncoupling, an experimental group should be statistically significantly different from both controls. Additionally, since all the experimental conditions resulted in a block of dye transfer, a structural change associated with the change in physiology cannot be interpreted in this study unless it is present in every experimental group. It is emphasized here that structural changes peculiar to some of the five uncoupling conditions were detected in this study, but we cannot interpret them since we cannot distinguish them from uncontrolled secondary effects of the experimental conditions.

Student's *t* test was used to make the comparisons, and *p* values were calculated both before and after a logarithmic transformation of the data. The transformation of the data did not change the pattern of the inferences drawn, but it did result in data distributions that were more symmetric (as judged by measures of skewness). The *p* values reported in Table III were obtained using the logarithmically transformed data. Frequency histograms were made, and served to demonstrate that the distributions of data were unimodal. Parameter 3 is a proportion of all-or-nothing occurrences; therefore, these data were analyzed using a χ^2 test with one degree of freedom.

RESULTS

Dye Transfer Experiments

Single embryonic lens epithelial cells were iontophoretically injected with the fluorescent dye Lucifer Yellow CH (59). Control injections invariably demonstrated dye transfer between adjacent cells (Fig. 2), even when the lenses were held in control medium for longer than 1 h (not shown). A panel of reagents was used to decrease the conductance of the low-resistance pathways as described in the Materials and Methods. When a lens was exposed to any of the uncoupling reagents for the specified time period (see Materials and Methods), an effective block in dye transfer was demonstrable (Fig. 2 and Table I). This change in the physiological state of the lens epithelial cells could be reversed by a return to control medium for three of the five uncoupling conditions (Fig. 2 and Table I). We were not able to detect reversal in the cells injected during the uncoupled condition.

Ultrastructural Experiments

Table II lists the number of lenses, the number of gap junctions, the total junctional area, and the number of connexons analyzed for each condition used.

Embryonic lenses were quick-frozen under conditions identical to those known to cause a block of dye transfer. Control lenses were quick-frozen either immediately after dissection (0-min control) or after a 40-min incubation in control medium (40-min control). Other lenses were aldehyde fixed, cryoprotected, and frozen conventionally. Micrographs of freeze-fractured gap junctions from each condition are presented in Fig. 3.

Five structural parameters that were measures of the degree of connexon–connexon association were developed, and each group of gap junctions was analyzed and quantitated. Each of the structural parameters was a ratio, and all five parame-

[2] Parameter 5 separates connexons in rows and pairs, or standing alone, from those that tend to crystallize in two dimensions, with the exception of a group of three connexons all contracting the other two, which do show two-dimensional grouping and yet have only two partners each.

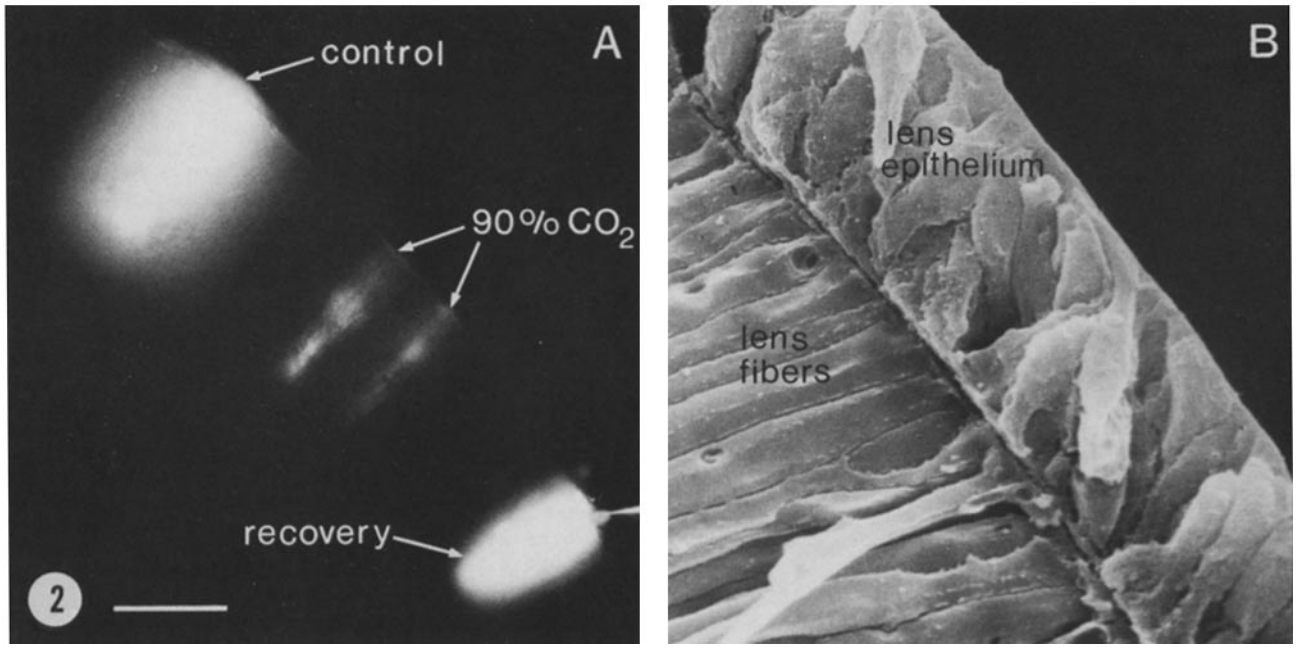

FIGURE 2 (*A*) Fluorescence micrograph of a living whole-mount lens (see Fig. 1) that has been through a series of experiments. A control injection was made first, then the control medium was replaced with the 90% CO_2-equilibrated medium. Two experimental injections were then made, each filling a single cell with dye. After the return of the lens to control medium, a fourth injection was made, demonstrating the recovery of dye transfer. A scanning electron micrograph of an embryonic lens is included in *B*, so that the orientation of the specimen in *A* can be better appreciated. Bar, 25 μm. × 600.

TABLE I. *Physiological Data**

Experimental condition	Dye transfer not blocked	Dye transfer blocked	Recovered (no. attempted)
CO_2	0	16	13 (14)
A23187	2	18	0
Heptanol	0	19	8 (10)
Acetate	0	16	13 (13)
Cyanide and iodoacetate	0	15	0

* No. of embryos.

TABLE II. *Quantity of Data Considered in the Ultrastructural Experiments*

Experimental condition	No. of lenses	No. of gap junctions	Total junctional area	No. of connexons
			μm^2	
0-min control	4	40	4.34	24,150
40-min control	6	67	9.23	48,849
CO_2	6	61	6.36	37,323
A23187	6	65	7.20	42,946
Heptanol	5	55	6.30	33,700
Acetate	5	56	4.63	27,326
Cyanide and iodoacetate	5	54	4.19	26,786
Aldehyde fixation	2	20	1.34	10,822

ters became numerically larger as the gap junction connexons became more condensed. Each parameter for each condition was compared with the same parameter for both controls, since variability between the two control conditions represented structural variability that was not related to any detectable changes in the physiological state of the channels. While any structural changes observed in this study may be significant, due to the nonspecific actions of the uncoupling reagents we have elected to search for changes in structural parameters common to all uncoupling reagents. If there were a structural change observed after only one of the uncoupling conditions, then this would represent a change that could not be directly related to a switch to high resistance, given the experimental design used in this paper.

A qualitative examination of the micrographs in Fig. 3, *A–G* gave the immediate impression that none of the quick-frozen gap junctions looked detectably different from the others. The aldehyde-fixed specimen, by contrast, demonstrated a markedly more condensed arrangement of the con-

FIGURE 3 Freeze-fractured lens epithelial gap junctions from each of the experimental groups used in this study. While these gap junctions should be considered to have structures typical of the group from which they were taken, the areas shown are not necessarily representative of the entire group for all of the structural parameters studied. (*A*) 0-min control; (*B*) 40-min control; (*C*) 90% CO_2; (*D*) A23187; (*E*) heptanol; (*F*) acetate; (*G*) cyanide and iodoacetate; (*H*) aldehyde fixation. There are no obvious structural differences between the quick-frozen gap junctions (*A–G*), but the connexons of the fixed gap junction (*F*) are arranged in a noticeably more condensed or crystalline configuration. Bar, 0.1 μm. × 150,000.

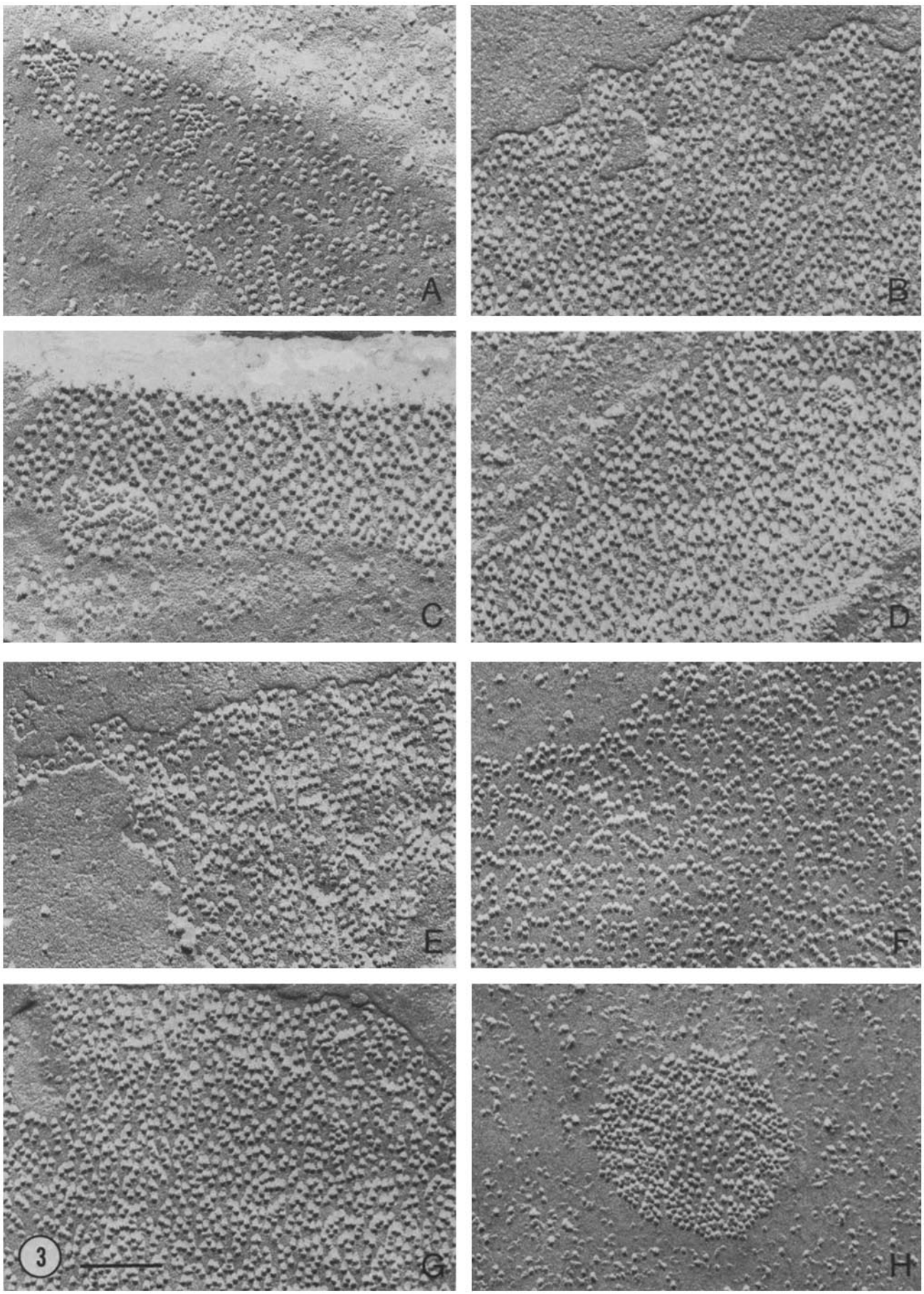
A
B
C
D
E
F
3
G
H

TABLE III. *Quantitative Analysis of Data*

Experimental condition	Structural parameters				
	1	2	3	4	5
0-min Control	6.01×10^3* (1.68×10^3)	9.90×10^{-2} (1.18×10^{-1})	0.40	3.18 (5.34)	9.78×10^{-2} (2.38×10^{-1})
	$0.6 < P < 0.7$‡	$0.1 < P < 0.2$	$0.1 < P < 0.2$	$0.01 < P < 0.05$	$0.1 < P < 0.2$
40-min Control	5.72×10^3 (1.09×10^3)	6.31×10^{-2} (6.07×10^{-2})	0.27	2.01 (1.21)	4.91×10^{-2} (6.51×10^{-2})
	$0.3 < P < 0.4$§	$0.1 < P < 0.2$	$0.1 < P < 0.2$	$P < 0.001$	$0.001 < P < 0.01$
CO_2	6.21×10^3 (1.86×10^3)	7.43×10^{-2} (8.86×10^{-2})	0.26	1.72 (8.65×10^{-1})	2.85×10^{-2} (4.16×10^{-2})
	$0.05 < P < 0.1$‖	$0.8 < P < 0.9$	$0.8 < P < 0.9$	$0.1 < P < 0.2$	$0.1 < P < 0.2$
	$0.1 < P < 0.2$	$0.05 < P < 0.1$	$0.6 < P < 0.7$	$0.3 < P < 0.4$	$0.2 < P < 0.3$
A23187	6.34×10^3 (1.31×10^3)	6.94×10^{-2} (1.06×10^{-1})	0.45	2.50 (3.08)	5.48×10^{-2} (8.25×10^{-2})
	$0.001 < P < 0.01$	$0.8 < P < 0.9$	$0.02 < P < 0.05$	$0.1 < P < 0.2$	$0.4 < P < 0.5$
	$0.5 < P < 0.6$	***0.001 < P < 0.01***	***P < 0.001***	$0.5 < P < 0.6$	$0.9 < P$
Heptanol	5.66×10^3 (1.27×10^3)	***4.59 × 10⁻²*** ***(8.87 × 10⁻²)***	***0.11***	2.93 (2.22)	1.41×10^{-1} (6.49×10^{-1})
	$0.7 < P < 0.8$	***0.01 < P < 0.05***	***0.02 < P < 0.05***	$0.001 < P < 0.01$	$0.05 < P < 0.1$
	$0.2 < P < 0.3$	$0.001 < P < 0.01$	$0.01 < P < 0.02$	$0.2 < P < 0.3$	$0.02 < P < 0.05$
Acetate	6.08×10^3 (1.29×10^3)	3.16×10^{-2} (3.97×10^{-2})	0.18	3.46 (8.71)	1.45×10^{-1} (7.53×10^{-1})
	$0.01 < P < 0.02$	$0.2 < P < 0.3$	$0.2 < P < 0.3$	$0.5 < P < 0.6$	$0.5 < P < 0.6$
	$0.01 < P < 0.02$	$P < 0.001$	***P < 0.001***	$0.001 < P < 0.01$	$0.001 < P < 0.01$
Cyanide and iodoacetate	**6.65×10^3** **(1.36×10^3)**	5.56×10^{-3} (2.30×10^{-3})	***0.04***	2.00 (2.19)	2.50×10^{-2} (3.75×10^{-2})
	$P < 0.001$	$0.05 < P < 0.1$	***P < 0.001***	$0.1 < P < 0.2$	$0.1 < P < 0.2$
	$P < 0.001$	**$0.001 < P < 0.01$**	**$P < 0.001$**	**$0.001 < P < 0.01$**	***P < 0.001***
Aldehyde fixation	**9.02×10^3** **(2.3×10^3)**	**2.52×10^{-1}** **(1.34×10^{-1})**	**1.00**	**6.88 (7.78)**	**5.38×10^{-1}** **(5.8×10^{-1})**
	$P < 0.001$	***P < 0.001***	***P < 0.001***	***P < 0.001***	***P < 0.001***

* Values given are mean (SD) for parameters 1, 2, 4, and 5; values of parameter 3 are proportions of all-or-nothing occurrences, and therefore do not include SD.
‡ *P* values that result from a comparison of the 0-min control with the 40-min control.
§ *P* values that result from a comparison of the experimental with the 0-min control.
‖ *P* values that result from a comparison of the experimental with the 40-min control.
Data in boldface type, significantly more condensed than both controls.
Data in boldface italic type, significantly less condensed than both controls.

nexons than did the quick-frozen gap junctions (Fig. 3*H*).[3]

The quantitative analysis of the data is presented in Table III. Included are means or values of the parameters, as well as the results of the statistical comparisons (see Materials and Methods for a precise definition of the structural parameters and an explanation of statistical methods). None of the structural parameters were significantly different from both controls for all five uncoupling conditions. Therefore, we have not been able to demonstrate any consistent, significant change in the in-plane packing of the connexons of gap junctions of embryonic lens epithelial cells that could be associated with a decrease in gap junctional conductance (block of dye transfer). However, examination of the data on fixed gap junctions revealed that after fixation all five parameters were significantly changed toward a more condensed morphology.

There were a few significant changes that did occur in the structures of the quick-frozen gap junctions. There was a decrease in both the amount of condensed area and the proportion of junctions with some condensed area in the heptanol group (as judged by parameters 2 and 3, see Table III). The metabolic inhibitors (cyanide and iodoacetate) affected two of the parameters in opposite ways. There was an increase in the number of connexons per unit area (parameter 1, see reference 27) and a decrease in the proportion of junctions with condensed area (parameter 3). Since these phenomena did not appear after the other uncoupling conditions, their significance was unclear, and they may be due to cellular responses to the reagents that are not directly associated with the change in conductance of gap junctions.

DISCUSSION

It has been proposed that the packing arrangement of gap junction connexons might reflect the physiological state of the gap junction (41), and that this correlation may be so strong as to permit physiological inferences to be taken from anatomical data (46). The present experiments were designed to critically test this hypothesis for the gap junctions of embryonic lens epithelial cells. The system used permitted precise control of experimental conditions in vitro, without disruption of the integrity of the organ studied. The embryonic chick lens epithelium is accessible for physiological experimentation, and it can be quick-frozen from the living state. The combination of these techniques has permitted an analysis of connexon packing arrangements in gap junctions that were in a known physiological state when quick-frozen. By quantitating five different structural parameters for each of five different treatments, all of which caused a block of junctional dye transfer, we have maximized the sensitivity of the experimental system for detecting structural changes that can be unequivocally related to the change in physiological state. A structural change that we could clearly interpret would then be manifest by a significant divergence of one or more structural parameters from control values for all five uncoupling treatments. Aldehyde-fixed specimens were also included for comparison with the quick-frozen samples.

Fig. 3 and Table III demonstrate that no consistent changes in the in-plane packing of connexons could be detected in gap junctions when the low-resistance intercellular channels were switched to an altered physiological state of low conductance. No structural parameters changed for all five conditions that blocked dye transfer. Aldehyde-fixed gap junctions were significantly more condensed than controls for all five structural parameters studied.

These results provide no support for the hypothesis that the in-plane packing of the connexons of a gap junction is strictly related to its physiology. It is clear that chemical fixation changed the structure of these junctions to such a great extent that they could no longer be compared with junctions frozen from the living state, and the fixed condition has no physiological counterpart. Conditions that resulted in a block of dye transfer did not change the structure of gap junctions in this system, but fixation did change their structure.

The conclusions drawn from this study must be limited to the lens epithelium of the chick, and they do not allow generalization to other tissues. Nonetheless, the lack of correlation found in this study, as well as the necessity of quick-freezing gap junctions from precisely known physiological states, indicate that until resolution can be increased so that the structure of individual channels can be examined, this type of approach to the study of structure/function correlations in gap junction–mediated intercellular communication is not fruitful.

We are indebted to John Rennie for his superb technical assistance.

This research was supported by grants EY02430 and GM18974 from the National Institutes of Health. Salary support from grant EY05481 is gratefully acknowledged.

Received for publication 25 June 1984, and in revised form 26 July 1985.

[3] Other reports have shown that gap junctions joining lens epithelial cells are partially crystallized after fixation (7, 20, 31, 43). Available evidence indicates that the relative positions of the connexons are more likely affected by the fixation rather than by cryoprotection (57).

REFERENCES

1. Asada, Y., G. D. Pappas, and M. V. L. Bennett. 1967. Alteration of resistance at an electrotonic junction and morphological correlates. *Fed. Proc.* 26:330a. (Abstr.)
2. Ashraf, M., and C. Halverson. 1978. Ultrastructural modifications of nexuses (gap junctions) during early myocardial ischemia. *J. Mol. Cell. Cardiol.* 10:263–269.
3. Azarnia, R., W. J. Larsen, and W. R. Loewenstein. 1974. The membrane junctions in communicating and noncommunicating cells, their hybrids and segregants. *Proc. Natl. Acad. Sci. USA.* 71:880–884.
4. Baldwin, K. M. 1979. Cardiac gap junction configuration after an uncoupling treatment as a function of time. *J. Cell Biol.* 82:66–75.
5. Barr, L., M. M. Dewey, and W. Berger. 1965. Propagation of action potentials and the structure of the nexus in cardiac muscle. *J. Gen. Physiol.* 48:797–823
6. Benedetti, E. L., and P. Emmelot. 1968. Hexagonal array of subunits in tight junctions separated from isolated rat liver plasma membranes. *J. Cell Biol.* 38:15–24.
7. Benedetti, E. L., I. Dunia, C. J. Bentzel, A. J. M. Vermorken, M. Kibbelar, and H. Bloemendal. 1976. A portrait of plasma membrane specializations in eye lens epithelium and fibers. *Biochem. Biophys. Acta.* 457:353–384.
8. Bennett, M. V. L., M. E. Spira, and G. D. Pappas. 1972. Properties of electrotonic junctions between embryonic cells of Fundulus. *Dev. Biol.* 29:419–435.
9. Bennett, M. V. L., J. E. Brown, A. L. Harris, and D. C. Spray. 1978. Electrotonic junctions between Fundulus blastomeres: reversible block by low intracellular pH. *Biol. Bull. (Woods Hole).* 155:428a–429a. (Abstr.)
10. Bernardini, G., and C. Peracchia. 1981. Gap junction crystallization in lens fibers after an increase in cell calcium. *Invest. Ophthalmol. & Visual Sci.* 21:291–299.
11. Caspar, D. L. D., D. A. Goodenough, L. Makowski, and W. C. Phillips. 1977. Gap junction structures. I. Correlated electron microscopy and x-ray diffraction. *J. Cell Biol.* 74:605–628.
12. Chalcroft, J. P., and S. Bullivant. 1970. An interpretation of liver cell membrane and junction structure based on observation of freeze-fracture replicas of both sides of the fracture. *J. Cell Biol.* 47:49–60.
13. Dahl, G., and G. Isenberg. 1980. Decoupling of heart muscle cells: correlation with increased cytoplasmic calcium and with changes of nexus ultrastructure. *J. Membr. Biol.* 53:63–75.
14. Deleze, J., and J. C. Herve. 1983. Effect of several uncouplers of cell-to-cell communication on gap junction morphology in mammalian heart. *J. Membr. Biol.* 74:203–215.
15. Dewey, M. M., and L. Barr. 1964. A study of the structure and distribution of the nexus. *J. Cell Biol.* 23:553–585.
16. Flagg-Newton, J., and W. R. Loewenstein. 1979. Experimental depression of junctional membrane permeability in mammalian cell culture. A study with tracer molecules in the 300–800 Dalton range. *J. Membr. Biol.* 50:65–100.
17. Gilula, N. B., O. R. Reeves, and A. Steinbach. 1972. Metabolic coupling, ionic coupling, and cell contacts. *Nature (Lond.).* 235:262–265.

18. Goodenough, D. A., and N. B. Gilula. 1974. The splitting of hepatocyte gap junctions and zonulae occludentes with hypertonic disaccharides. *J. Cell Biol.* 61:575–590.
19. Goodenough, D. A., and J. P. Revel. 1970. A fine structural analysis of intercellular junctions in the mouse liver. *J. Cell Biol.* 45:272–290.
20. Goodenough, D. A., J. S. B. Dick II, and J. E. Lyons. 1980. Lens metabolic cooperation: a study of mouse lens transport and permeability visualized with freeze-substitution autoradiography and electron microscopy. *J. Cell Biol.* 86:576–589.
21. Green, C. R., and N. J. Severs. 1984. Gap junction connexon configuration in rapidly frozen myocardium and isolated intercalated discs. *J. Cell Biol.* 99:453–463.
22. Hamberger, V., and H. L. Hamilton. 1951. A series of normal stages in the development of the chick embryo. *J. Morphol.* 88:49–92.
23. Hanna, R. B., G. D. Pappas, and M. V. L. Bennett. 1984. The fine structure of identified electrotonic synapses following increased coupling resistance. *Cell Tissue Res.* 235:243–249.
24. Hanna, R. B., T. S. Reese, R. L. Ornberg, D. C. Spray, and M. V. L. Bennett. 1981. Fresh frozen gap junctions: resolution of structural detail in the coupled and uncoupled states. *J. Cell Biol.* 91 (2, Pt. 2):125a. (Abstr.)
25. Hertzberg, E. L., D. C. Spray, and M. V. L. Bennett. 1985. Reduction of gap junctional conductance by microinjection of antibodies against the 27-kDa liver gap junction polypeptide. *Proc. Natl. Acad. Sci. USA.* 82:2411–2416.
26. Heuser, J. E., T. S. Reese, M. J. Dennis, Y. Jan, L. Jan, and L. Evans. 1979. Synaptic vesicle exocytosis captured by quick-freezing and correlated with quantal transmitter release. *J. Cell Biol.* 81:275–300.
27. Hirokawa, N., and J. Heuser. 1982. The inside and outside of gap-junction membranes visualized by deep-etching. *Cell.* 30:395–406.
28. Johnston, M. F., and F. Ramon. 1981. Electrotonic coupling in internally perfused crayfish segmented axons. *J. Physiol.* 317:509–518.
29. Johnston, M. F., S. A. Simon, and F. Ramon. 1980. Interaction of anaesthetics with electrical synapses. *Nature (Lond.).* 286:498–500.
30. Kistler, J., and S. Bullivant. 1980. The connexon order in isolated lens gap junctions. *J. Ultrastruct. Res.* 72:27–38.
31. Kuszak, J. R., J. L. Rae, B. V. Pauli, and R. S. Weinstein. 1982. Rotary replication of lens gap junctions. *J. Ultrastruct. Res.* 81: 249–256.
32. Lee, W. M., D. G. Cran, and N. J. Lane 1982. Carbon dioxide induced disassembly of gap junctional plaques. *J. Cell Sci.* 57:215–228.
33. Loewenstein, W. R., M. Nakas, and S. J. Socolar. 1967. Junctional membrane uncoupling. Permeability transformations at a cell membrane junction. *J. Gen. Physiol.* 50:1865–1891.
34. McNutt, N. S., and R. S. Weinstein. 1970. The ultrastructure of the nexus. A correlated thin-section and freeze-cleave study. *J. Cell Biol.* 47:666–688.
35. Meda, P., I. Findlay, E. Kolod, L. Orci, and O. H. Petersen. 1983. Short and reversible uncoupling evokes little change in the gap junctions of pancreatic acinar cells. *J. Ultrastruct. Res.* 83:69–84.
36. Nagano, T., Y. Toyama, and F. Suzuki. 1982. Further observations on the Sertoli cell junctions of the mouse testis after metal contact freeze-fracture, and comparisons with cellular junctions of other epithelial cells. *Am. J. Anat.* 163:47–58.
37. Nakas, M., S. Higashino, and W. R. Loewenstein. 1966. Uncoupling of an epithelial cell membrane junction by calcium ion removal. *Science (Wash. DC).* 151:89–91.
38. Page, E., T. Karrison, and J. Upshaw-Earley. 1983. Freeze-fractured cardiac gap junctions: structural analysis by three methods. *Am. J. Physiol.* 244:H525–H539.
39. Pappas, G. D., Y. Asada, and M. V. L. Bennett. 1971. Morphological correlates of increased coupling resistance at an electrontonic synapse. *J. Cell Biol.* 49:173–188.
40. Payton, B. W., M. V. L. Bennett, and G. D. Pappas. 1969. Temperature-dependence of resistance at an electrotonic synapse. *Science (Wash. DC).* 165:594–597.
41. Peracchia, C. 1973. Low resistance junctions in crayfish. I. Two arrays of globules in junctional membranes. *J. Cell Biol.* 57:54–65.
42. Peracchia, C. 1977. Gap junctions. Structural changes after uncoupling procedures. *J. Cell Biol.* 72:628–641.
43. Peracchia, C. 1978. Calcium effects on gap junction structure and cell coupling. *Nature (Lond.).* 271:669–671.
44. Peracchia, C., and A. F. Dulhunty. 1976. Low resistance junctions in crayfish. Structural changes with functional uncoupling. *J. Cell Biol.* 70:419–439.
45. Peracchia, C., and L. L. Peracchia. 1980. Gap junction dynamics: reversible effects of divalent cations. *J. Cell Biol.* 87:708–718.
46. Peracchia, C., and L. L. Peracchia. 1980. Gap junction dynamics: reversible effects of hydrogen ions. *J. Cell Biol.* 87:719–727.
47. Politoff, A. L., S. J. Socolar, and W. R. Loewenstein. 1969. Permeability of a cell membrane junction. Dependence on energy metabolism. *J. Gen. Physiol.* 53:498–515.
48. Raviola, E., D. A. Goodenough, and G. Raviola. 1980. Structure of rapidly frozen gap junctions. *J. Cell Biol.* 87:273–279.
49. Reed, P. W., and H. A. Lardy. 1972. A23187: a divalent cation ionophore. *J. Biol. Chem.* 247:6970–6977.
50. Revel, J. P., and M. J. Karnovsky. 1967. Hexagonal array of subunits in intercellular junctions of the mouse heart and liver. *J. Cell Biol.* 33:C7–C12.
51. Revel, J. P., A. G. Yee, and A. J. Hudspeth. 1971. Gap junctions between electrotonically coupled cells in tissue culture and in brown fat. *Proc. Natl. Acad. Sci. USA.* 68:2924–2927.
52. Robertson, J. D. 1963. The occurrence of a subunit pattern in the unit membranes of club endings in Mauthner cell synapses in goldfish brains. *J. Cell Biol.* 19:201–221.
53. Rose, B., and W. R. Loewenstein. 1975. Permeability of cell junction depends on local cytoplasmic calcium activity. *Nature (Lond.).* 254:250–252.
54. Schuetze, S. M., and D. A. Goodenough. 1982. Dye transfer between cells of the embryonic chick lens becomes less sensitive to CO_2-treatment with development. *J. Cell Biol.* 92:694–705.
55. Shibata, Y., and E. Page. 1981. Gap junctional structure in intact and cut sheep cardiac Purkinje fibers: a freeze-fracture study of Ca^{2+}-induced resealing. *J. Ultrastruct. Res.* 75:195–204.
56. Shibata, Y., K. Nakata, and E. Page. 1980. Ultrastructural changes during development of gap junctions in rabbit left ventricular myocardial cells. *J. Ultrastruct. Res.* 71:258–271.
57. Sikerwar, S., and S. Malhotra. 1981. Structural correlates of glutaraldehyde-induced uncoupling in mouse liver gap junctions. *Eur. J. Cell Biol.* 25:319–323.
58. Spray, D. C., R. L. White, A. Campos de Carvalho, A. L. Harris, and M. V. L. Bennett. 1984. Gating of gap junction channels. *Biophys. J.* 45:219–230.
59. Stewart, W. W. 1978. Functional connections between cells as revealed by dye-coupling with a highly fluorescent naphthalimide tracer. *Cell.* 14:741–759.
60. Turin, L., and A. Warner. 1977. Carbon dioxide reversibly abolishes ionic communication between cells of early amphibian embryos. *Nature (Lond.).* 270:56–57.
61. Warner, A. E., S. C. Guthrie, and N. B. Gilula. 1984. Antibodies to gap-junctional protein selectively disrupt junctional communication in the early amphibian embryo. *Nature (Lond.).* 311:127–131.