

# Gap Junction Connexon Configuration in Rapidly Frozen Myocardium and Isolated Intercalated Disks

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**ABSTRACT** By using two ultrarapid freezing techniques, we have captured the structure of rat and rabbit cardiac gap junctions in a condition closer to that existing *in vivo* than to that previously achieved. Our results, which include those from fully functional hearts frozen *in situ* in the living animal, show that the junctions characteristically consist of multiple small hexagonal arrays of connexons. In tissue frozen 10 min after animal death, however, unordered arrays are common. Examination of junction structure at intervals up to 40 min after death reveals a variety of configurations including dispersed and close-packed unordered arrays, and hexagonal arrays. By use of an isolated intercalated disk preparation, we show that the configuration of cardiac gap junctions *in vitro* cannot be altered by factors normally considered to induce functional uncoupling.

These experiments demonstrate that, contrary to the conclusions of some earlier studies (Baldwin, K. M., 1979, *J. Cell Biol.*, 82:66–75; Peracchia, C., and L. L. Peracchia, 1980, *J. Cell Biol.*, 87:708–718), the arrangement of gap junction connexons, in cardiac tissue at least, cannot be used as a reliable guide to the functional state of the junctions.

Gap junctions form specialized regions of intercellular contact at which communication can occur between cells. They have been implicated in many biological processes including electrical conduction, development and differentiation, and hormone transmission (8, 11, 14, 18, 20, 30, 42–44). There are now many communications that document the morphology and physiology of these junctions, and particular interest has been focused on the morphological correlates of differing functional states (2, 4, 6, 7, 11, 13, 16, 19, 22, 24–29, 32, 33, 40). Several of these authors have proposed that an uncoupling of cells connected by gap junctions (and hence loss of intercellular permeability) is accompanied by an increase in the regularity of packing and a decrease in the center-to-center spacing of the connexons, the functional subunits that make up the junctions (2, 6, 24–29, 32).

Others have shown, however, that the relationship between morphology and functional state may be more complex, and have therefore tried either to define their experimental conditions more rigorously (22) and/or to use ultrarapid freezing techniques (13, 22, 33, 39) in an attempt to get closer to the true functional (or nonfunctional) configuration of the junction. Recently there have even been indications that coupling in some cell types may be associated with the presence of

ordered arrays of connexons (22, 39), a complete reversal of the conclusions in the works noted above.

In the present study, we have attempted to clarify the relationship between gap junction structure and function in the mammalian heart. Cardiac muscle cells have large numbers of gap junctions that link adjacent myocytes, forming low-resistance pathways along which the action potential can spread from one cell to the next (8, 23). They are not scattered at random over the plasma membrane, but are generally confined to the intercalated disks, specialized membrane junctional areas located at or near the ends of neighboring cells (36, 41).

The direct freezing of tissues (without pretreatments) offers the best available method for preserving the *in vivo* configuration of potentially labile structures. To date however, ultrarapid freezing techniques (9, 15, 21, 31, 34), when applied to tissue samples, have been limited by the need to excise and mount specimens before freezing, thereby introducing the possibility of time- and damage-related alterations to membrane structure in the samples. To reduce these variables, we have developed two techniques for freezing tissue in a condition as close as possible to that existing in the living animal (37, 38), and at set intervals after animal death. In this way

we have been able to ascertain the structure of gap junctions in the functioning heart, and to follow structural alterations that take place postmortem. In addition, we have studied gap junction structure in isolated intercalated disks in an attempt to determine the factors that might control gap junction connexon configuration.

Our results, which include those obtained by freezing fully functional hearts in live animals, show that connexons of cardiac gap junctions *in vivo* have an ordered arrangement, and that unordered arrays can be formed some time after animal death. They also indicate that, in cardiac tissue at least, the arrangement and spacing of gap junction connexons cannot, unfortunately, be used as a reliable guide to the functional state of the junctions.

## MATERIALS AND METHODS

Hearts from Sprague-Dawley rats (250–300 g) and New Zealand White rabbits (2–2.5 kg) were used for the experiments. Animals were anaesthetized with an intraperitoneal injection of pentobarbitone for *in situ* freezing of hearts or were killed by dislocation of the neck for papillary muscle and disk isolation experiments.

**Rapid Freezing of Heart Papillary Muscles:** Each animal was stunned, its neck broken, and its heart quickly removed and plunged into ice-cold Krebs-Henseleit buffer (1 mM MgCl<sub>2</sub>, 0.435 mM NaH<sub>2</sub>PO<sub>4</sub>, 114 mM NaCl, 28 mM NaHCO<sub>3</sub>, 5 mM KCl, 1.8 mM CaCl<sub>2</sub>, 11 mM glucose). After a brief rinsing, the left ventricle was slit open and pinned back to reveal the papillary muscles. A muscle was selected and held by its tendon (chorda tendina) with fine forceps. The tendon (which consists largely of collagen with some elastic elements, but no cardiac muscle cells) was cut above the forceps, allowing the muscle to be raised for clamping between thin, prealigned copper support plates (BUO12-056-T, Balzers High Vacuum Ltd., Berkhamsted, England) temporarily glued onto fine curved forceps. The muscle itself was then cut free at its base, and the sandwich was immediately plunged rapidly by hand into liquid propane under conditions designed to achieve optimal cooling rates (10, 35, 37). After transfer into liquid nitrogen, the sandwich was freed from the forceps and stored. Each copper support plate was equipped with a hemicylindrical groove to accommodate the muscle in the sandwich without compressing it. By using this method of mounting the specimen before excision, we routinely froze undamaged papillary muscles within 2 s of cutting the muscle proper, and thus had them ready mounted for freeze-fracture electron microscopy. Any preparation that took longer than 2 min to complete from the time the animal was stunned was discarded. The time taken from stunning the animal to removal of the heart averaged 20 s. Details of the freezing technique used for this part of the study have been described elsewhere (37).

For postmortem experiments, the procedure above was followed except that rats were left for 10, 20, or 40 min before removal of the heart. In some cases, 2 min before the heart was removed, the thorax was punctured and a thermometer was placed into the body cavity adjacent to the heart to check body temperature.

**Rapid Freezing of Whole, Beating Rat Hearts *in Situ*:** In the majority of these experiments, anaesthetized rats were quickly opened by a surgical incision running up the body and through the diaphragm so that the rib cage could be spread to reveal the heart *in situ*. As soon as the pleural cavity was broken open, the lungs were artificially ventilated by use of a rubber bulb. The beating heart was then supported on a specially designed, spoon-shaped spatula with a keyhole slot cut into it through which the major blood vessels fitted. The object of this spatula was to hold the heart gently in place and to separate it from underlying organs.

A highly polished, liquid nitrogen-precooled copper block was then slammed down by hand onto the heart. The block was guided by using two cylinders, linked by a crossbeam, sliding down two upright shafts. The block was clamped to the crossbeam so that it could be slammed accurately and with considerable speed onto the prepositioned heart. Immediately upon freezing, the spoon, heart, and copper block, held together to prevent rewarming of the specimen, were raised, and the heart was "snapped" away from the body and dropped into liquid nitrogen.

In a further refinement of the technique, we used a surgical operating procedure by which the heart was not only beating normally, but the rat was breathing unaided (i.e., without the need for artificial ventilation) up until the instant of impact of the copper block. To do this, a small section of the sternum was first removed from an anaesthetized rat. The pericardial membrane was punctured and the heart was then gently lifted up, using a specially designed,

curved spatula, to lie externally to the animal. This had to be done with extreme care to avoid a pneumothorax. The keyhole-slot spatula was placed under the heart which was then frozen as above. A diagram of the freezing apparatus and a detailed description of its use is given elsewhere (38).

**Isolated Intercalated Disk Experiments:** Isolated intercalated disks were prepared by the method of Green and Severs (12). In brief, this method involves differential centrifugations and KCl extraction of myofibrils to obtain a fraction enriched in intercalated disks. The disks were studied by freeze-fracture electron microscopy after various treatments as listed below: (a) disks were isolated in 1 mM NaHCO<sub>3</sub> and centrifuged into a firm pellet, and samples were frozen either unfixed or after fixation and glycerination; (b) disks, isolated with 1 mM NaHCO<sub>3</sub> buffer containing 3 mM EDTA (pH 7.2), were fixed, centrifuged into a pellet, and glycerinated before freezing; (c) disks, isolated in 1 mM NaHCO<sub>3</sub>, were incubated for 40 min (37°C) in either 1 mM NaHCO<sub>3</sub> and 3 mM EDTA (pH 7.2 or 5.5), 1 mM NaHCO<sub>3</sub> and 1.5 mM CaCl<sub>2</sub> (pH 7.2 or 5.5), or in Krebs-Henseleit buffer before fixing, centrifuging into a pellet and glycerinating for freeze-fracture. In these experiments, pre-warmed fixative was added with the aim of stabilizing the junctions in their postincubation configurations. The concentration of these mixtures was ~0.2 g of tissue per 80 cm<sup>3</sup> buffer.

In addition, hearts were homogenized into 1 mM NaHCO<sub>3</sub> or 1 mM NaHCO<sub>3</sub> and 3 mM EDTA (both at pH 7.2) and fixative was added within 3 min of animal death. In this case tissue concentration was 1 g per 80 cm<sup>3</sup>. After 30 min the crude homogenate was filtered, centrifuged into a pellet, and glycerinated, and samples were frozen for freeze-fracture.

In all of these pretreated specimens, the fixative used was glutaraldehyde added to the buffer containing the heart material or intercalated disks to a final concentration of 3%. Glycerination was carried out for 20–30 min immediately before freezing, using 25% glycerol in the appropriate buffer.

For freezing of both fixed and unfixed disks, small samples of material were sandwiched between copper specimen support plates and frozen by plunging into liquid propane held at –180°C.

**Freeze-Fracture Electron Microscopy:** Mounted papillary muscles or isolated disks were fractured and replicated using a double (i.e., complementary) replica device in a Balzers BAF 400T freeze-fracture apparatus. Replication was at 10<sup>-6</sup> to 10<sup>-7</sup> mbar, –115°C to –120°C, with platinum-carbon evaporated from 45°C and carbon from 85°C. Specimens were rotated as the carbon was applied to minimize fragmentation of replicas during cleaning. Specimens from whole frozen hearts were prepared by "cutting" pieces off the flattened, rapidly frozen portion of the heart under liquid nitrogen and mounting them, well-frozen side uppermost, on a modified specimen table. To achieve this the clamp portion of the standard table was replaced with a specially-designed toothed clamp, which held the specimen firmly onto the stage when the side screws were gently tightened. Specimens so held were cut by microtome as close as possible to their top surface (i.e., that touched first by the copper block freezer) before replicating as above.

Replicas were cleaned in chromic acid and viewed in a Philips EM 301 electron microscope. All junctions observed were photographed and printed to a final magnification of ×75,500 for later analysis.

## RESULTS

### *Assessment of Connexon Arrangement*

For experiments on intact tissue, 1,830 freeze-fractured gap junctions were photographed for subsequent analysis. All of these junctions were from cardiac tissue frozen without prior fixation or cryoprotection using one or other of the two rapid-freezing techniques developed for this study. In addition, 261 junctions were studied in isolated intercalated disks; most of these were fixed and glycerinated before freeze-fracturing.

In counting junctions, we have not included very small groups of connexons, or larger junctions in which the connexon arrangement was unclear. The latter group includes junctions obscured by folding of the replica, those lying at an unfavorable angle to the direction of shadowing, or those badly distorted during fracturing and replication. In cases where two or more groups of connexons separated by cytoplasmic cross-fracturing were clearly from the same junction, they were counted as one. Where possible, we have considered the appearance of the E-face arrangement of the junction more significant than that of the P-face (5, 16), though junc-

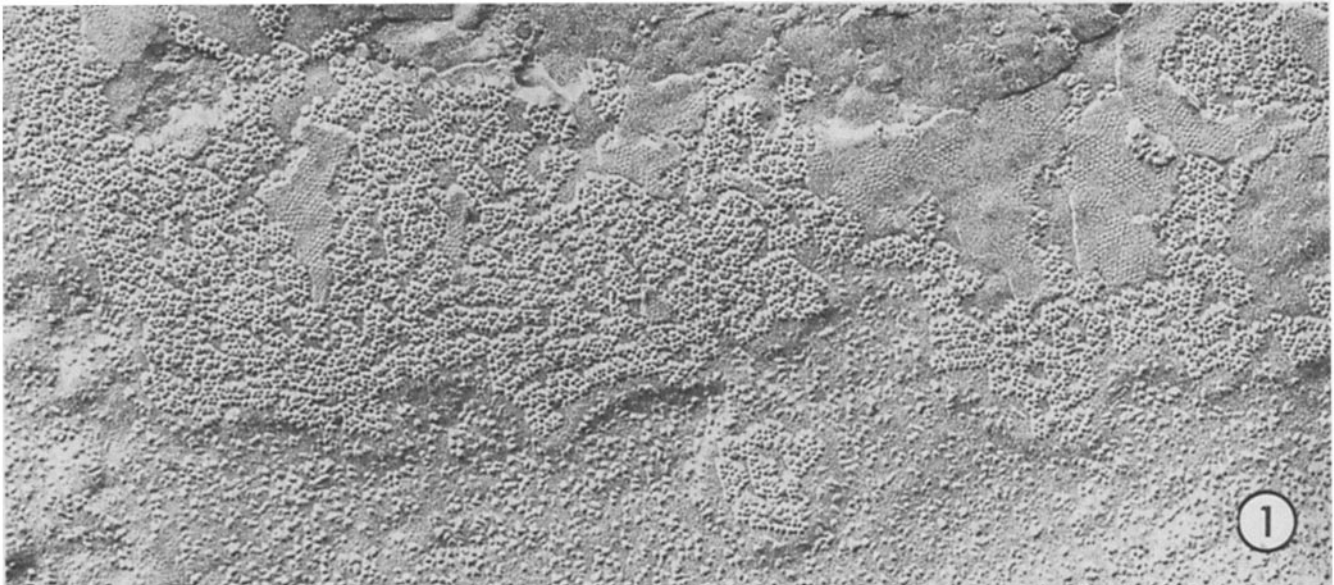
tions showing only P-faces were not excluded if of satisfactory quality.

### *Junction Classification*

Gap junction connexon configuration was found to be highly variable in the myocardial specimens examined. However, two categories of arrangement, (a) ordered and (b) unordered, were readily distinguishable from one another. We retained this simple classification of previous workers partly

because of the difficulty in further subclassification and partly because it is on this basis that the morphological correlates of gap junction function have previously been discussed.

**ORDERED CONFIGURATIONS:** Two extremes of ordered junction were observed: a clumped hexagonal array and a homogeneous hexagonal form. The former junctions consist of multiple, small groups of hexagonally arranged connexons separated by particle-free aisles of smooth membrane (Figs. 1 and 2). The P-face (Fig. 1) is characterized by arrays of

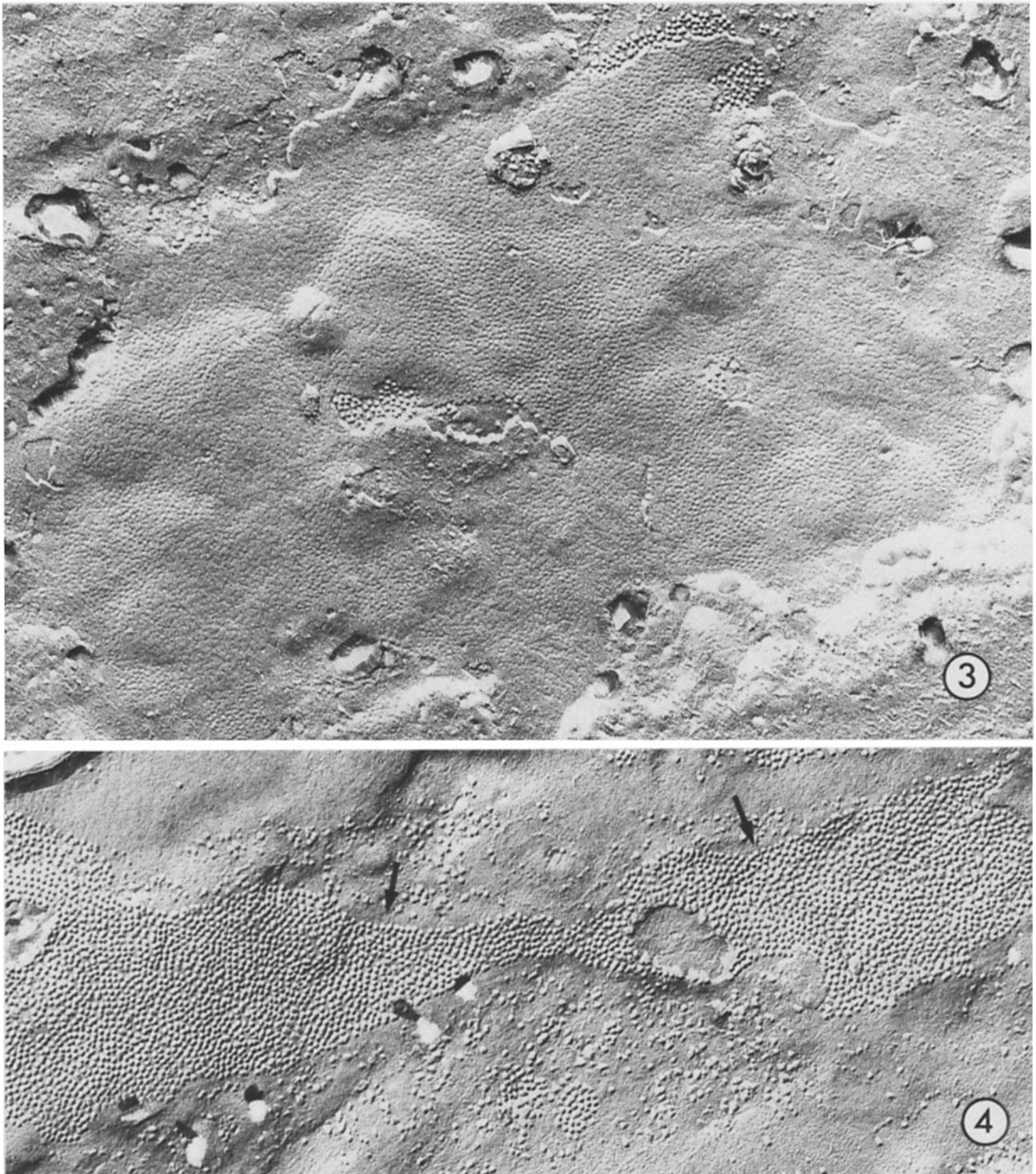


FIGURES 1 and 2 Gap junctions in rat heart papillary muscles directly frozen immediately after animal death using the copper sandwich technique. On both the P-face (Fig. 1) and E-face (Fig. 2), the connexons are clearly in clumped, hexagonal arrays typical of the "0 min" group in the papillary muscle experiments. The junctions occur within smooth patches of membrane and have an irregular outline. The number of connexons in each clump is variable; in places individual ones are seen but, in others up to ~80 occur within a clump.  $\times 95,000$ .

particles and the E-face (Fig. 2) by arrays of pits; usually both arrays are observed within patches of smooth membrane. The junction overall has an irregular outline and seldom is distinct, clearly delineated, and with outer edge apparent. The number of connexons within each clump is variable; in places individual connexons are observed, but there may be up to ~80 within an individual clump (Fig. 2). The homogeneous junc-

tion form, by contrast, comprises a tightly compressed plaque of hexagonally arranged connexons. The junction nevertheless generally consists of multiple small arrays, but there are no smooth membrane patches or aisles between them, and the outer edge of the junction is delineated by a distinct, rounded border.

UNORDERED CONFIGURATIONS: A large variety of



FIGURES 3 and 4 An E-face (Fig. 3) and a P-face (Fig. 4) view of gap junctions in rat heart papillary muscles directly frozen 10 min after animal death. The connexons are randomly arranged within patches of smooth membrane. While the junction in Fig. 3 has an irregular outer edge, parts of the edge of the junction in Fig. 4 are clearly delineated (arrows). (Fig. 3)  $\times 110,000$ . (Fig. 4)  $\times 130,000$ .

junctions containing randomly arranged connexons was observed. A typical unordered configuration is seen in Fig. 3, in which E-face pits are readily seen in an irregular pattern. Fig. 4 shows a P-face view of a similar junction. Looser packing was also observed, and the center-to-center spacing of connexons often varied considerably within junctions. A very tight packing was evident in many junctions, and connexons can be so tightly packed that it becomes difficult to differentiate hexagonally arrayed regions from tight-packed random regions (Fig. 5). The outer edge of random array junctions varied considerably, ranging from an indistinct, irregular outline (Fig. 3) to a smoothly contoured, clearly delineated edge (Figs. 4 and 5). This feature did not differ among samples;

junctions with or without distinct borders were found in all three papillary muscle experiment postmortem groups.

#### *Papillary Muscle Experiments*

The readily reproducible results of the copper-sandwich, propane-plunge, freezing technique has allowed accumulation of data from a large number of specimens. Only one papillary muscle was used from each animal, and freezing was always carried out within 2 s of excision from the heart. Specimens were frozen as rapidly as possible after cervical dislocation (designated "0 min") or left for periods of 10, 20, or 40 min before preparation for freezing (See Materials and Methods). In the postmortem experiments, the body temperatures of the

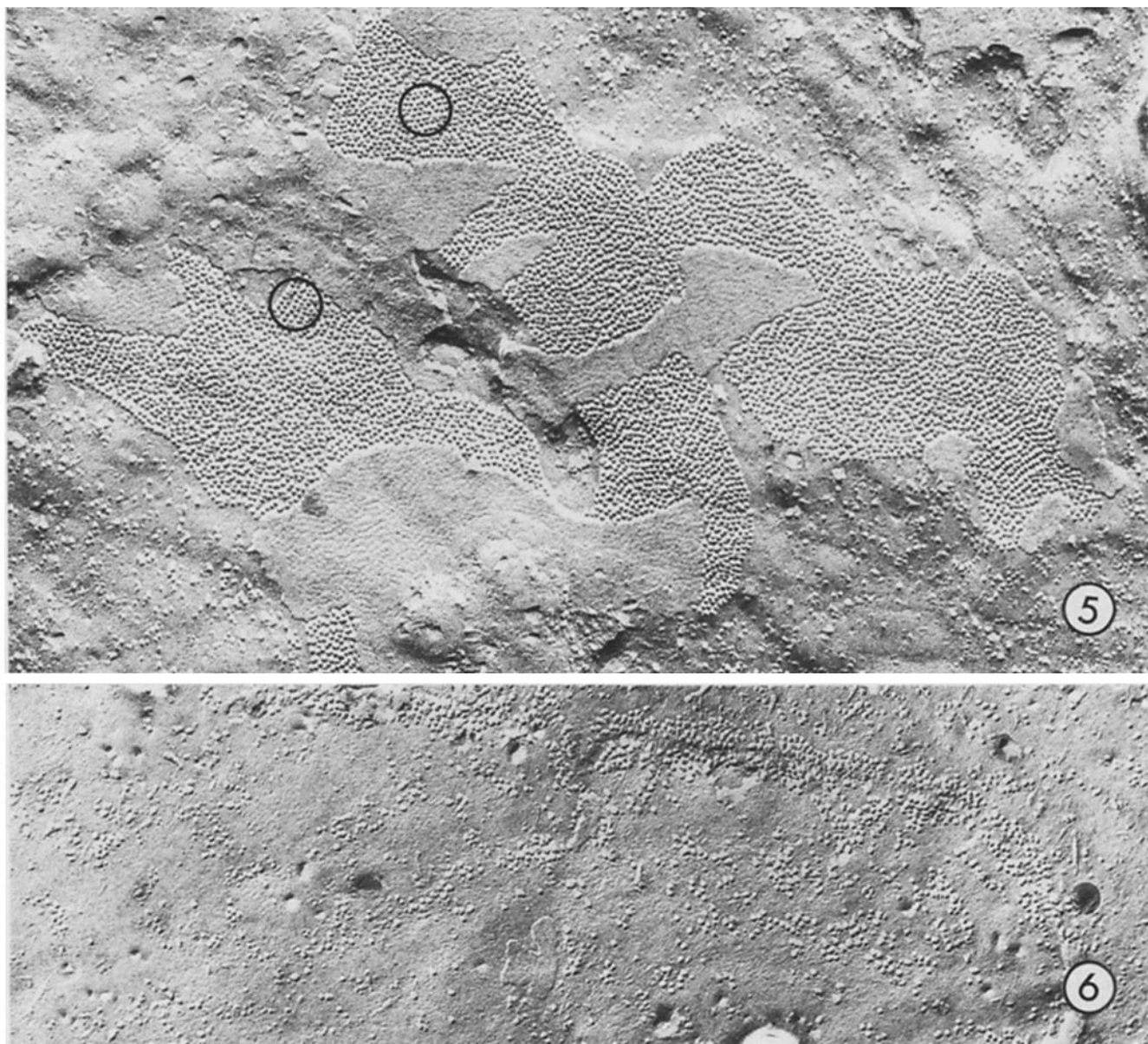


FIGURE 5 A gap junction in a rat heart papillary muscle directly frozen 40 min after animal death. The connexons in this example are tightly packed together, and it is difficult to distinguish between random and hexagonally arrayed regions. Some areas where connexons are clearly hexagonally arranged have been circled. This junction has an irregular shape but its outer edge is clearly delineated.  $\times 104,000$ .

FIGURE 6 An example of connexon "streaming" in a rat heart papillary muscle directly frozen 10 min after animal death. The junctional components appear to be undergoing long distance migration within the membrane, a type of connexon configuration only seen in the 10-min postmortem group.  $\times 98,000$ .

10- and 20-min samples remained at 37°C. After 40 min, the temperature had fallen by about 5°C.

Correlation of freeze-fracture and thin-section images made it possible to estimate the distance from the surface at which the gap junctions routinely examined were located. Transverse thin sections of rat papillary muscles fixed in a state of stretch corresponding to that of the freeze-fractured specimens showed that thickness of the surface endocardial layer covering the muscle cells varies from 0.7 to 2.8  $\mu\text{m}$ . This comprises a thin endothelium (50–200 nm in nonnuclear regions) seated on a connective tissue layer which contains occasional fibroblasts, smooth muscle, and nerve cells. The most superficial layer of cardiac muscle cells extends down to a depth of between 4 and 12  $\mu\text{m}$  from the tissue surface, and intercalated disks are common in this zone. Our papillary muscle replicas typically showed extensive views of endothelial plasma membrane, with periodic breaks into the underlying layer of cardiac muscle cells. From this it was clear that the intercalated

disks routinely examined were located between 0.7 and 12  $\mu\text{m}$  from the tissue surface.

Only specimens from which >10 junctions were obtained are included in our results. An overall average of 5–6 junctions from each intercalated disk were located although 10–15 were not unusual. In general, all the junctions from any one disk had the same configuration; there was little variation within disks. Our results are summarized in Table I.

Of nine animals sampled immediately after death (0 min group: seven rats, two rabbits), all had gap junctions with predominantly hexagonally arrayed connexons. The maximum number of unordered junctions in any one specimen reached only 20% (specimen 8). An overall average of 94% of the junctions observed had ordered arrays, the vast majority of these being of the clumped hexagonal form (Figs. 1 and 2). A notable feature of this group was the lack of variability among animals.

In the 10-min postmortem group, four of the five specimens

TABLE I  
Analysis of Gap Junction Arrangement in Propane Plunge-frozen Papillary Muscles, Copper Block-frozen Beating Hearts In Situ, and Isolated Intercalated Disks

Experiment	Heart No.*	Intercalated	Gap junc-	Junctions in	Junctions in
		disks found	tions ana-	hexagonal	random array
		<i>n</i>	<i>n</i>	%	%
Papillary muscles "0 min" (i.e., frozen as rapidly as possible after animal death)*	1	16	72	100	0
	2	16	137	98.5	1.5
	3	4	29	93	7
	4	23	158	97.5	2.5
	5	3	12	91.5	8.5
	6	3	14	100	0
	7	3	16	87.5	2.5
	8*	19	112	80	20
	9*	21	102	97	3
Subtotals		108	652	94 <sup>†</sup>	6 <sup>†</sup>
Papillary muscles "10 min" postmortem <sup>‡</sup>	10	5	19	89.5	10.5
	11	16	88	88.5	11.5
	12	6	36	80.5	19.5
	13	10	62	100	0
	14	54	195	8	92
Subtotals		106	400	73 <sup>†</sup>	27 <sup>†</sup>
Papillary muscles "20 min" postmortem <sup>‡</sup>	15	7	27	100	0
	16	20	126	93	7
	17	15	78	8	92
	18	25	138	49	51
Subtotals		67	369	62.5 <sup>†</sup>	37.5 <sup>†</sup>
Papillary muscles "40 min" postmortem <sup>‡</sup>	19	6	27	52	48
	20	19	33	94	6
	21	20	138	69	31
	22	25	160	34	66
Subtotals		70	358	62 <sup>†</sup>	38 <sup>†</sup>
Beating hearts, frozen in situ <sup>§</sup>	23	1	17	100	0
	24	1	1	100	0
	25	9	31	100	0
	26	1	2	100	0
Subtotals		12	51	100	0
Isolated intercalated disk experiments			261	98	2

\* All specimens are from rat material with the exception of those marked (\*), which are from rabbit.

<sup>‡</sup> In all papillary muscle experiments, the specimens were frozen within 2 s of excision by manual plunging into liquid propane.

<sup>§</sup> Copper block slam-frozen beating hearts in situ in animals which had either artificially-ventilated lungs, or were breathing freely.

<sup>†</sup> Average values.

sampled had junctions that remained in an ordered configuration, i.e., predominantly clumped hexagonal. An exception, however, was specimen 14, in which 92% of the junctions contained random arrays (Figs. 3 and 4). The overall average number of ordered junctions in this group was 73%. The random-array junctions often had irregular shapes with widely spaced connexons. In many cases there were indications of junction disassembly, with a dispersed scattering of connexons within certain areas of the membranes and apparent "streaming" of particles (Fig. 6). These effects were not observed in any other group.

Samples taken 20 min after animal death showed striking variation not only among, but also within specimens. Specimen 18 had approximately half of its gap junctions in an ordered configuration, with the other half unordered. The other specimens in this group had junctions either predominantly of the hexagonal form or predominantly of the random type. The total average of ordered junctions in this group was only 62.5%, over one-third of the junctions being unordered at this stage.

The 40-min postmortem samples were similar to those in the 20-min group and, although there was a tendency toward increased variability within specimens, the overall average of ordered junctions remained at ~62%. However, in specimen 22, the connexons of virtually all the junctions were extremely tightly packed (Fig. 5), and their classification into the ordered or unordered configurations becomes somewhat arbitrary. In some cases, collections of small, hexagonally arrayed junctions were located (Fig. 7). Although small junctions were seen in all groups, clusters of this type were only observed in the 40-min postmortem specimens.

### *Whole Heart Experiments*

The standard of cryopreservation obtained using the copper-block slammer was less satisfactory than that achieved by the propane-plunge technique, but was nonetheless surprisingly good considering the size of the samples being frozen. Large areas of well-frozen sarcolemma, as judged by smoothly contoured fracture faces with randomly distributed nonjunctional particles (e.g., Fig. 8*b*), were located in areas in which the most superficial cardiac muscle cells had been successfully fractured. Below this level, however, ice crystal size rapidly increased with depth from the surface making most of the sample unusable. Within the well-frozen zone, the structural details of gap junctions were clearly discernible. Of the 51 junctions viewed by use of this technique, all were in ordered arrays of the clumped, hexagonal type (Fig. 8) within clear patches of the sarcolemma.

### *Isolated Intercalated Disk Experiments*

The isolation technique used gives a fraction enriched with gap junctions which are still surrounded by intact regions of the disk sarcolemma (Fig. 9). In these experiments a total of 261 junctions were recorded and analyzed. From a total of 114 in the standard NaHCO<sub>3</sub> buffer isolated disk preparations, only five small junctions had connexons in random arrays. The great majority (95%) had a hexagonal configuration whether replicated in a fixed (and glycerinated) or unfixed state (Fig. 10). All 31 junctions from hearts homogenized, or disks incubated in 3 mM EDTA (pH 7.2 or 5.5), had a similar hexagonal configuration as did those incubated with calcium present (28 junctions). Incubation in standard Krebs-Henseleit buffer (50 junctions) induced no change.

In the crude homogenate (to which fixative was added within 3 min of animal death), the junctions were in hexagonal arrays, whether the hearts were homogenized into 1 mM NaHCO<sub>3</sub>, pH 7.2 (15 junctions) or into 1 mM NaHCO<sub>3</sub>, 3 mM EDTA, pH 7.2 (23 junctions).

## DISCUSSION

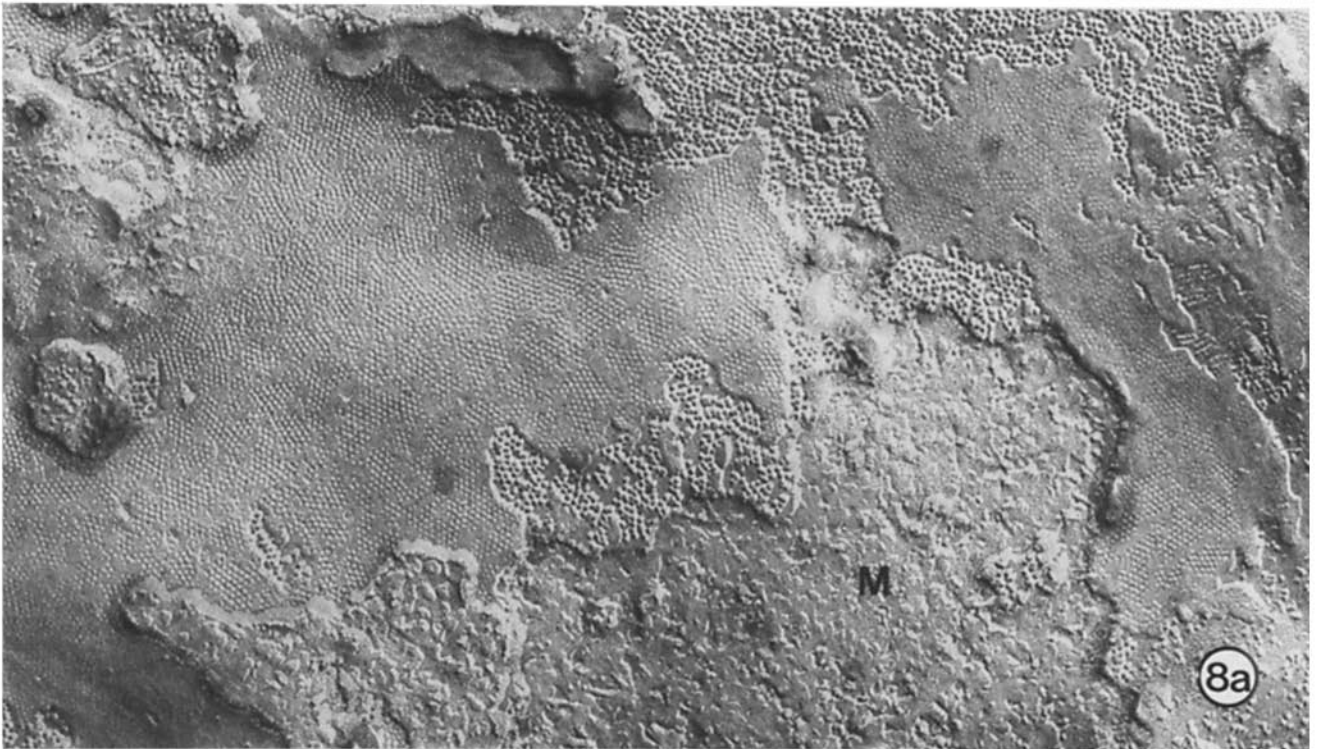
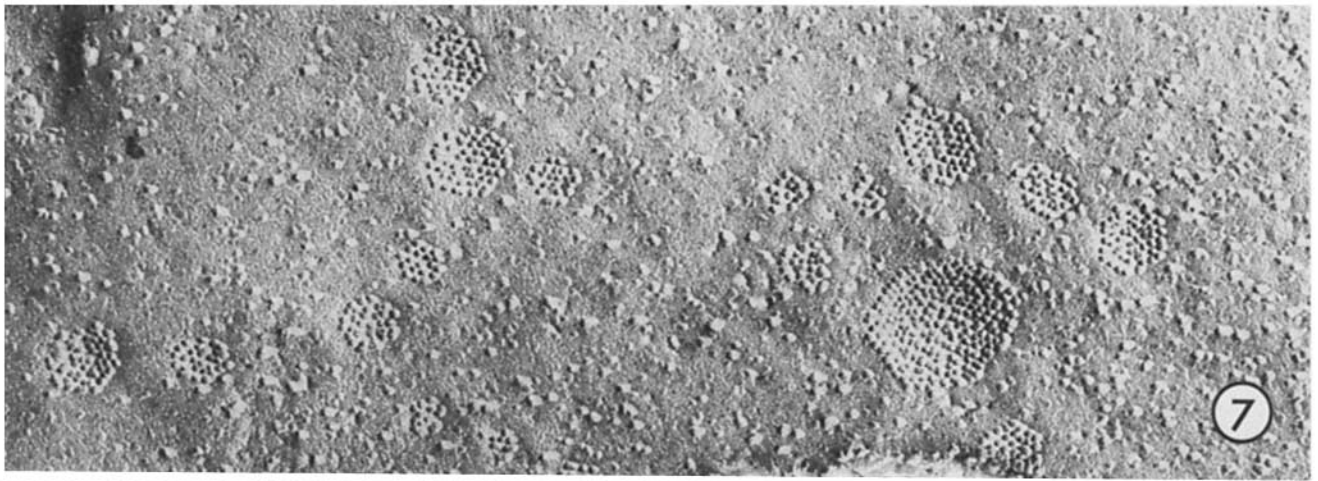
Rapid freezing techniques offer a means of stabilizing labile cell structures in their native configuration. However, this advantage is lost if excessive time is taken or damage caused in preparing samples for freezing. It is necessary to balance these factors against the technical difficulties involved in freezing large specimens, and most techniques to date have relied upon carefully trimmed specimens of small size in order to obtain adequate tissue preservation (9, 15, 21, 31, 34).

The papillary muscle sandwich technique used in the present study routinely gave good cryopreservation of cardiac tissue which had been cut from the heart only 2 s before completion of freezing. Furthermore, the region of the specimen damaged by excision lies well outside the area which was subsequently fractured and replicated. However, this method does not entirely overcome the problem of time delay and possible alterations such as excision-induced partial depolarization of the cells. The aim of the copper block in situ experiments was, therefore, to verify the results obtained from the 0-min papillary muscles. The size of ice crystals formed during freezing by this technique was, on average, larger, but its outstanding feature was that it enabled the direct freezing of fully functional hearts, beating normally, in live animals. The two techniques in combination have therefore permitted some new insights into the structure of cardiac gap junctions in vivo and of postmortem induced changes.

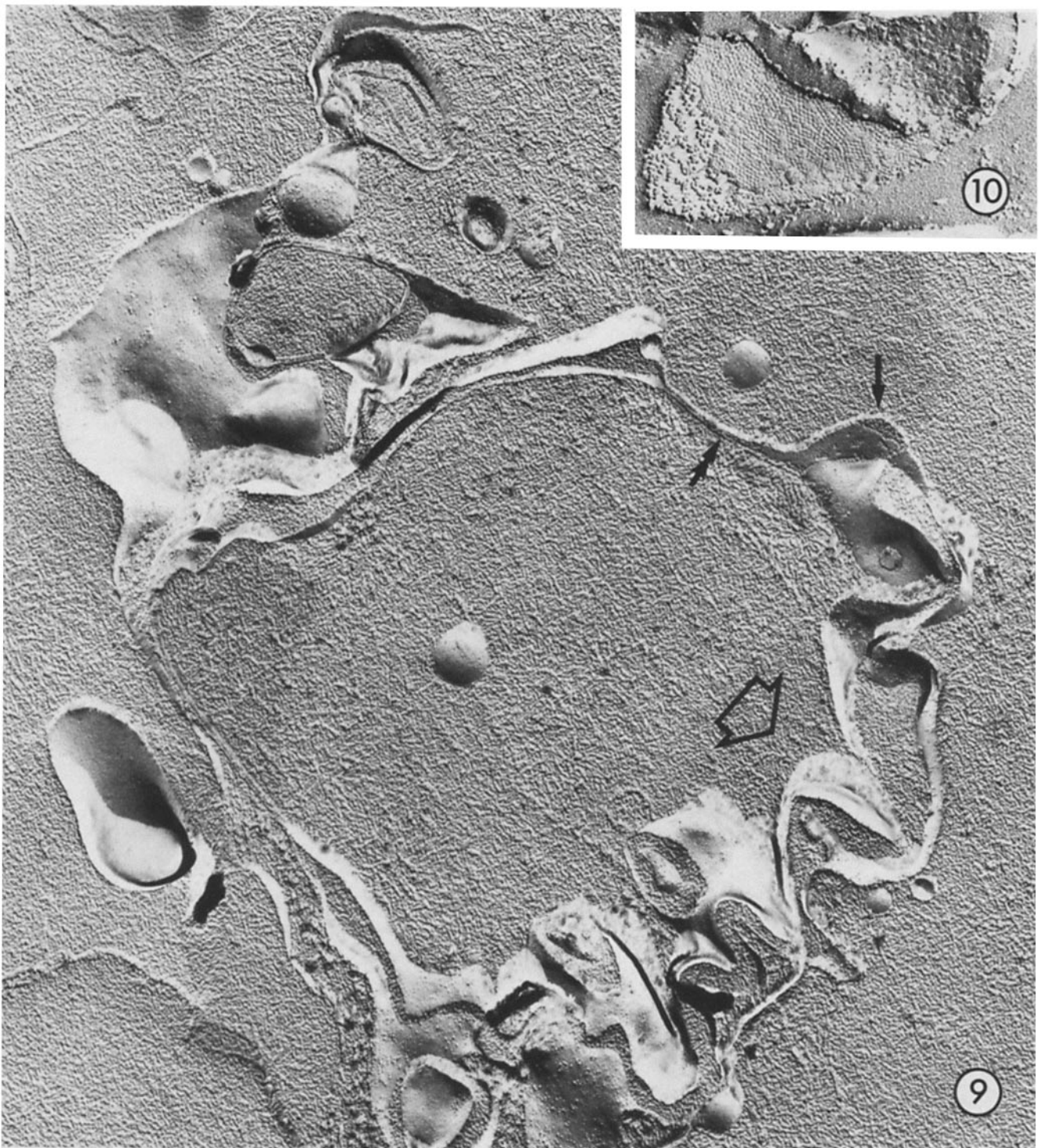
Both the papillary muscle and whole heart experiments indicate that the connexons of the majority of gap junctions in the normal functioning cardiac myocyte are in ordered arrays. All 13 hearts studied by these techniques gave the same result, and most (nine specimens) had 97% or more of their junctions in this configuration. As these results are consistent in samples of papillary muscle and ventricle wall from two species (rat and rabbit), they would appear to reflect features common to mammalian cardiac tissue in general.

It was only after animal death that random arrays were seen in significant numbers. Four of the five 10-min postmortem samples were similar to those in the 0-min group, but the fifth showed a remarkably different junction organization. By the 20-min postmortem stage, over one-third of all the junctions were in a random configuration. This overall percentage did not change in the 40-min postmortem group. While there did appear to be a tendency toward mixed populations of the junction configurations with increased time, rather than the predominance of one form or another within a given sample, it is necessary to recognize that, within the longer time-period experiments, a wide range of postmortem changes may alter or arrest connexon rearrangement.

In the 10-min postmortem sample containing junctions predominantly of the random type (specimen 14), there was not only a disarrangement of the connexons, but also images suggestive of longer-distance particle migration were commonly observed. The resulting configurations seen were not unlike those reported during gap junction disassembly and connexon streaming induced by high carbon dioxide levels (17). This is not entirely unexpected; cardiac tissues very rapidly become anoxic once the flow of oxygenated blood is







FIGURES 9 and 10 Fig. 9: An intercalated disk isolated from a rat heart using the standard procedure. The two intact disk membranes of the adjacent cells are seen running side by side, converging together at a gap junction which has been fractured to reveal tangential (filled arrow) and cross section (filled double arrow) views. The irregular, pleated portion of the disks (hollow arrow) forms part of a fascia adherens junction.  $\times 78,500$ . Fig. 10: A gap junction in an intercalated disk isolated from a rat heart and fixed with glutaraldehyde. The connexons are hexagonally packed in an arrangement typical of all gap junctions in the isolated disk experiments carried out. This arrangement could not be induced to change when disks were isolated or incubated in media of varying compositions.  $\times 112,000$ .

FIGURES 7 and 8 Fig. 7: A group of small gap junctions with hexagonally packed connexons in a rat papillary muscle directly frozen 40 min after animal death. Such an arrangement could be the result of a reaggregation of connexons from a single, original junction which has passed through a dispersed, random array stage.  $\times 135,000$ . Fig. 8: (a and b) Gap junctions in hearts frozen using the copper block technique. In each example, the heart was beating in a living, anaesthetized rat up until the moment of impact by the liquid nitrogen-cooled block. The connexons of each junction are in clumped, hexagonal arrays set in a clear, smooth area of the sarcolemma. *M*, mitochondrial membrane. In *b*, note smoothly contoured membranes with randomly distributed nonjunctional particles, indicating satisfactory cryopreservation. (a)  $\times 84,000$ . (b)  $\times 112,000$ .

arrested. This particle movement appeared to be short-lived and was not seen to be continued in any of the longer term postmortem samples.

The simple junction classification system we have used does not distinguish between the different types of ordered junction present and, although junctions at time 0 appeared to be almost entirely of the clumped hexagonal type, many in later samples were in a homogeneous hexagonal array. One of the 40-min postmortem specimens (heart 22), for example, had all its junctions with very tightly packed connexons. It may be that junctions can undergo a change from the *in vivo* hexagonal arrangement to a secondary hexagonal form via the unordered configurations. The clusters of small hexagonally arrayed junctions seen at the 40-min postmortem stage could be the result of such a reorganization from widely dispersed connexons. However, similar clusters of small junctions have also been reported in oxygenated hearts contracting spontaneously *in vitro* (22) and may not, therefore, necessarily reflect an uncoupled state.

The isolated intercalated disk experiments were designed to give some insight into factors that might be responsible for the connexon rearrangements observed in whole tissue experiments. The disks, in which the gap junctions remain surrounded by sarcolemma, were intended to provide a model system analogous to that previously used to study the effect of divalent cations and pH on eye lens junction structure (16, 25, 26, 28, 29). After isolating or incubating disks in the various media, it was necessary to stabilize the gap junctions with glutaraldehyde to prevent structural changes during the subsequent pelleting necessary for electron microscopy. Aldehyde fixation is known to induce uncoupling and alterations in gap junction structure in some tissues (3, 33) but apparently has little effect on connexon configuration in the heart (2, 22, 25). In the homogenized hearts where there are no tissue penetration problems, fixation would be expected to be rapid and had no detectable effect on the structure of disks isolated using the standard technique and frozen with or without pretreatment.

As might be expected from the results obtained with papillary muscles and whole hearts, gap junctions in crude homogenates, to which fixative had been added <3 min after animal death, were in hexagonal arrays. In our isolated disks, however, we were unable to obtain gap junctions in random arrays or to induce them by incubation of the disks. In all our experiments the junctions were hexagonally arrayed regardless of calcium concentration, pH, or the addition of ions which normally maintain cardiac activity in perfused whole heart preparations (Kreb-Henseleit buffer was tried although its calcium concentration is relatively high compared with normal intracellular levels). These results would suggest that in contrast to the situation proposed for eye lens junctions (25, 28, 29), the connexon arrangement in cardiac gap junctions appears not to be controlled by the same factors that are considered to induce functional modulation. However, no isolated junction preparation can be considered to reflect accurately the *in vivo* state of functionally intact junctions, and the possibility that the response of the junctions is impaired after isolation cannot be completely excluded.

We have not attempted to measure the spacing of connexons for any of our experiments. Although this variable is commonly quoted in gap junction studies relating structure to function, (1, 2, 4, 6, 7, 19, 22, 24, 25, 28, 29, 40), the

dynamic nature of the connexon configuration changes observed during our experiments makes such measurements of dubious value. There were clearly a wide range of spacings, varying from very tight packing to widely dispersed random arrays, and even variation within junctions could be equally as large. It would appear that a continuum exists between hexagonal and random packing which persists right through to the scattering of individual connexons induced during longer-distance connexon migration. It was not possible to define distinct connexon-spacing classes within our results.

It is most unlikely that our results can be attributed to damage caused during tissue preparation and freezing. They were consistent in all 13 samples taken immediately after animal death. Although ice-crystal damage was sometimes a problem in the copper block-frozen material, the better preserved regions showed hexagonally arrayed junctions which appeared identical to those obtained from samples frozen using the propane-plunge sandwich technique where ice crystal size approaches that of standard cryoprotected material (37). There was also no correlation between ice crystal size and junctional configuration in the postmortem experiments which showed both the hexagonal and random forms. Furthermore, in our isolated disk experiments, gap junctions were of similar appearance in samples prepared with or without cryoprotection, these samples being frozen in the same manner as that used for papillary muscles.

It has been suggested that junctions containing clumped hexagonal arrays (with particle-free aisles between the arrays of connexons) are in intermediate stage during a change from random to hexagonal configurations brought about by uncoupling (2). Such a junctional configuration has also been considered characteristic of embryonic tissue (4, 39). Our results would however indicate that this junction form is in fact normal in functional adult myocardium, as has also been suggested from some recent *in vitro* studies (22). A corollary to this is that a reevaluation of some gap junction-structure function relationships might be desirable. Conclusions drawn from changes in cardiac gap junction configuration attributed to injury-induced uncoupling (2), for example, would appear to be unwarranted in the light of our present data.

Although functional cardiac gap junctions *in vivo* appear to have hexagonally packed connexons, coupled cells in general may not necessarily always have this gap junction configuration. Raviola et al. (33) were able to freeze samples of stomach from anaesthetized rats within 20 s of the suspension of blood supply, and the junctions from that organ showed random arrays. Their samples of similarly prepared liver had junctions in hexagonal arrays. It also seems from combined physiological and structural studies that gap junctions (including myocardial ones) may be able to exist with their connexons in random arrays while apparently retaining functional properties (see, for example, references 4, 6, 13). Conversely, in our postmortem experiments, in which acidosis and anoxia would be expected to bring about an uncoupling of cells, we observed a significant increase in the number of gap junctions that contained random arrays.

In conclusion, our findings demonstrate that the arrangement and spacing of gap junction connexons cannot be used as a reliable guide to the functional state of the junction. In cardiac tissue at least, the connexons of gap junctions *in vivo* would appear to have an ordered arrangement, the unordered arrays observed developing as a postmortem event.

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