

# MEMBRANE JUNCTIONS IN THE INTERMEMBRANE SPACE OF MITOCHONDRIA FROM MAMMALIAN TISSUES

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## ABSTRACT

There have been several reports describing paracrystalline arrays in the intermembrane space of mitochondria. On closer inspection these structures appear to be junctions of two adjoining membranes. There are two types. They can be formed between the outer and inner mitochondrial membranes (designated outer-inner membrane junctions) or between two cristal membranes (intercristal membrane junctions). In rat heart, adjoining membranes appeared associated via a central dense midline approximately 30 Å wide. In rat kidney, the junction had a ladder-like appearance with electron-dense "bridges" approximately 80 Å wide, spaced 130 Å apart, connecting the adjoining membranes. We have investigated the conditions which favor the visualization of such structures in mitochondria. Heart mitochondria isolated rapidly from fresh tissue (within 30 min of death) contain membrane junctions in approximately 10–15% of the cross sections. This would indicate that the percentage of membrane junctions in the entire mitochondrion is far greater. Mitochondria isolated from heart tissue which was stored for 1 h at 0°–4°C showed an increased number of membrane junctions, so that 80% of the mitochondrial cross sections show membrane junctions. No membrane junctions are observed in mitochondria in rapidly fixed fresh tissue or in mitochondria isolated from tissue disrupted in fixative. Thus, the visualization of junctions in the intermembrane space of mitochondria appears to be dependent upon the storage of tissue after death. Membrane junctions can also be observed in mitochondria from other stored tissues such as skeletal muscle, kidney, and interstitial cells from large and small intestine. In each case, no such junctions are observed in these tissues when they are fixed immediately after removal from the animal. It would appear that most studies in the literature in which isolated mitochondria from tissues such as heart or kidney were used were carried out on mitochondria which contained membrane junctions. The presence of such structures does not significantly affect normal mitochondrial function in terms of respiratory control and oxidative phosphorylation.

## INTRODUCTION

There have been numerous reports of crystalline structures or inclusion bodies within the matrix space of mitochondria. Several recent reports (1, 4, 7, 8, 10) deal with a new structural element in

the intermembrane space<sup>1</sup>, i.e., membrane junctions which contain dense material between them.<sup>2</sup>

<sup>1</sup> The intermembrane space is used to refer to the compartment enclosed between the inner and outer

Such junctions in the intermembrane space of mitochondria are of two types. They can be formed between the outer and inner mitochondrial membranes, designated "outer-inner membrane junctions", or they can be formed between two adjacent cristal membranes designated "intercristal membrane junctions." The membrane junctions resemble "desmosomes" which are frequently observed in the association of plasma membranes of two adjoining cells in that the junction comprises two adjoining membranes with central dense material. The role of the membrane junctions in mitochondria is unclear. This study is concerned with defining the conditions which favor the visualization of such structures in isolated mitochondria and fixed tissue.

#### MATERIALS AND METHODS

All reagents, unless designated otherwise, were reagent grade. Protein was estimated by the method of Lowry et al. using crystalline bovine serum albumin (Armour Pharmaceutical Co., Chicago, Ill.) as a standard (5). The Clark oxygen electrode was used to measure oxygen uptake. Respiratory control and P/O ratios were calculated from the enhanced oxygen utilization resulting from the addition of known amounts of ADP (2). Male Sprague-Dawley rats of the Holtzmann strain (200-300 g) were used in these studies.

#### *Electron Microscopy*

**FIXATION AND EMBEDDING:** Samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at 4°C and post-fixed in 1% OsO<sub>4</sub> in 0.1 M Veronal-acetate buffer (pH 7.4) containing 2.4 mM CaCl<sub>2</sub> and 0.06 M NaCl for 2 h at 4°C. In the case of heart and kidney tissue, small cubes, 0.3 mm on a side, were fixed. Isolated mitochondria were fixed as pellets formed by centrifuging 10 min at 20,000 rpm in a Spinco Ti-50 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). All samples were block stained with 0.5% uranyl acetate in Veronal-acetate buffer (pH 6.0) for 2 h at room temperature before dehydration (3) and dehydrated in a series of increasing ethanol concentrations. The ethanol was replaced with propylene oxide and the samples were

mitochondrial membranes. It is sometimes referred to as the intracristal space.

<sup>2</sup>The term membrane junction is used to indicate the close apposition of two membranes in a region having distinctive electron microscope structure (3). It is not intended to imply special electrical conductivity across the junction.

embedded in Araldite or Epon 812 (6). Thin sections were cut on an LKB Ultratome (LKB Instruments, Inc., Rockville, Md.) with diamond knives (E. I. DuPont de Nemours & Co., Wilmington, Del.). The mounted sections were doubly stained with 1% uranyl acetate followed by lead (9) and were examined in a Hitachi HU-11B electron microscope. The same solutions for fixation and embedding were used for all samples.

#### *Preparation of Normal Rat Heart and Rat Kidney Mitochondria*

The heart and kidneys were rapidly removed from a 250-g Holtzmann strain Sprague-Dawley rat sacrificed by decapitation. They were immediately cooled in 0.25 M sucrose at 0°C. Processing for both heart and kidneys was similar. The tissue was suspended in 25 ml of 0.25 M sucrose-10 mM HEPES-1 mM EDTA, pH 7.5, in a J-20 centrifuge tube. The tissue was homogenized with a Brinkmann Polytron 20ST (obtained from Brinkmann Instrument, Inc., Westbury, N. Y.) set at 4.5 for 7 s. The homogenate was diluted to 40 ml with the same medium and centrifuged for 10 min at 5,000 rpm in a J-20 rotor using a model J-21 Beckman centrifuge. The supernatant was recentrifuged for 10 min at 20,000 rpm. The mitochondrial pellet was resuspended with a Potter-Elvehjem glass-Teflon homogenizer and centrifuged as before. The lower rich brown pellet of mitochondria was sampled for electron microscopy. When mitochondria from aged tissue were prepared, the tissue was wrapped in Parafilm and stored on ice for the indicated times before homogenization of the tissue and isolation of the mitochondria. This procedure, designed for rapid isolation, takes approximately 30 min.

#### *Preparation of Beef Heart Mitochondria*

Two beef hearts were transported from the slaughterhouse in plastic bags packed in ice. The red meat was trimmed from the left ventricle and was cubed approximately 1 inch on a side. The meat was ground with a Hamilton Beach "gourmet" grinder. The ground meat, 200-g portions, was slurried with 800 ml of 0.25 M ultrapure sucrose (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.), 20 mM HEPES pH 7.5, and 1 mM EDTA. The mixture was homogenized with the Polytron 20ST, set at 5.0, for 45 s. As the homogenization proceeded, 0.7 ml of 45% KOH was added to maintain the pH at 7. The homogenate was centrifuged in a J-14 rotor of a Beckman J-21 centrifuge for 10 min at 5,000 rpm. The supernatant was carefully decanted and recentrifuged for 20 min at 14,000 rpm in the same rotor. The pellets were resuspended in 250 ml of

0.25 M sucrose containing 10 mM HEPES, pH 7.5, and 1 mM EDTA using a Potter-Elvehjem glass-Teflon homogenizer and recentrifuged for 20 min at 14,000 rpm. The upper light layer of the pellet was split off by means of a fine stirring rod and gentle swirling and discarded. The lower dark brown layer was resuspended in 45 ml of the same medium and centrifuged for 10 min at 20,000 rpm in a JA-20 rotor. The upper light layer of the pellet was again discarded. The dark brown lower layer was separated from any black button at the bottom of the tube and was resuspended in 0.25 M sucrose. The resulting mitochondria typically exhibited respiratory control ratios of greater than four using glutamate, malate, and malonate as substrate mixture. All operations were performed at 0°-4°C.

### *Isolation of Mitochondria from Rapidly Fixed Tissue*

Heart and kidneys were quickly removed from a 250-g rat. Each tissue was suspended in 25 ml of 2.5% glutaraldehyde, 0.25 M sucrose, 0.1 M cacodylate, 10 mM HEPES pH 7.4, and 1 mM EDTA at 0°C and rapidly homogenized with a Brinkmann Polytron 20ST set at 4.5 for 7 s. The homogenate was centrifuged for 10 min at 5,000 rpm in a JA-20 rotor. This produced a heavy mat floating on top of the liquid. The mat, which is a consequence of glutaraldehyde fixation, was removed with a spatula and the supernatant recentrifuged for 10 min at 20,000 rpm in a JA-20 rotor. The pellet was resuspended in the glutaraldehyde-containing medium and recentrifuged before the resulting pellet was sampled for electron microscopy.

### RESULTS

Beef heart mitochondria, isolated approximately 3 h after death of the animal and then fixed with glutaraldehyde, contained membrane junctions in the intermembrane space in approximately 45% of the cross sections of mitochondria; most junctions are the outer-inner membrane type junctions (cf. Figs. 1, 3, and 4; Table I). It takes about 1 h from the time the animals are killed in the slaughterhouse until the hearts are received in the laboratory. During the first 0.5 h, the heart remained within the animal at above room temperature; it was cooled in crushed ice the remainder of the time. The isolation procedure took about 1.5-2.0 h and was carried out in the cold.

At low magnification, the membrane junctions appear as regions of increased electron density approximately 0.2-0.3  $\mu\text{m}$  long; the junctions frequently approximate a straight line. The outer membrane of the outer-inner membrane junction

appears smooth in contrast with normal outer membranes which frequently appear rippled (Fig. 1 and cf. Fig. 2 for rat heart mitochondria). Occasionally several intercrystal membrane-type junctions (Fig. 3) appear parallel to one another (Fig. 1).

Both types of membrane junctions from bovine or rat heart have a similar appearance at higher magnification (Figs. 3-5). The membranes participating in the junction retain a trilaminar appearance. The two adjoining membranes of the junction have a midline dense band between them with more translucent regions between the midline and the membranes. The midline band is more electron opaque than the matrix space. The membrane junction is about 230 Å wide of which the membranes themselves are each about 50 Å wide, the midline dense band is about 30 Å wide, and the more translucent regions are each about 50 Å wide (Figs. 3-5).

Rat heart mitochondria isolated rapidly, about 30-min postmortem, contained a lower frequency of membrane junctions in the intermembrane space; only about 10-15% of the cross sections of mitochondria contained such junctions (Tables I and II). The smaller percentage of membrane junctions in mitochondria isolated from rat heart as compared with bovine heart suggested that the visualization of these structures was related to changes occurring postmortem. This was tested by rapid fixation of fresh heart tissue. The rat heart was quickly removed after decapitation, rapidly immersed, and finely cubed or homogenized by means of a Polytron 20ST in the glutaraldehyde fixative solution. No membrane junctions were observed in mitochondria either in rapidly fixed tissue sections (not shown) or in isolated mitochondria from tissue which had been homogenized directly in glutaraldehyde (Fig. 6).

The frequency of occurrence of membrane junctions in mitochondria isolated from rat heart tissue was studied as a function of the time of storage of the tissue at 0°C (Fig. 11) before disruption of the tissue to release the mitochondria (Table I). The zero time point is obtained by blending the tissue in fixative immediately upon removal from the animal. Membrane junctions were not observed in the tissue section (not shown) or in the mitochondria isolated from glutaraldehyde-fixed tissue (Fig. 6). Mitochondria prepared by rapid isolation (0.5 h) from fresh heart and then fixed showed membrane junctions in 10-15% of the sections. These junctions were entirely of the

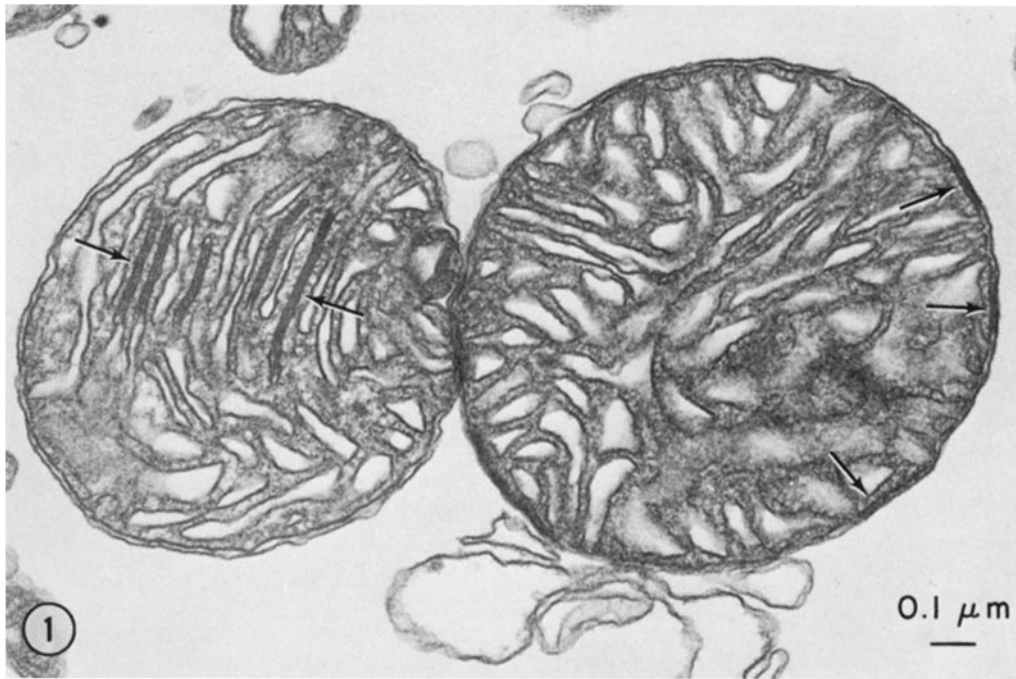


FIGURE 1 Beef heart mitochondria, fixed immediately after isolation, approximately 3-3.5 h post-mortem. Two types of membrane junctions are observed in the intermembrane space. An outer-inner membrane junction can be observed in the mitochondrion on the right (arrows) (cf. Figs. 4 and 5). Inter-cristal membrane type junctions also can be observed in the mitochondrion on the left (arrows) (cf. also Fig. 3).  $\times 60,000$ .

FIGURE 2 Rat heart mitochondria isolated from tissue which was first stored at 0-4°C for 1 h post-mortem. The mitochondria were fixed approximately 1.5 h postmortem. Membrane junctions in the intermembrane space are observed with great frequency. Most junctions are of the outer-inner membrane type.  $\times 60,000$ .

TABLE I  
*Membrane Junctions in the Intermembrane Space of Mitochondria as a Function of the Time of Tissue Aging*

	Fixation Time	Percentage			Total
		OI*	IC*	Both	
	<i>h</i>				
A. Rat Heart	0	0	0	0	0
	0.5	15	0	0	15
	1.5	72	10	3	85
	5.5	37	4	1	42
B. Bovine Heart	3.0	40	5	1	46

The results are expressed as percentage of membrane junctions in a total of 500 mitochondrial cross sections which were studied for each sample. The zero time point is obtained by blending heart tissue obtained immediately after death directly in glutaraldehyde fixation solution using a Polytron 20ST as described in the isolation of mitochondria from rapidly fixed tissue (cf. Materials and Methods). The other time points were: 0.5 h, rapid isolation from the fresh tissue; 1.5 h, tissue stored on ice for 1 h before isolation of mitochondria; 5.5 h, tissue stored on ice for 5 h before isolation of mitochondria. The isolation of rat heart mitochondria took about 30 min. Bovine heart mitochondria were isolated from slaughterhouse material as discussed in the text. Fixation time refers to the time of initiation of fixation postmortem.

\* OI and IC are abbreviations for outer-inner membrane type and intercrystal membrane type junctions, respectively.

outer-inner membrane type. After storage for 1.0 h before isolation, i.e. fixative added 1.5 h postmortem (cf. Fig. 2), the tissue showed membrane junctions in greater than 80% of the mitochondrial cross sections, 72% of which are of the outer-inner membrane type junction and 10% of the intercrystal type junction. More prolonged storage of the tissue resulted in destruction of the normal tissue morphology as well as a decrease in observable membrane junctions. Beef heart mitochondria isolated 3.5 h after death show membrane junctions in 45% of the cross sections. An increase in the percentage of membrane junctions results as well from storage of the isolated mitochondria in the cold (Table II). The increase is greater at pH 6.0 than pH 7.4.

Membrane junctions in the intermembrane space of mitochondria were also observed in other tissues after storage, such as kidney, skeletal

muscle, and intestine (interstitial cells of both small and large intestines). We did not, however, observe membrane junctions in liver tissue after storage. Kidney tissue was studied to see whether the membrane junctions appear after storage as they do in heart tissue.

Kidney tissue to which fixative was added immediately postmortem was devoid of membrane junctions both in tissue sections (not shown) and in the isolated mitochondria (Fig. 8). "Normal" mitochondria, i.e. isolated rapidly from sucrose homogenates (isolation time was approximately 30 min), contained membrane junctions in 8% of the cross sections (Fig. 11). In mitochondria from tissue which had been "aged" on ice for 1 h before fixation (Fig. 9), the number of membrane junctions rose to 30% (Figs. 10, 11). After 5 h, the morphology of the kidney mitochondria was very poor and the number of membrane junctions again seemed to decrease, in this case to about 10%. The membrane junctions in kidney were of the outer-inner membrane type exclusively (Fig. 10), i.e., no intercrystal membrane junctions were observed.

The percentages of membrane junctions ob-

TABLE II  
*Membrane Junctions in the Intermembrane Space of Rat Heart Mitochondria as a Function of Time of Storage of Isolated Mitochondria*

Time of fixation	pH 7.4	pH 6.0
<i>h</i>	%	%
0.5	11	—
1.5	31	59
3.5	36	50

These results are expressed as percentage of membrane junctions in a total of 500 mitochondrial cross sections which were studied for each sample. The sample fixed at 0.5 h refers to mitochondria prepared as rapidly as possible from fresh rat heart and then fixed. The 1.5- and 3.5-h samples refer to the rat heart mitochondria which were stored for 1 and 3 h, respectively, at 0-4°C before glutaraldehyde fixation. The mitochondria were stored as a suspension of approximately 5 mg protein/ml at two different pH's: either at pH 7.4 in 0.25 M sucrose containing 10 mM HEPES and 1 mM EDTA, pH 7.5, or at pH 6.0 in 0.25 M sucrose containing 10 mM PIPES and 1 mM EDTA. All of the membrane junctions were of the outer-inner membrane type. Fixation time refers to the time of initiation of fixation post mortem.

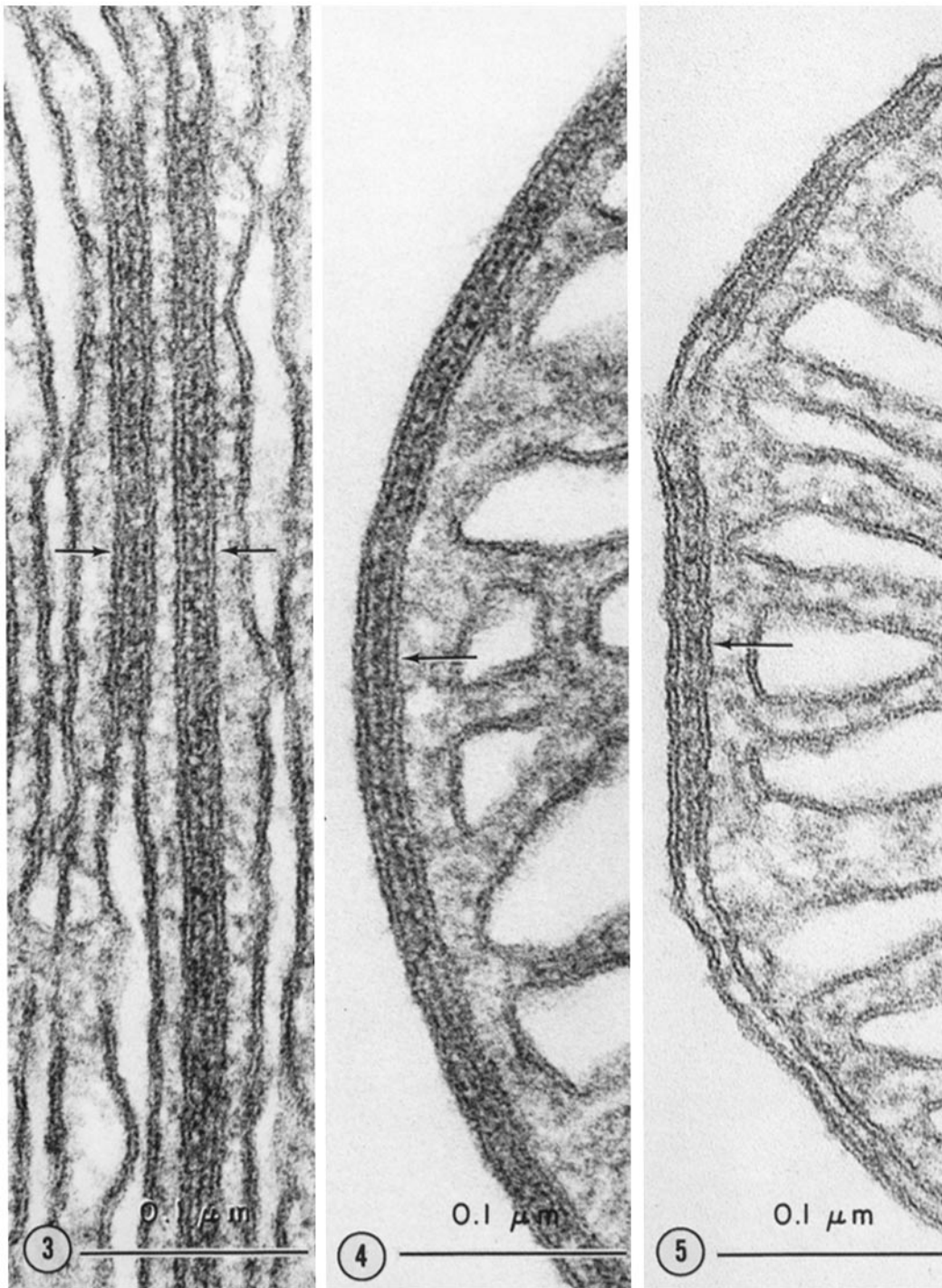


FIGURE 3 An enlarged section of a beef heart mitochondrion showing a typical intercrystal membrane type junction (arrows). The conditions for isolation of mitochondria and storage of tissue were the same as those described in Fig. 1.  $\times 330,000$ .

FIGURE 4 An enlarged section of a beef heart mitochondrion showing a typical membrane junction of the outer-inner membrane type (arrow). The conditions for isolation of mitochondria and "aging" of tissue were the same as those described in Fig. 1.  $\times 330,000$ .

FIGURE 5 An enlarged section of a mitochondrion from rat heart showing a typical junction of the fused outer-inner membrane type (arrow). The aging and conditions for isolation were the same as those described in Fig. 2.  $\times 330,000$ .

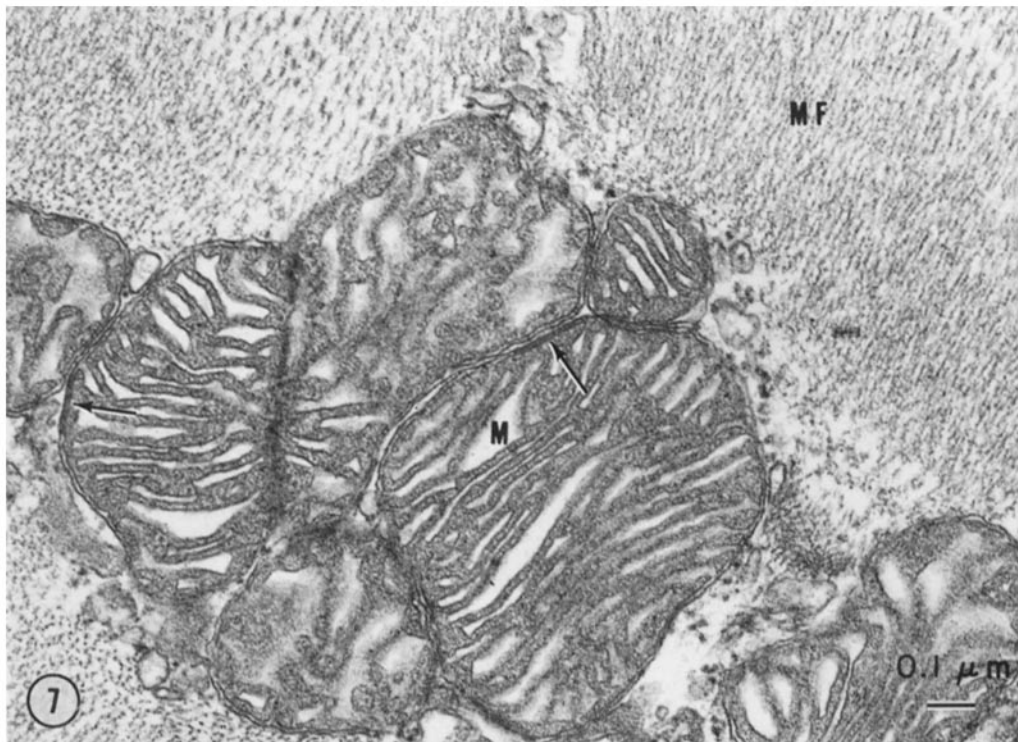


FIGURE 6 Mitochondria isolated from rat heart tissue and fixed immediately postmortem. The tissue was fixed by homogenization in the isolation medium containing glutaraldehyde. No membrane junctions were seen.  $\times 60,000$ .

FIGURE 7 Rat heart tissue kept at  $0^{\circ}$ - $4^{\circ}\text{C}$  for 1 h before glutaraldehyde fixation. Membrane junctions mostly of the outer-inner membrane type (arrows) are observed in the intermembrane space of the mitochondria. *M*, mitochondria; *Mf*, muscle fiber.  $\times 60,000$ .





FIGURE 8 Mitochondria isolated from rat kidney tissue which was fixed immediately postmortem by homogenization in the isolation medium containing glutaraldehyde. No membrane junctions were observed in the intermembrane space.  $\times 60,000$ .

FIGURE 9 Rat kidney tissue kept at  $0^{\circ}\text{--}4^{\circ}\text{C}$  for 1 h before glutaraldehyde fixation. Membrane junctions of the outer-inner type, exclusively, are visible in the intermembrane space of the mitochondria (arrows). *M*, mitochondria; *PM*, plasma membrane.  $\times 100,000$ .



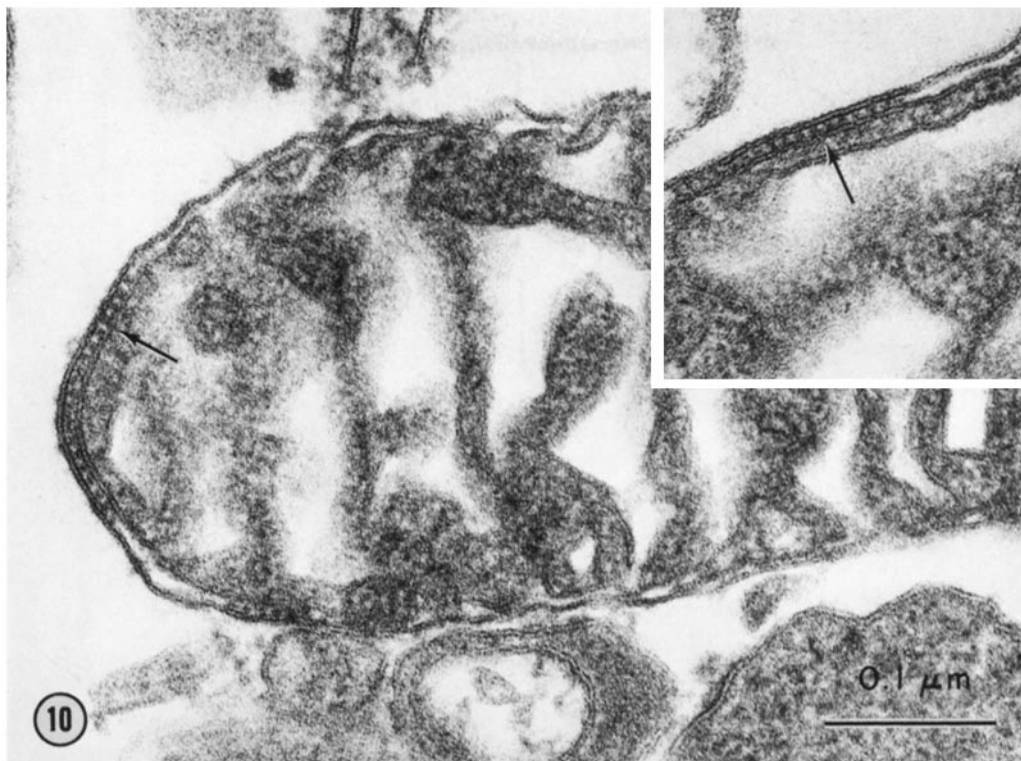


FIGURE 10 A portion of a kidney mitochondrion showing detail of the membrane junctions in the intermembrane space. Only outer-inner membrane type junctions are observed. The membrane junctions in kidney mitochondria have a ladder-like appearance and are distinctive from those of heart mitochondria (cf. Figs. 4 and 5). The *inset* shows another membrane junction with less curvature.  $\times 220,000$ .

served in cross sections of rat heart and kidney mitochondria were compared as a function of aging of the tissue in the cold (Fig. 11). The same general picture is obtained for both types of mitochondria, but the percentage of membrane junctions resulting from storage is greater in heart mitochondria than in kidney mitochondria.

The outer-inner membrane junctions of kidney mitochondria are different in appearance from those of heart mitochondria. While the width of the membrane junctions was similar to that of the membrane junctions of heart mitochondria (approximately  $230 \text{ \AA}$ ), the junction did not have a continuous  $30\text{-\AA}$  central band but was bridged by "rungs"  $80 \text{ \AA}$  wide and spaced  $130 \text{ \AA}$  apart giving a ladder-like appearance (Fig. 10).

#### DISCUSSION

The first description of membrane junctions in mitochondria from mammalian tissue was made by Hall and Crane (4) approximately 3 yr ago.

They observed junctions of the intercrystal membrane type in isolated beef heart mitochondria which bore some resemblance to membrane junctions in mitochondria of plant tissue which were previously described by Newcomb and co-workers in 1968 (8). These atypical cristae in bean root mitochondria were likened to the tight junctions between plasma membranes of animal cells. Wakabayashi and co-workers (10) observed membrane junctions of both the outer-inner membrane type and the intercrystal membrane type in beef heart mitochondria. Membrane junctions were observed in heart mitochondria in a variety of configurations including orthodox, condensed, and "energized-twisted." Two recent studies deal with the induction of membrane junctions in the intermembrane space of mitochondria by prolonged aging of tissue (24–48 h) (1) and by use of high pressures (7).

The thrust of our studies is that membrane junctions in the intermembrane space of mito-

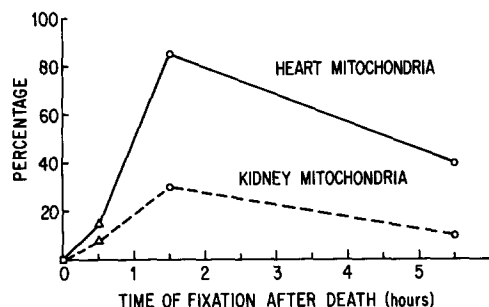


FIGURE 11 Membrane junctions in the intermembrane space of isolated heart and kidney mitochondria as a function of tissue storage time. The symbols represent:  $\square$ , mitochondria isolated from tissue which was fixed immediately post mortem (cf. text);  $\Delta$ , mitochondria isolated from fresh tissue and then fixed;  $\circ$ , tissue kept at  $4^{\circ}\text{C}$  for 1 or 5 h and then the mitochondria were isolated and fixed. The isolation time was 0.5 h. The ordinate refers to the percentage of the sections of mitochondria which contain membrane junctions in the intermembrane space of the mitochondria. Each point represents the percentage of membrane junctions observed in a total of 500 mitochondrial cross sections. The time of fixation after death refers to the time when the glutaraldehyde fixative was added to the tissue or isolated mitochondria.

chondria are normally not apparent in rapidly fixed tissue and that their visualization appears to be due to changes resulting from aging of tissue or isolated mitochondria after death. The presence of membrane junctions in the intermembrane space of mitochondria does not severely alter general mitochondrial function, as we know it, in that mitochondria containing membrane junctions are still capable of oxidative phosphorylation as well as good respiratory control. It would appear, thus, that most studies on isolated heart mitochondria were carried out on mitochondria containing such membrane junctions in approximately 10% of the mitochondrial cross sections observed by electron microscopy. This would mean that the percentage of membrane junctions in the entire mitochondrion would be far greater. The influence of such membrane junctions on mitochondrial function cannot fully be evaluated at the present time. It has been the general experience that heart tissue can yield good quality mitochondria with regard to function even after storage of the heart for several hours in the cold. It is ironic that a change in morphology in the isolated mitochondria, that is, the appearance of membrane junctions in the intermembrane

space, is more indicative of aging than sensitive parameters of mitochondrial function such as P/O and respiratory control ratios.

All of the electron micrographs shown here are of samples which were fixed and processed for electron microscopy in a similar manner (cf. Materials and Methods). Hence, the frequency of appearance of membrane junctions is directly referable to the time of storage of the tissue or of the isolated mitochondria and was not due to differences in sample preparation. The method of sample preparation used to visualize membrane junctions can be varied. The membrane junctions are observable in samples fixed with glutaraldehyde either in the cold or at room temperature for intervals of 15 min to overnight. Fixation with osmium tetroxide alone also allows visualization of the structures but with less contrast.

Lowering the pH seems to increase the number of membrane junctions (Table II). A drop in pH would be expected when tissue is stored, in that the cells would become anaerobic with time and glycolysis would give rise to production of hydrogen ions. There is, however, a difference to be noted between the storage of tissue and the storage of isolated mitochondria in that no intercrystal membrane junctions were observed in isolated mitochondria after storage.

Membrane junctions in mitochondria were found in other rat tissues as well after storage, including skeletal muscle, kidney, and interstitial cells of the small and large intestine. In each case, after rapid fixation of the fresh tissue there was a complete absence of membrane junctions in the mitochondria. We did not observe membrane junctions in the mitochondria of liver.

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