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# Is the Nexus Necessary for Cell-to-Cell Coupling of Smooth Muscle?

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*Summary.* Electronmicroscopic study of electrically coupled smooth muscles was undertaken to determine the distribution of nexuses in various types of smooth muscle. The study revealed that while nexal structures were commonplace in some types of smooth muscle, they were very rare or absent in others, even though in some cases these cells were only a few nanometers distant from one another. The persistence in thin section of these structures in the main circular muscle of dog intestine after poor fixation, fixation under strain, cell shrinkage, and metabolic damage of various sorts seems to rule out the thesis that they are labile. The absence of nexuses in longitudinal muscle of dog intestine examined both by thin section and by freeze fracture suggests that in this tissue they are absent or very rare *in vivo* and cannot account for electrical coupling.

Nexuses were discernible in thin sections of main circular muscle after a variety of experimental conditions of fixation. Metabolic inhibition or *in vitro* permanganate fixation partially destroyed nexal contacts. These procedures induced tissue, membrane apposition and an accompanying increase in the number of structures which resemble nexuses at low magnification (nexus-like structures). "Nexus-like" structures occurred in all smooth muscle fixed by *in vitro* permanganate associated with apposition of membranes and poor preservation of basement membrane. A technique of *in vitro* permanganate fixation was developed which prevented tissue swelling; consequently "nexus-like" structures were absent in tissues so treated. The suggestion is made that some structures described in the literature as nexuses, following permanganate fixation, may represent "nexus-like" structures.

The balance of evidence suggests that nexuses need not be present for electrical coupling of some smooth muscle cells, in which other types of cell-to-cell contacts must be invoked.

It is generally accepted that the nexus or gap junction provides the basis for electrical coupling between smooth muscle cells [15, 16, 3]. This is usually interpreted to imply that the nexus represents a region

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of low electrical resistance between cells. Nexuses in smooth muscle are now recognized to be gap junctions rather than tight junctions [17, 47] since they show a 2 nm gap after staining *en bloc*. While no one has yet made direct measurements of the resistance of these junctions in smooth muscle, indirect estimates have been made for the nexus of the intercalated disc of heart muscle [37, 52], a structure similar in appearance to the nexus of smooth muscle. These estimates are consistent with the view that nexuses represent regions of low resistance, but are based on the assumption either that all current passes through the nexus [53], or that the junctions consist of aqueous pores over 0.33% of their area [37]. These studies are based on the frequency and distribution of nexuses in heart muscle; no estimation has been reported for smooth muscle.

Electrical coupling has been demonstrated [34, 51, 50, 56] in certain smooth muscles in which nexal structures appear by thin section electron-microscopy (EM) to be totally absent [30, 54, 42], or exceedingly rare [21, 22], suggesting that these structures are labile or invisible in this technique or that nexuses are not necessary for coupling. Their absence in thin section EM studies was also demonstrated in the dense layer of the circular muscle of the intestine [17], although demonstration of electrical coupling has not been achieved in this layer. In all coupled smooth muscles, there are other types of specialized regions of close approach between cells, which may act as regions for current flow between cells [30, 22, 42, 31].

The failure to demonstrate nexal structures in thin sections may be due to inadequate techniques; e.g., Dewey and Barr [16] reported that in their preparations glutaraldehyde fixation failed to preserve nexuses, whereas permanganate fixation did so. Stress from contraction during fixation might also disrupt nexuses. However, the nexus (gap junctions) are easily characterized in freeze-fracture replicas even after the junction has been split experimentally [27].

The purpose of these studies was to determine whether or not nexal structures could be found using thin section EM techniques and freeze-fracture techniques in smooth muscle in which electrical coupling has been demonstrated, evaluate whether or not the absence of such structures was in some cases a technical artifact, and evaluate the hypothesis that the nexus is the structure necessary for electrical coupling among smooth muscle cells. That the nexus is sufficient for electrical coupling when present is not questioned. It should be emphasized that this study deals with the preservation of nexal structures using various EM techniques;

the absence of such structures does not imply the absence of electrical coupling and may not prove the absence of functional nexuses *in vivo*. However, the extent to which the absence of nexuses in thin section EM studies implies the absence of functional nexuses *in vivo* is a fundamental issue to be considered. Freeze fracturing has helped to resolve this issue since this technique should allow visualization of small, rare nexuses [48], and allow detection of nexuses even if they were to split experimentally [27].

## Materials and Methods

### *Tissues and Fixation*

Dog intestine was usually fixed by intra-arterial perfusion of a Krebs Ringer (K.R.) solution containing glutaraldehyde as previously described [14, 29]. If required, as in experiments on the effects of hypertonic solutions or metabolic inhibitors, intestinal segments were first perfused with various Ringer solutions before fixation. The procedures used for perfusion of hypertonic solutions have been described [14]. In some cases, intestinal muscle was isolated, separated from the mucosa, and incubated for varying times at 37 °C in K.R. solution aerated with 95% $O_2$ – 5% $CO_2$, with or without added iodoacetate ($10^{-3}$ M) and dinitrophenol ($10^{-3}$ M). Most of the pieces were then fixed by immersion in 5% glutaraldehyde in Millonig's buffer. Some of the pieces were fixed in permanganate as previously described [30]. A technique for intra-arterial fixation with permanganate was also developed, which involved perfusion of the tissue with glucose-free Krebs solution for 5 min, followed by permanganate solution (*see text*).

Dog stomach was fixed by intra-arterial perfusion of K.R. solution followed by K.R. + glutaraldehyde, through a cannula inserted *via* one iliac artery and placed with its tip near the coeliac artery. The aorta was occluded proximal to the coeliac artery, and the renal arteries and the other iliac artery were occluded distally. This procedure results in fixation of the intestines, liver and other visceral organs as well as the stomach. In some cases phosphate was substituted for the bicarbonate content of the K.R. buffer.

Rat myometrium was fixed either by *in situ* perfusion of Millonig's buffered 5% glutaraldehyde through the aorta, or by immersion of the dissected tissues in the same fixative. The dissection removed the endometrium as well as part of the circular muscle. Some pieces of the myometrium were incubated in K.R. buffer as described above, and the ATP content of the tissue was determined. It was found that the ATP content of the tissue so incubated was identical to that of fresh or incubated undissected tissues [11–13, 46]. In some cases, incubated strips of uterine muscle were fixed in a freshly prepared mixture of 1 part K.R. solution and 1 part 2% permanganate.

In two sets of experiments, tissues were either (1) incubated 30 min in hypotonic K.R. solution (normal Ringer diluted 1:3 with distilled water to yield a solution of almost 100 mosmols) and then fixed in a hypotonic solution of 1% glutaraldehyde with pH adjusted to 7.4 by NaOH; or (2) incubated for 30 min in hypertonic K.R. solution (sucrose added to yield ca. 600 mosmols) and fixed in the same solution with 1% permanganate.

Human fallopian tube (either ampulla or ithmus) was removed at hysterectomy or during tubal ligation, and placed immediately in K.R. solution with 5% glutaraldehyde in Millonig's buffer.

*Preparation for Electron Microscopy*

Glutaraldehyde-fixed tissues were stored at least overnight in cold fixative and then postfixed for 1 hr in 1% $OsO_4$ in Millonig's phosphate buffer; $OsO_4$ postfixation was omitted for permanganate-fixed tissues. The samples were dehydrated in graded ethanols and propylene oxide, and embedded in Epon 812 in flat molds in order to obtain the desired orientation for sectioning. The muscles to be studied were usually oriented longitudinally in the block. Silver to gray sections were cut on a Porter-Blum MT2 ultra-microtome, and mounted without a supporting film on specimen grids. They were stained with uranyl acetate and lead citrate, and viewed in a JEM-7A electronmicroscope. Nexuses can be quantitated with great reliability through examination of cells cut in cross section [30], and we routinely carried out studies on cells so sectioned. In all cases some sections of longitudinally cut cells were also examined.

*Viewing*

The scanning of grid squares for nexal structures was usually carried out by two observers simultaneously (G.D. and E.E.D.) and an agreement was reached on all equivocal structures. Our definition of a nexus structure required that a distinct 5 or 7 lined (depending on staining procedures) structure be present. Furthermore, several control specimens, as well as those subjected to metabolic inhibition, were counted independently by different observers, and there was agreement between them. Double-blind techniques were not generally feasible since tissues fixed with permanganate and glutaraldehyde can be readily distinguished, as can longitudinal and circular muscle layers fixed by nearly any technique.

*Procedure for Freeze Fracture*

Dog intestine, which had been fixed by intra-arterial perfusion with K.R. solution containing 1.5% glutaraldehyde, was stored overnight in cold fixative (5% glutaraldehyde in Millonig's buffer). For freeze-fracturing, circular and longitudinal muscle was dissected from fixed intestine and allowed to stand in Millonig's buffer containing 30% glycerol for at least one hr. Tissue samples were frozen in liquid Freon 22, routinely freeze-fractured at $-100$ °C and Pt-C replicas made in a Balzers BA 360M high vacuum freeze-etch unit [36, 55].

## Results

### *Intestinal Muscle*

Nexal structures in longitudinal *vs.* circular muscle have been studied in 66 animals, and in ileum as well as in duodenum and jejunum (Figs. 1 and 2). In preparations fixed with glutaraldehyde by intra-arterial perfusion, we have never failed to find in the main circular muscle layer numerous nexuses in approximately the same range of frequency (6 to

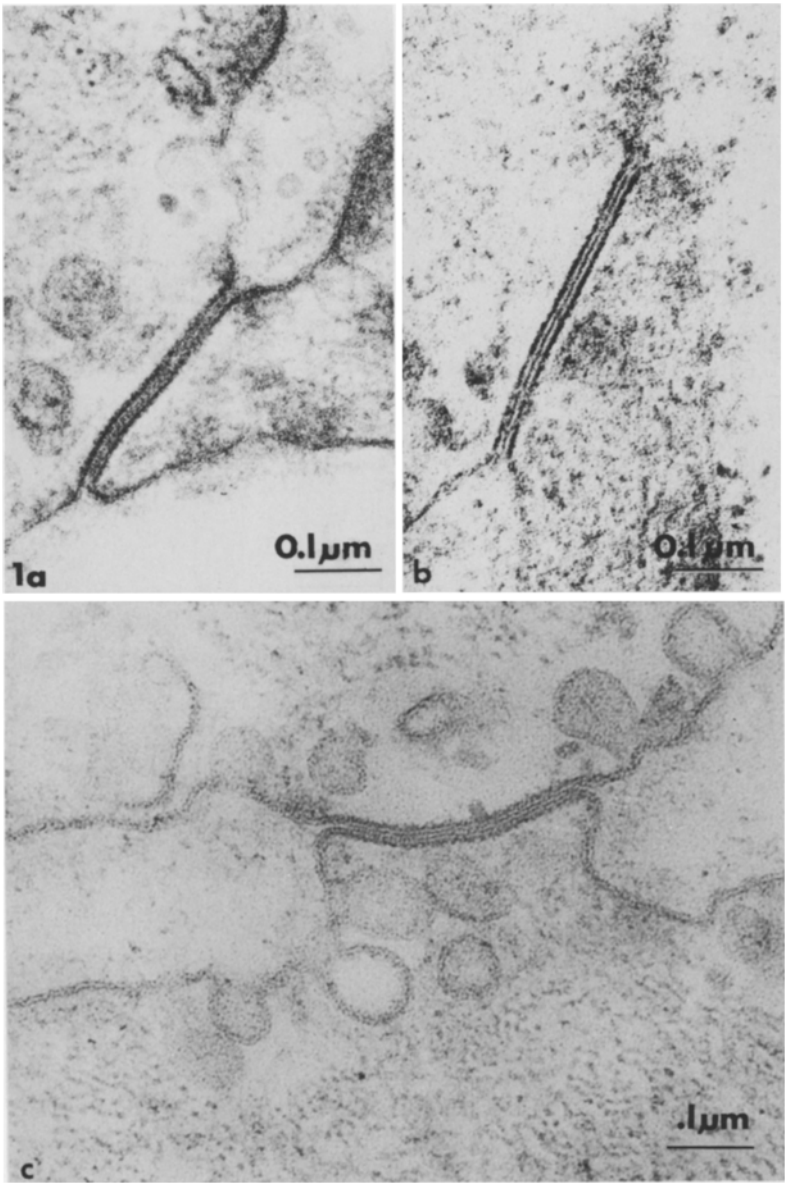


Fig. 1. Nexuses in the main circular layer of dog intestine after fixation by intra-arterial perfusion with (*a*) buffered glutaraldehyde; (*b*) permanganate; (*c*) buffered glutaraldehyde and *en bloc* staining with uranyl acetate

15 per 400-mesh grid square containing about 40 cells cut in cross section, 0.15 to 0.38 per cell) in accordance with our earlier findings [7, 14, 30, 17]. When stained *en bloc* [47, 39], these junctions have a clear 2 nm gap between the plasma membranes; thus they are gap junctions (Fig. 1 *c*).

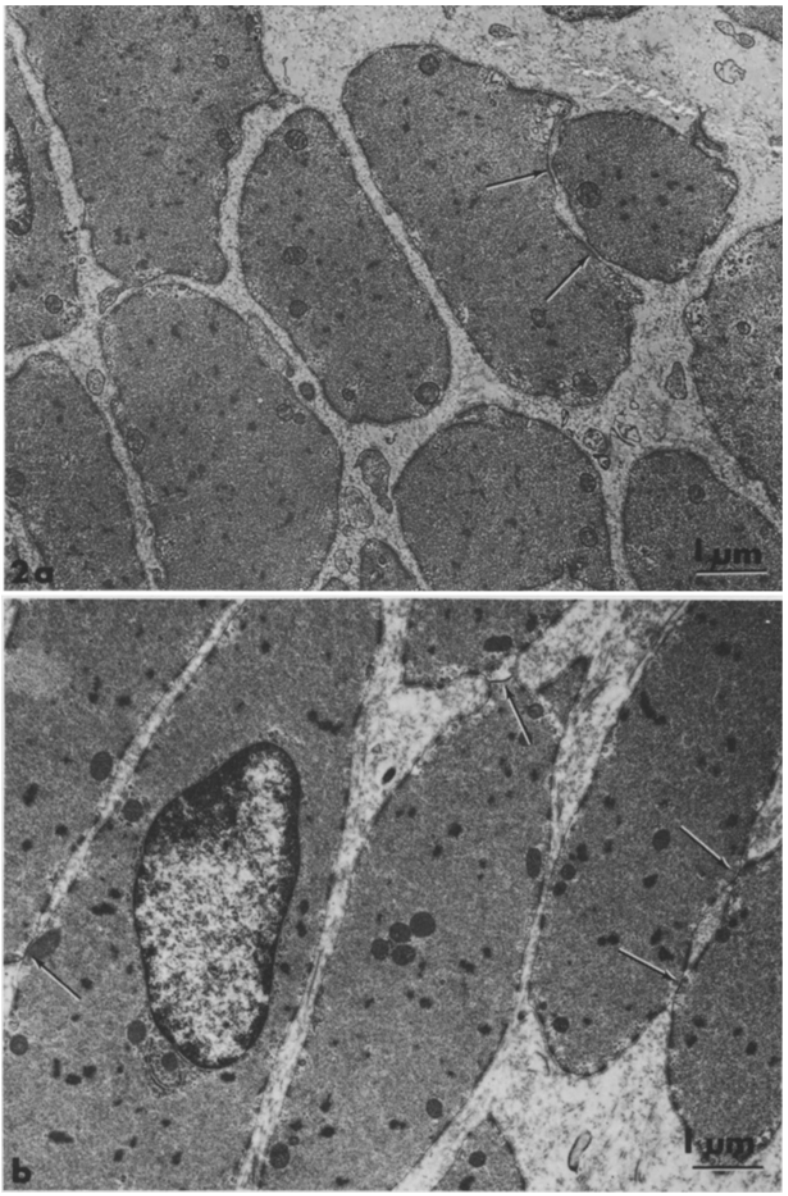


Fig. 2. Smooth muscle of dog intestine after fixation by intra-arterial perfusion with buffered glutaraldehyde. (*a*) Longitudinal muscle layer; no nexuses are present. The arrows are pointing to intermediate contacts. (*b*) Main circular muscle layer with several nexuses in the field. The arrows point to nexuses

Absence or rarity of nexal structures in longitudinal muscle and their presence in circular muscle applies not only to those preparations in which fixation led to ideal results by our criteria (good cell preservation,

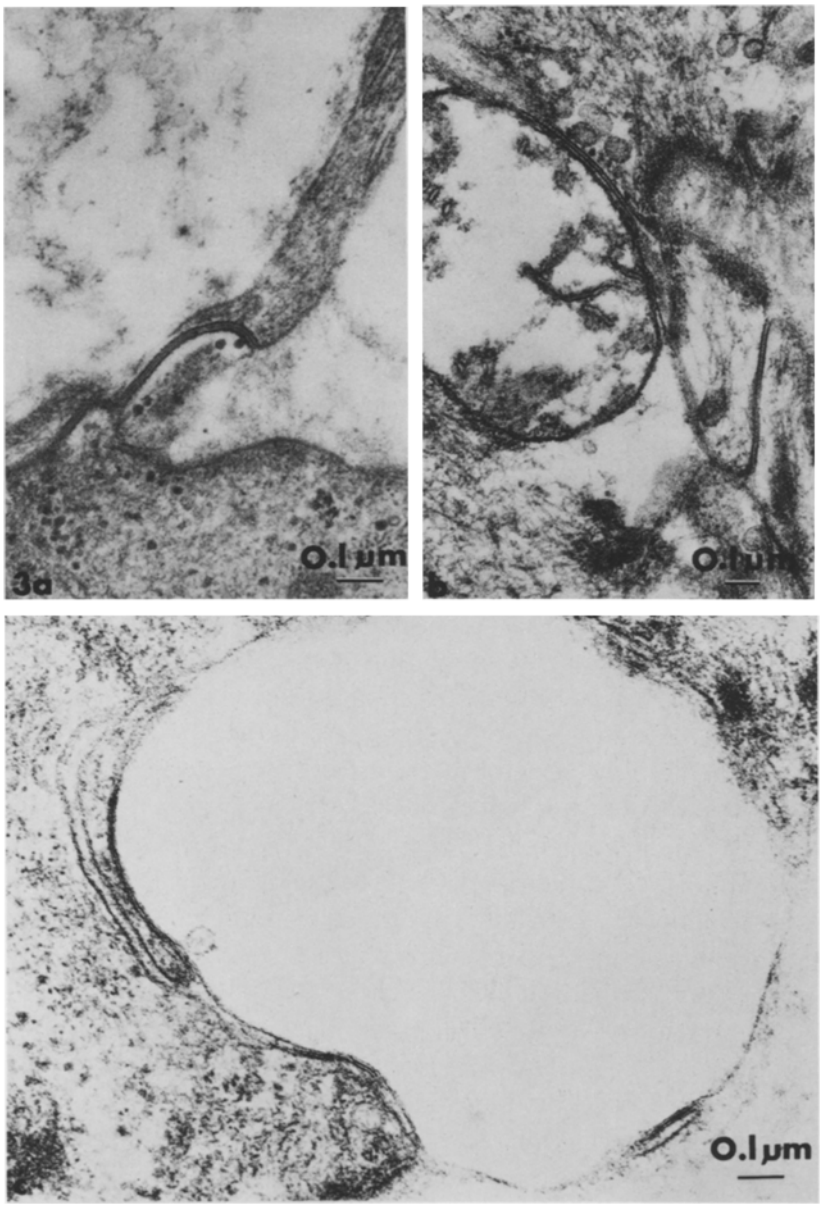


Fig. 3. (*a*) Tissue was perfused with hypertonic solution which resulted in uncoupling of electrical control activity of overlying longitudinal muscle. The cells in circular muscle became grossly shrunken, and were held together with long processes which regularly terminated in nexuses. (*b*) A example of poor *in vitro* fixation of circular muscle of dog intestine. Despite the poor general appearance of the tissue (swollen mitochondria, loss of organized myofilaments in the cytoplasm and damage to the plasma membrane) the nexuses are well preserved. (*c*) Duodenal circular smooth muscle of dog intestine, incubated *in vitro* for 30 min in iodoacetate and dinitrophenol. As a result of swelling of intracellular compartments, the membranes are pushed close together, giving the five-layered appearance of a nexus. These nexus-like structures under permanganate fixation could be misinterpreted as nexuses

mitochondria in condensed state, many membrane vesicles, etc.), but also to cells fixed after hypertonic solutions (Fig. 3*a*) or after ischemia (Fig. 3*b*). After hypertonic perfusion to the point of electrical uncoupling of intestinal control potentials or slow waves as previously described [14], there were, in one experiment, 97 nexal structures in 699 cells from circular muscle cut in cross section (0.132 nexus/cell), while in control muscles perfused with isotonic solutions there were 39 nexuses in 250 cells (0.156 nexus/cell).

Similarly, treatment of the muscle with KCl, acetylcholine, or other contraction stimulants or with relaxants such as adrenaline or EDTA prior to fixation did not alter the number or distribution of nexal structures. However, very severe and prolonged lack of blood supply and other damaging treatments did decrease the number of nexal structures in circular muscle. Politoff *et al.* [44] have already shown that metabolic inhibition produce electrical uncoupling, probably by increasing intracellular $Ca^{++}$ [*see* 43, 35] and we have shown that Ca gain occurs in smooth muscle after metabolic inhibition [4, 59]. Conceivably, structural nexuses may persist after cells are functionally uncoupled. If so, it would argue against the notion that the structural nexus is labile and easily disrupted. Therefore, we examined the effect of metabolic inhibitors on nexuses in the dog intestine.

Treatment of smooth muscle with $10^{-3}$ M iodoacetate and $10^{-3}$ M dinitrophenol rapidly depletes cells of ATP, and causes K loss, Na and $H_2O$ gain, and then Ca gain [4, 59]. It also alters mitochondrial configuration, destroys membrane vesicles, and creates "light cells" [11–13, 23–25, 4]. We therefore studied the effect of these metabolic inhibitors on the structural integrity of intestinal nexuses. The initial experiments were carried out *in vitro*. Tissues were removed, dissected, and allowed to recover in aerated K.R. solution. After recovery, they were incubated in the presence of inhibitors for different lengths of time, and then fixed by immersion in buffered glutaraldehyde. Only experiments in which the control tissues, without inhibitors, were well preserved were accepted for analysis. The results of one such experiment are shown in Table 1.

As a result of 5 or 10 min *in vitro* incubation of tissues in IAA + DNP, the cells were swollen, membrane vesicles were diminished in number, and mitochondria were no longer in the condensed configuration. Nevertheless, some nexal structures were still present, though the number was clearly diminished after 5 min inhibition, and reduced to a very low level after 10 min inhibition. No such structures were found after periods of inhibition. Already after 5 min inhibition, many regions of membrane

Table 1. Effects of metabolic inhibitors on nexal structures after *in vitro* incubation

| Experiment | No. (grids squares) | Nexal structures/ grid square | Nexus-like/ grid square | Total/ grid square |
|---|---|---|---|---|
| Glutar. fixation | | | | |
| Control | | | | |
| 20 min K.R. | 8 | 11.5±0.6 | 0 | 11.5±0.6 |
| IAA+DPN ($10^{-3}$ M) | | | | |
| 5 min | 7 | 6.6±0.7[a] | 4.0±0.8[a] | 10.6 |
| 10 min | 7 | 0.6±0.3[a] | 2.8±0.5[a] | 3.4 |
| 20 min | 7 | 0.0[a] | 0.0 | 0.0 |
| $KMnO_4$ effect | | | | |
| 30 min KR+ 5 min $KMnO_4$ | 5 | 4.4±1.5[a] | 14.4±2.8[a] | 18.8±2.0[a] |
| 30 min KR+10 min $KMnO_4$ | 7 | 6.7±0.7[a] | 16.3±1.4[a] | 23.0±1.7[a] |
| 30 min KR+20 min $KMnO_4$ | 2 | 4.0 | 22.0 | 26.0 |
| 30 min KR+60 min $KMnO_4$ | 11 | 1.1±0.2[a] | 14.1±1.8[a] | 15.2±1.9 |

[a] Significantly different from control. Grids were from same specimen in each category.

approximation were found, but neither a five-lined structure characteristic of nexal structures, nor distinct membranes from adjacent cells, could be discerned at high magnification. The regions of membrane apposition (Fig. 3*c*) could have been produced by disrupting nexal structures or by cell swelling: we have designed them as "nexus-like" structures. The sum of nexal and nexus-like structures was less after brief periods of metabolic inhibition than the number of nexuses in control tissues (Table 1). Nexus-like structures were found even after prolonged inhibition. Thus metabolic inhibition disrupts nexal structures, but there may be a lag between ATP depletion, with resultant $Ca^{++}$-gain, junctional uncoupling and cell swelling, and complete disappearance of nexal structures. Nexus-like structures are produced by metabolic inhibition with cell swelling.

In addition to studies *in vitro*, we perfused various segments of the intestine intra-arterially for varying periods with K.R. solution at 24 °C, containing metabolic inhibitors. Controls were similarly perfused in the absence of inhibitors. Since prolonged perfusion of tissues resulted in extracellular edema, it was decided to express the data in terms of nexal structures per cell, rather than per grid square. The results of these experiments are shown in Table 2.

In perfused control tissues, the number of nexal structures per cell

Table 2. Effects of metabolic inhibitors on circular muscle nexuses after *in vivo* perfusion (glutaraldehyde fixation)

| Experiment | | No. grids squares | Cells/grid square | Nexal structures/ grid square | Nexal structures/ cell |
|---|---|---|---|---|---|
| #1 | Control[a] | | | | |
| | 15 min KR | 6 | 44.8±1.1 | 12.0±1.0 | 0.267±0.022 |
| | IAA+DNP ($10^{-3}$ M) | | | | |
| | 5 min | 6 | 27.8±2.7[b] | 6.2±1.1[b] | 0.218±0.020 |
| | 10 min | 6 | 30.3±2.4[b] | 5.0±1.3[b] | 0.192±0.034 |
| | 15 min | 6 | 26.3±1.9[b] | 5.0±0.8[b] | 0.164±0.015[b] |
| #2 | Control | | | | |
| | 30 min KR | 6 | 104 ±2 | 14.0±1.1 | 0.14 ±0.01 |
| | IAA+DNP ($10^{-3}$ M) | | | | |
| | 10 min | 6 | 116 ±7.5 | 16.3±1.4 | 0.145±0.01 |
| | 15 min | 6 | 77 ±2.4[b] | 4.8±0.8[b] | 0.06 ±0.01[b] |
| | 30 min | 6 | 72 ±2.3[b] | 5.8±1.1[b] | 0.08 ±0.01[b] |
| #3 | Control | | | | |
| | 30 min KR | 6 | 105 ±3 | 14.5±1.8 | 0.14 ±0.02 |
| | IAA+DNP ($10^{-3}$ M) | | | | |
| | 30 min | 6 | 116 ±4 | 5.2±1.5[b] | 0.04 ±0.01[b] |

[a] In Experiment 1, 400 mesh grids were used; in experiments 2 and 3, 300 mesh grids were used.
[b] Significant difference from control in each experiment.

was similar to values for tissues fixed without prolonged perfusion. This was true despite marked extracellular edema which reduced the number of cells per grid square (Table 2) from about 70 to about 45 for 400-mesh grids in controls, and from 70 to about 25 in inhibited tissues. This presumably produced mechanical strain on nexal junctions. The disappearance of nexal structures during metabolic inhibition was slower than *in vitro* probably because the perfusion temperature was 24 °C; some persisted after 30 min exposure to metabolic inhibitors. There was, in fact, little decrease in the number of such structures until 15 min of perfusion with metabolic inhibitors had elapsed. Changes related to metabolic inhibition had occurred in mitochondria after 5 min; marked swelling of endoplasmic reticulum and damage to membrane vesicles occurred before nexal structures had disappeared (Fig. 4). There was also at least

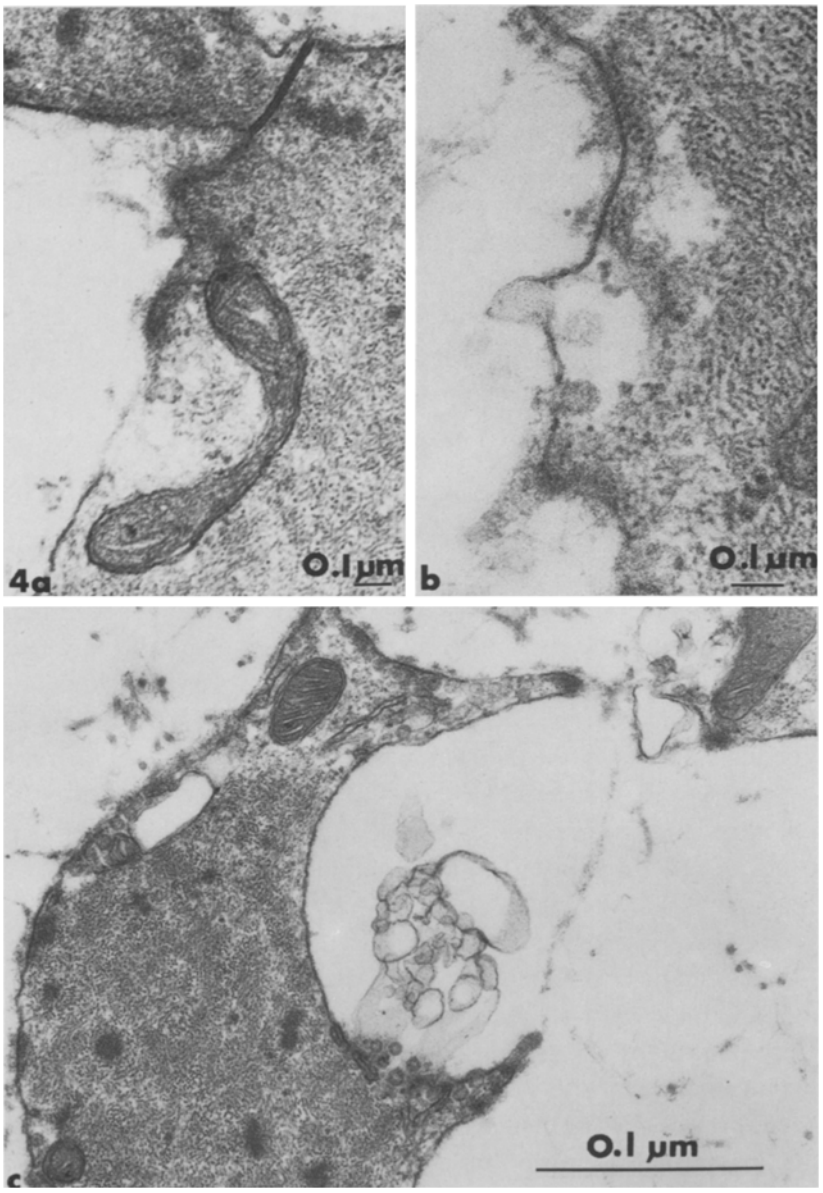


Fig. 4. Intestinal smooth muscle was perfused for 30 min with Krebs solution containing $10^{-3}$ M each of iodoacetate and dinitrophenol. (*a*) Note the altered shape and state of the mitochondria, and damage to plasma membrane, while the nexus appears to be intact. V or dumbell shaped mitochondria were very common in these muscles, but very rare in controls (*cf.* Figs. 2 and 9). (*b*) Single membrane vesicles "popped" out by treatment. (*c*) Note swollen sarcoplasmic reticulum and extruded membrane with vesicles

as much swollen endoplasmic reticulum in the perfused controls as in metabolically inhibited tissues. This suggests that the increased perfusion pressure or the decreased osmotic pressure of the perfusate caused swelling of endoplasmic reticulum. However, altered mitochondrial shapes (Fig. 4*a*) and the appearance of many regions in which membrane appeared "blown out" (Fig. 4*c* and *b*) occurred only in inhibited tissues. These experiments show clearly that extensive membrane damage had occurred while nexal structures still persisted. No nexus-like structures were found as nexal structures disappeared since apposition of plasma membranes did not occur as cells swelled, owing to the presence of extracellular swelling provoked by perfusion.

### *Permanganate Fixation and Nexal Structures in Intestine*

We wished to test the hypothesis that the overall distribution of nexal structures in the intestine is the same after permanganate fixation as after glutaraldehyde fixation, but that there is generally poorer and more variable preservation of these structures with permanganate. If this hypothesis is correct, we would expect to find a smaller average and greater variation of nexal structures per cell in circular muscle fixed with permanganate than in muscle fixed in glutaraldehyde, but the maximum value of theis parameter should be the same. Further, there should be no nexal structures in either longitudinal muscle or the inner dense of circular muscle.

To test this hypothesis, we initially used the conventional approach: isolation of small segments of muscle, followed by variable amounts of incubation *in vitro* in aerated physiological solutions, and then fixation for 10 to 30 (usually 20) min in permanganate. Thin sections from such material are notoriously variable in appearance [29]. In many cases, cells were grossly damaged, and no nexuses were observed in any layer. In others, a few nexal structures were found, exclusively in the circular muscle layer. Gross swelling of cells occurred, producing apposition of cell membranes (Fig. 5*a*), but not five- or seven-lined nexal structures [30], despite a recent misinterpretation of our findings [45]. A variety of nexus-like artifacts can be produced when smooth muscle cells are swollen in such a way that two opposing plasma membranes (Fig. 5*a*) or even intracellular membranes (Fig. 5*b*) are pressed together. After glutaraldehyde fixation, tissues incubated *in vitro* showed typical nexuses (*see* tables control).

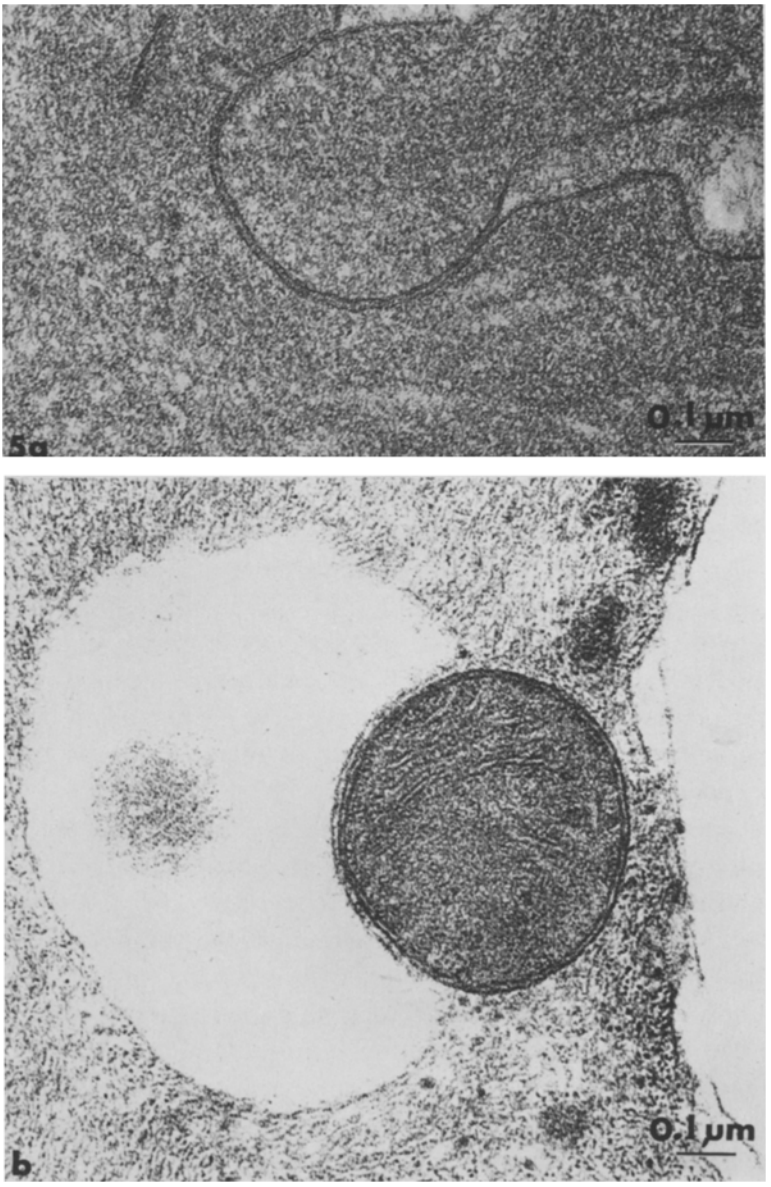


Fig. 5. Nexus-like structures in intestinal smooth muscle. (*a*) Produced by *in vitro* fixation with permanganate. Note that the membrane of the two cells are distinct in some regions but not in others. Note the membrane vesicle, an organelle not usually associated with nexal junctions. (*b*) Produced by *in vitro* treatment with $10^{-3}$ M iodoacetate and dinitrophenol followed by glutaraldehyde fixation. Note the close relationship between mitochondrial membrane and an intracellular membrane, probably sarcoplasmic reticulum

In some experiments (Table 1) we treated permanganate in the same way as a metabolic poison. Tissues were exposed to it *in vitro* for varying periods of time, rinsed in 0.9% saline and were then fixed as usual in glutaraldehyde. The number of nexal structures decreased with prolonged permanganate exposure, but a substantial number of nexus-like structures were found. It was impossible to rule out that these were originally nexal structures.

Better permanganate fixation was obtained by perfusing a segment of the intestine for 5 min with glucose-free Krebs solution at 24 °C to eliminate glucose and other organic oxidizing substances; then it was perfused for 5 min with permanganate at 24 °C, which resulted in uniform fixation of the tissue. The segments were removed, cut up into small pieces, washed, and placed in glucose-free Krebs solution at 4 °C until embedded. In some cases, the permanganate perfusion was preceded or followed by 5 min perfusion with the Krebs-glutaraldehyde solution, and placed in 5% glutaraldehyde buffer after dissection; while in other cases the tissues were perfused with only permanganate as a fixative, and were placed in 5% buffered glutaraldehyde. Glutaraldehyde was used to determine if (a) it reduced the number of nexuses preserved by permanganate alone; or (b) it helped preserve nexuses when permanganate fixation was inadequate. Whenever permanganate was used as a fixative, post-osmication was omitted.

In tissues perfused with permanganate as described above, the cells were better preserved and not so grossly swollen as when permanganate fixation was applied *in vitro* (Fig. 6*a*). There was also the usual poor preservation of basement membrane material; membrane vesicles were fewer and smaller, mitochondria were in the orthodox state, endoplasmic reticulum was swollen, etc. The nexuses which were preserved in circular muscle had the same general structure as in glutaraldehyde-fixed tissues (e.g., they were of the simple abutment type, and no peg-and-socket type nexuses were found); *see* Fig. 6*b*.

When the tissues were fixed *in situ* by permanganate, at low magnification some structures had the overall appearance of a nexus; but the five- or seven-layered structure could not be resolved at higher magnification, nor could the separate components of the two individual cell membranes be resolved as in "close apposition" contacts (Fig. 6*b*). Conceivably the nature of these structures might have been resolved with a tilt-stage; we suspect many may be nexuses. These types of junction we also called "nexus-like", although they may differ in origin from those described above. If these structures represented poorly preserved

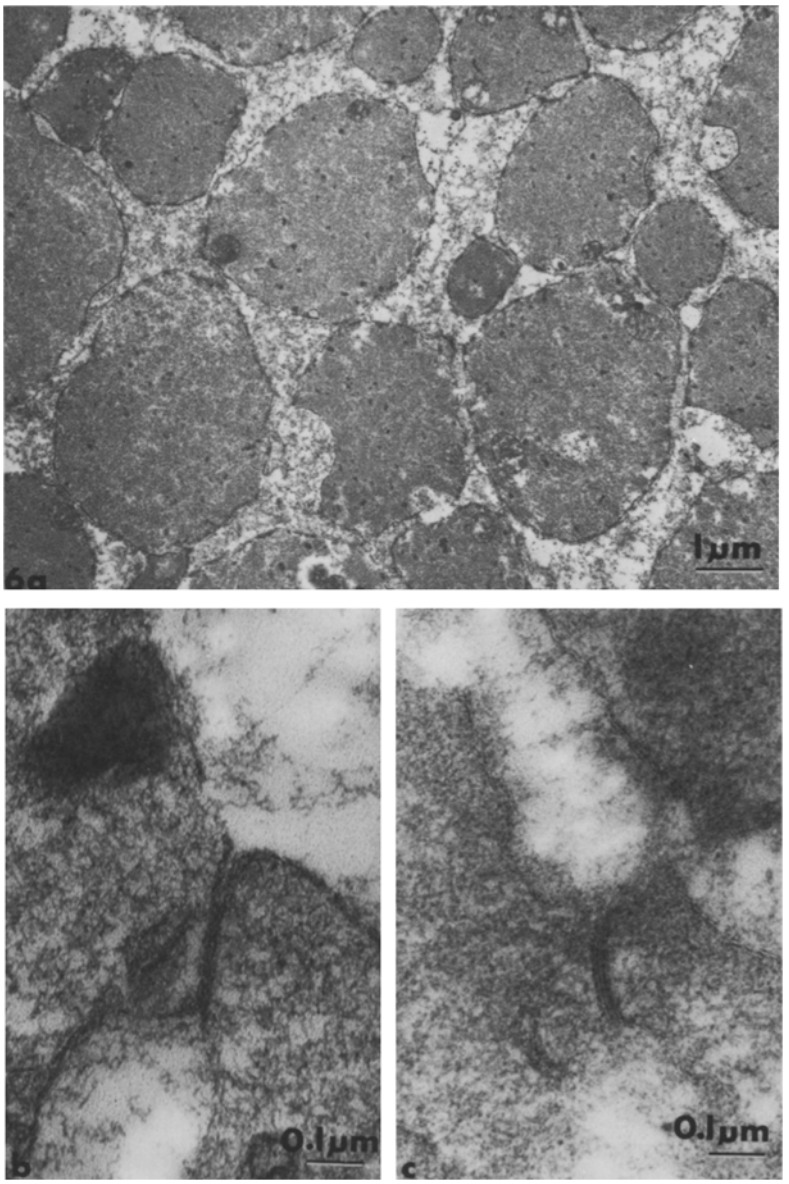


Fig. 6. Intestinal smooth muscle was fixed by intra-arterial perfusion with permanganate in glucose-free solution. (*a*) Low power view, indicating that although the overall fixation of the tissue is much inferior to glutaraldehyde, perfusion fixation at least prevented gross swelling of the tissue, in contrast to the case with *in vitro* permanganate fixation. (*b*) Nexus-like structure. Note that the five-layered structure is not discernible. (*c*) Nexus, showing the five-layered structure

Table 3. Effects of various procedures on circular muscle nexal structures after *in vivo* perfusion

| Procedure | No. grids[a] squares | Cells/ grid squares | Nexal structures/ cell | Nexus-like/ cell | Total/cell |
|---|---|---|---|---|---|
| KR-gluc[b], 5 min; $KMnO_4$, 5 min; into KR-gluc | 7 | 35±1.5 | 0.05 ±0.02 (0.00 to 0.17) | 0.15 ±0.025 (0.06 to 0.23) | 0.20 ±0.04 (0.06 to 0.40) |
| KR-gluc, 5 min; $KMnO_4$, 5 min; into Glutar | 7 | 36±2.3 | 0.05 ±0.01 (0.00 to 0.09) | 0.02 ±0.01 (0.00 to 0.08) | 0.08 ±0.01 (0.03 to 0.12) |
| KR-gluc, 5 min; $KMnO_4$, 5 min; Glutar, 5 min; into Glutar | 7 | 37±3.8 | 0.09 ±0.02 (0.00 to 0.16) | 0.07 ±0.02 (0.03 to 0.13) | 0.16 ±0.03 (0.03 to 0.24) |
| KR-gluc 5 min; Glutar, 5 min; $KMnO_4$, 5 min; into Glutar | 8 | 50±5.3 | 0.004±0.004 (0.00 to 0.03) | 0.005±0.005 (0.00 to 0.02) | 0.008±0.008 (0.00 to 0.03) |
| KR-gluc, 5 min; $KMnO_4$, 5 min; KR-gluc, 5 min; into KR-gluc | 6 | 46±8.0 | 0 | 0 | 0 |

[a] 400 mesh grids were used.
[b] Krebs Ringer solution without glucose.

nexuses after permanganate fixation, the total of these plus true nexal structures per cell should not exceed the total number of nexal structures per cell after glutaraldehyde fixation. The average number of nexal structures plus nexus-like structures was less in the circular muscle layer than after glutaraldehyde fixation, but the maximum number sometimes approached the usual average number found after glutaraldehyde fixation (Table 3). More nexal structures were found when permanganate was used as the sole fixation or with subsequent perfusion with glutaraldehyde. No nexal or nexus-like structures were preserved after perfusion of permanganate-fixed tissues with Krebs solution or with glutaraldehyde before permanganate perfusion. In the latter tissues, permanganate was unable to penetrate tissues distant from the blood vessels and these permanganate perfused areas lacked nexal structures.

Thus these results conform to the predictions from the hypothesis

that permanganate fixation is more variable than glutaraldehyde fixation in preserving nexal structures in intestinal circular muscle. They are inconsistent with any hypothesis that permanganate fixation preserves such structures better than does glutaraldehyde fixation.

From these results, we concluded that intra-arterial glutaraldehyde fixation followed by post-osmication is most reliable in preserving nexal structures in circular muscle under the entire range of procedures from the least to the most damaging.

The reduction in the number of such structures of the metabolically inhibited tissue is most likely due to the damage caused by these agents to the tissue, rather than by any fixation problems, since the remaining nexal structures appeared to be well preserved structurally. It was therefore evident that they are not highly labile structurally, since damage to mitochondria, plasma membrane (shape change and eversion), endoplasmic reticulum (swelling), membrane vesicles, etc., was marked and even electrical uncoupling may have occurred before nexal structures disappeared.

Nexus-like structures were produced when the plasma membranes of two neighboring cells were brought into close proximity to each other due to swelling after *in vitro* permanganate fixation or after metabolic inhibition with subsequent glutaraldehyde fixation. These regions of membrane apposition were not seen when the tissue was perfused *in situ* and fixed with permanganate or with glutaraldehyde and extracellular edema prevented membrane apposition. When permanganate fixation was carried out *in vivo*, there were no nexal structures in longitudinal muscle, nor in the dense circular muscle layer but these were present in circular muscle. Even after permanganate fixation *in vitro*, nexal structures were found only in circular muscle. Thus, the distribution of these structures after permanganate fixation is qualitatively like that following glutaraldehyde fixation.

Owing to the swelling following permanganate fixation *in vitro*, not only membrane apposition occurs, but also in some instances peg-and-socket type areas of membrane apposition were produced (Fig. 6*a*). These peg-and-socket type structures, however, were not apparent in samples fixed *in vivo* by permanganate fixation. Peg-and-socket structures were never seen after glutaraldehyde fixation, unless cells were incubated in hypotonic saline, in which case the cells became swollen and consequently some peg-and-socket type area could be observed (Fig. 11*a*). These artifactual structures, however, could in no case be identified as nexal structures.

*Freeze Fracture of Main Circular Muscle*

The presence of nexuses in the circular muscle was ascertained in the freeze-fractured replicas. Every one of the 10 replicas prepared from 7 dogs revealed many typical junctions which could be casily recognized by their characteristic close packing of particles on the fractured face (labelled *PF* in Fig. 7) viewed from the intercellular space and corresponding groups of depressions on the complementary face (labelled *EF* in

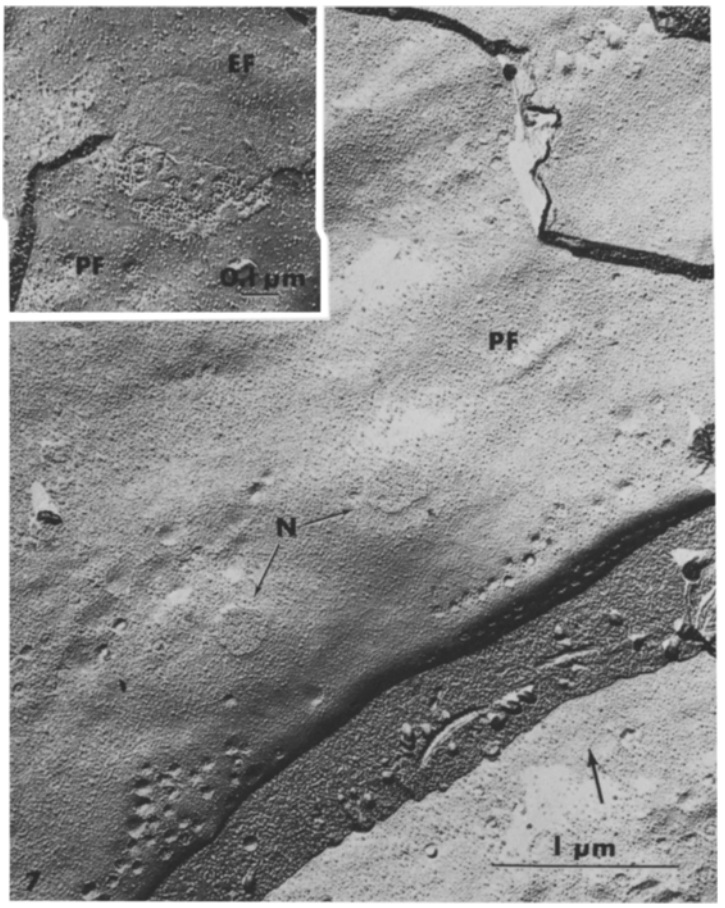


Fig. 7. Freeze fracture replica of circular muscle from dog small intestine. Two nexuses can be seen as aggregates of membrane particles on the *PF* face. Insert shows a nexus in which both the cluster of particles on the *PF* face and the corresponding series of pits on the *EF* face are apparent. Shadow angle, noted by the large arrow, is the same in both photographs. *N*, nexus; *EF*, extracellular fracture face; *PF*, protoplasmic fracture face. The terms *EF* and *PF* correspond to the *A* or concave face and the *B* or convex face, respectively

Fig. 7) viewed from within the cell. All of the nexuses recognized by this technique had the same relation to the cell structure; none seemed to be part of a peg-and-socket junction between cells.

### *Absence of Nexal Structure in Longitudinal Muscle by Thin Section*

In longitudinal muscle fixed by perfusion with glutaraldehyde (31 dogs), nexuses have never been observed (Fig. 2*a*). After fixation *in vitro* (6 dogs), nexus-like structures, but no nexuses, were found in some experiments in which cells were swollen because tissue preservation was inadequate or in others because metabolic inhibitors were used (Figs. 3 and 5*a*). After *in vitro* fixation with permanganate (7 dogs), nexuses were never seen but nexus-like structures were (Fig. 6*a*). After *in vivo* fixation with permanganate (5 dogs), nexuses were absent and nexus-like contacts were very rare. Thus we conclude either that nexuses are absent or extremely rare in thin sections of longitudinal muscle. We have used freeze-fracture studies to aid in resolving whether they are absent in thin sections because they are invisible or labile under the best conditions we can apply.

### *Freeze Fracture of Longitudinal Muscle*

The study of freeze-fractured replicas of the longitudinal muscle indicates the absence of junctions characterizable as typical nexuses (gap junctions) and which are easily discernible in the circular muscle (see p. 224). None of the 15 replicas of the longitudinal intestinal muscle obtained from 7 dogs showed any junction that could be interpreted as a nexus. Except for the absence of the nexuses, the fractured faces of the longitudinal muscle were comparable to the corresponding fractured faces of the circular muscle (Fig. 8).

If nexuses are absent in longitudinal muscle of the intestine, the question arises what structures do provide for coupling between cells in this layer. This will be examined in detail elsewhere. There are, however, two candidate structures which occur in these and other smooth muscles [30, 14, 31]: (1) long intermediate contacts (desmosome-like) structures which are characterized by a 40–60 nm gap between parallel plasma membranes with an intermediate dense line or row of particles in the intervening basement membrane and increased electron density

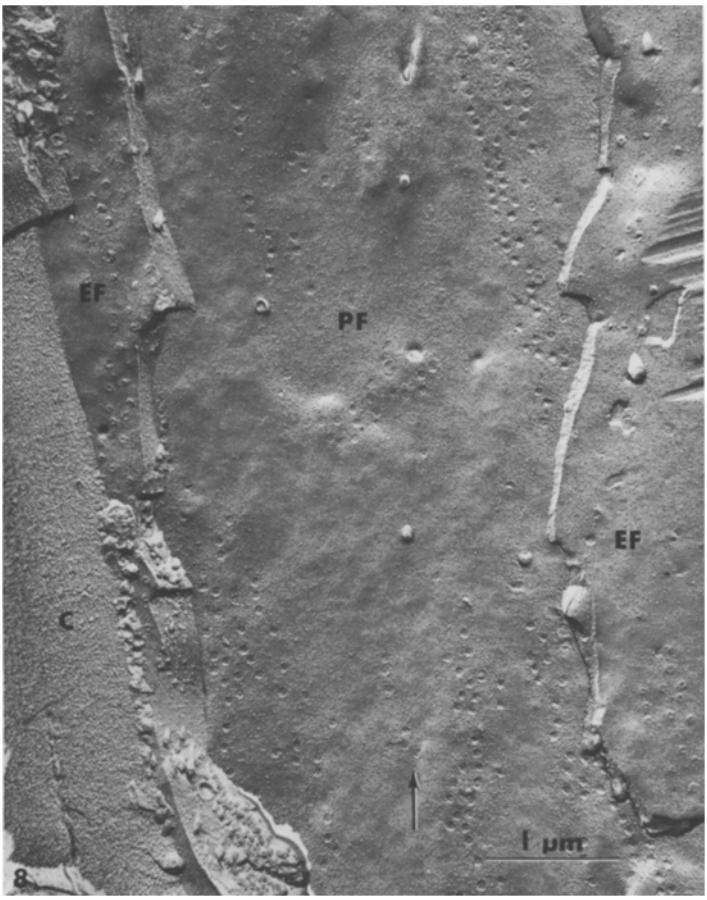


Fig. 8. Typical freeze fracture replica of longitudinal muscle from dog small intestine showing a large area of a *PF* face. No nexuses can be seen in this replica nor were they seen in any others examined. *C*, cytoplasm. Other figure markings as in Fig. 7

of the subadjacent cytoplasm; (2) close appositions with a gap of 10–30 nm between apposed, structurally undifferentiated membranes. Intermediate contacts are often found between an appendage of one cell and the surrounding membrane of another cell into which it is inserted. Close appositions occur mainly between small appendages of two cells.

### *Absence of Nexuses in the Inner Dense Layer of Circular Muscle*

More strain may be exerted in longitudinal muscle nexuses during fixation compared to circular muscle nexuses. However, circular muscle of

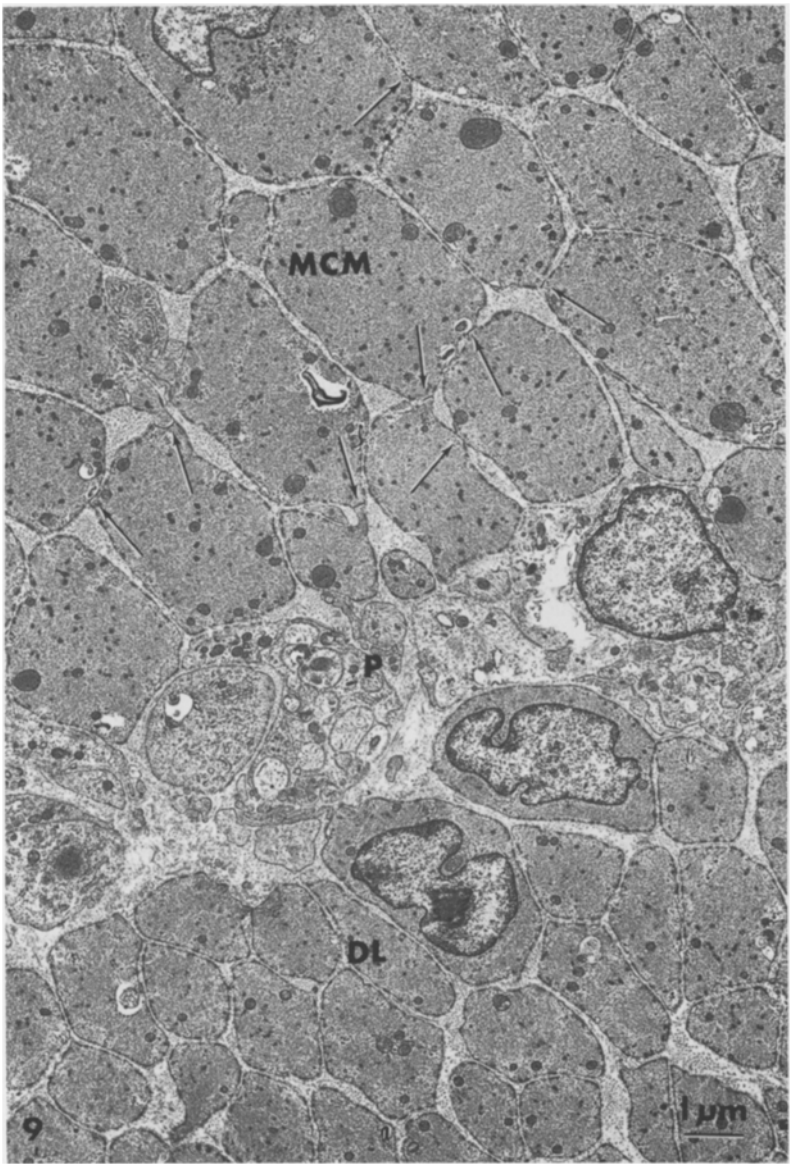


Fig. 9. Overall view of circular muscle in the dog intestine after intra-arterial glutaraldehyde fixation. The dense circular muscle layer *DL* is separated from the main circular muscle *MCM* by the plexus muscularis profundus *P*. Note that while nexuses are plentiful in the main layer, they are absent in the dense layer

the dog small intestine is separated by a nerve plexus into an inner thin, dense layer (6 to 12 cells thick, small, closely-packed cells) and an outer main portion (Fig. 9). In the inner dense layer, examination of segments

from 20 dogs revealed no nexal structure, while in the same grids (Fig. 9) many such structures were found in the main circular muscle layer. This result was obtained in all regions (duodenum to ileum) of the dog intestine, and after permanganate fixation *in vivo* as well as after glutaraldehyde fixation (*see above*). No nexuses were found after freeze fracture of this region. However, in thin sections, nexuses were found between "interstitial" cells in the nerve plexus separating the two circular muscle layers, and between these cells and circular muscle of the main (outer) layer, but not between interstitial cells and circular muscle of the dense inner layer [*see also* 17].

Differential disruption of nexal structures by differential mechanical strain was further eliminated by fixing segments of intestine either after relaxation with epinephrine (1 μg/ml) or contraction (acetylcholine, 1 μg/ml) or high potassium Ringer solution (119 mM $K^+$). The distribution of nexuses was unaffected. These results rule out differential mechanical strain as a basis for disruption of longitudinal muscle nexal structures when they are preserved in circular muscle. They also argue against

Table 4. Occurrence of nexal structures and close apposition contact in stomach muscle layers[a]

| Area | Longitudinal | | Circular | |
|---|---|---|---|---|
| | Nexus | Close | Nexus | Close |
| Proximal | | | | |
| Electrical control activity absent | | | | |
| 1 | 0 | – | 0 | 0.20 |
| 2 | 0 | 0.38 | 0 | 0.18 |
| 3 | 0 | 0.13 | 0.02 | 0.25 |
| 4 | 0 | 0.36 | 0.03 | 0.22 |
| 5 | 0 | 0.48 | – | – |
| Distal | | | | |
| Electrical control activity present | | | | |
| 1 | 0 | – | 0.18 | 0.16 |
| 2 | 0 | 0.45 | 0.35 | 0.31 |
| 3 | 0 | 0.49 | 0.40 | 0.20 |
| 4 | 0 | – | 0.20 | 0.36 |
| 5 | 0 | 0.54 | 0.30 | 0.27 |
| 6 | <0.05 | 0.20 | 0.10 | 0.16 |
| 7 | <0.05 | 0.13 | 0.13 | 0.13 |
| 8 | <0.05 | 0.25 | 0.28 | 0.20 |

[a] Data expressed as contacts per cell in cross-section with a minimum of 80 cells examined. These data are for one stomach; ten stomachs were similar.

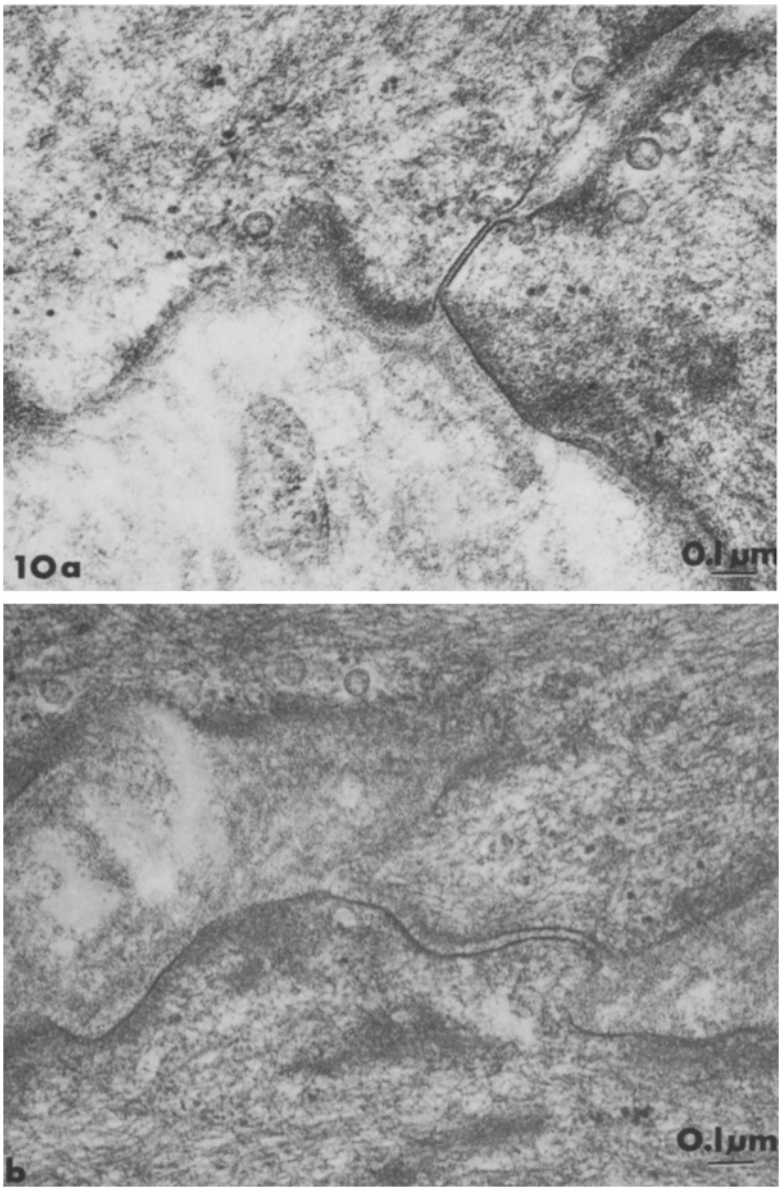


Fig. 10. Dog stomach: *in vivo* glutaraldehyde fixation. (*a*) A typical nexus which has regular occurrence in the circular muscle layer. (*b*) Close apposition which is present in both muscle layers, but the only contact in the longitudinal muscle

any difference between preservation of nexuses in muscle layers owing to different geography or orientation. There still might be some chemical difference between longitudinal muscle or inner dense circular muscle and the main circular muscle layer.

*Dog Stomach.* We have examined 10 stomachs for cell-to-cell connections [42]. There were numerous nexuses in circular muscle of the electrically active and coupled region of the dog stomach (Table 4), but none in most regions of the longitudinal muscle, and only a few (<1 per 40 cells) in the longitudinal muscle of the antrum. In the nonelectrically active region (fundus) there were no or very few nexuses in either layer, but this region may not be electrically coupled. Table 4 also contains data about the distribution of close apposition contacts in the stomach, and Fig. 10 illustrates both types of structures. There were almost no intermediate contacts in either layer of the dog stomach.

*Uterine Muscle.* Longitudinal muscle cells of rat myometrium are well known to be electrically coupled; they conduct action potential spikes [6, 40], applied voltages [58, 1, 2, 8], etc.

However, in extensive studies of many estrogen treated and pregnant rats prior to term, we were unable to find nexal structures between cells in either the longitudinal or circular muscle layers. Table 5 contains a summary of some of the experimental procedures and conditions studied. This quantitative study represents only a small fraction of the uterine tissues we have examined.

On the other hand, we found nexal structures regularly in the fibrocytes which separate the same muscle bundles which lack them (Fig. 11).

Table 5. Effects of various procedures on occurrence of nexal structures in myometrium

| Tissues | Fixation | Procedures | $n$[a] | Cell types | Nexal structures |
|---|---|---|---|---|---|
| Estrogen dominated | Glutaraldehyde *in vitro* | Controls 1 hour Krebs Ringer | 24 | Smooth muscle | − |
| | | | | Fibrocytes | + |
| | | Metabolic inhibitors 1 hour IAA + DNP | 4 | Smooth muscle | − |
| | | | | Fibrocytes | − |
| | Glutaraldehyde *in situ* by i.a. perfusion | | 6 | Smooth muscle | − |
| | | | | Fibrocytes | + |
| | Permanganate *in vitro* | | 4 | Smooth muscle | (Nexus-like) |
| | | | | Fibrocytes | − |
| Pregnant | Glutaraldehyde *in situ* | | 10 | Smooth muscle | − |
| | | | | Fibrocytes | + |

[a] $n$ = Number of tissues examined.

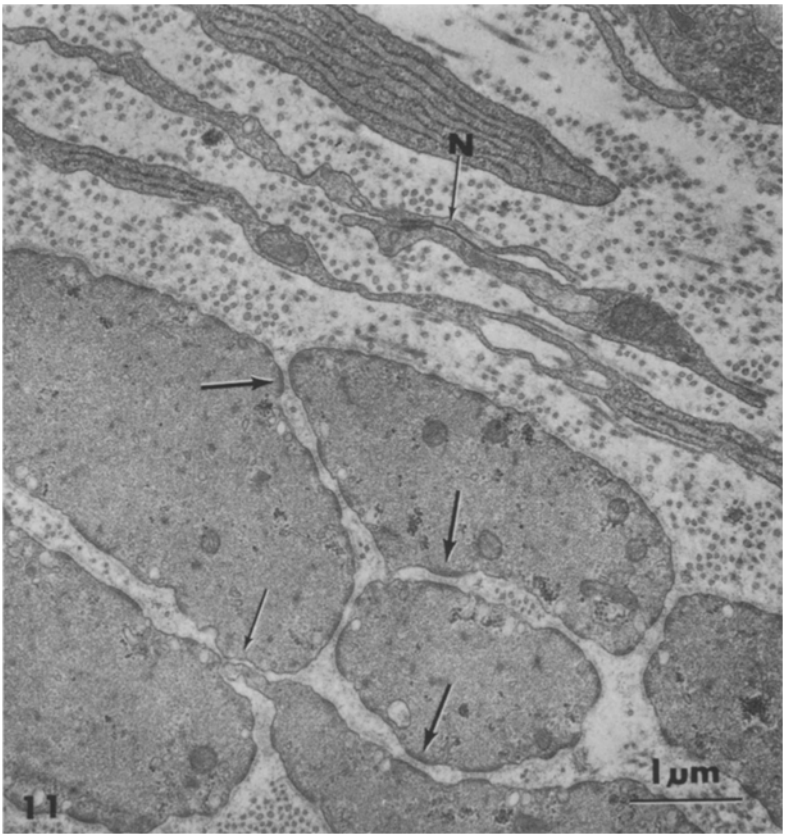


Fig. 11. Rat myometrium, showing presence of nexal contacts between fibrocytes *N*. There are several intermediate contacts (large arrows) and close appositions (small arrow), which structures are prevalent throughout the tissue

Hashimoto *et al.* [29] found structures which appear to be nexuses between fibroblasts in skin. Nexal structures between fibrocytes of uterine muscle may have been mistaken for gap junctions between smooth muscle cells, since at high magnification portions of fibrocytes, especially after permanganate fixation, may closely resemble smooth muscle cells (e.g., membrane vesicles, filamentous cytoplasm, etc.). A selective preservation of nexal structures in fibroblasts due to their lack of contractile properties, which would protect them from mechanical strain during fixation, seems unlikely in view of the fact that they are closely associated with muscle bundles and are thenselves able to contract [41]. Furthermore, the lack of nexal structures between smooth muscle cells which showed no sign of contraction (smooth cell boundaries, etc.) as well as the use – in some

cases—of a relaxant solution (noradrenaline $10^{-7}$ M) prior to fixation seems to negate this argument.

The one report of nexal structures in rat myometrium [5] used permanganate as a fixative. In the present study, as well as in previous reports [30], we found that *in vitro* permanganate fixation caused gross swelling of smooth muscle, which led to poor preservation of a variety of structures, as well as bringing some areas of adjacent plasma membranes into close apposition to each other. If swelling creates nexus-like structures, then swelling of the cells prior to glutaraldehyde fixation might create nexus-like structures, while shrinking prior to permanganate fixation might prevent their formation. These predictions were tested in the estrogen-stimulated rat uterus.

When isolated myometrium was exposed to hypotonic solution and then fixed in hypotonic glutaraldehyde, the cells were subsequently found to be markedly swollen but still separated by basement membrane and collagen (Fig. 12*a*). When cells were fixed in permanganate, irrespective of whether or not they had been shrunk prior to fixation, they were found to be swollen together (Fig. 12*b*). In addition, some peg-and-socket type regions of apposition, never seen after conventional glutaraldehyde fixation, were produced by both swelling procedures (Figs. 12*a* and 5*a*). Thus permanganate fixation destroys membrane integrity, and in so doing causes marked swelling; but comparison of cells swollen and fixed in glutaraldehyde with those swollen in permanganate suggests that permanganate allows very close cell membrane apposition by failing to preserve collagen as well as basement membrane and other ground substances. Basement membrane contains a collagen type protein [33], possibly accounting for similar fixation interactions. If this explanation is correct, the ability of permanganate fixation to yield nexus-like structures may be explained in terms of its ability to provide fixation conditions leading to cell-to-cell apposition. It should be emphasized that the typical five-lined structure observed after fixation of intestine with permanganate or glutaraldehyde was never observed in these nexus-like regions of membrane apposition.

*Human Fallopian Tube.* We studied electrical activity and ultrastructure of the ampulla of the human fallopian tube [9, 10]. We found propagated spikes and numerous close apposition contacts between muscle cells. There were no nexuses in specimens from 63 women in either ampulla or isthmus, in any muscle bundle or layer.

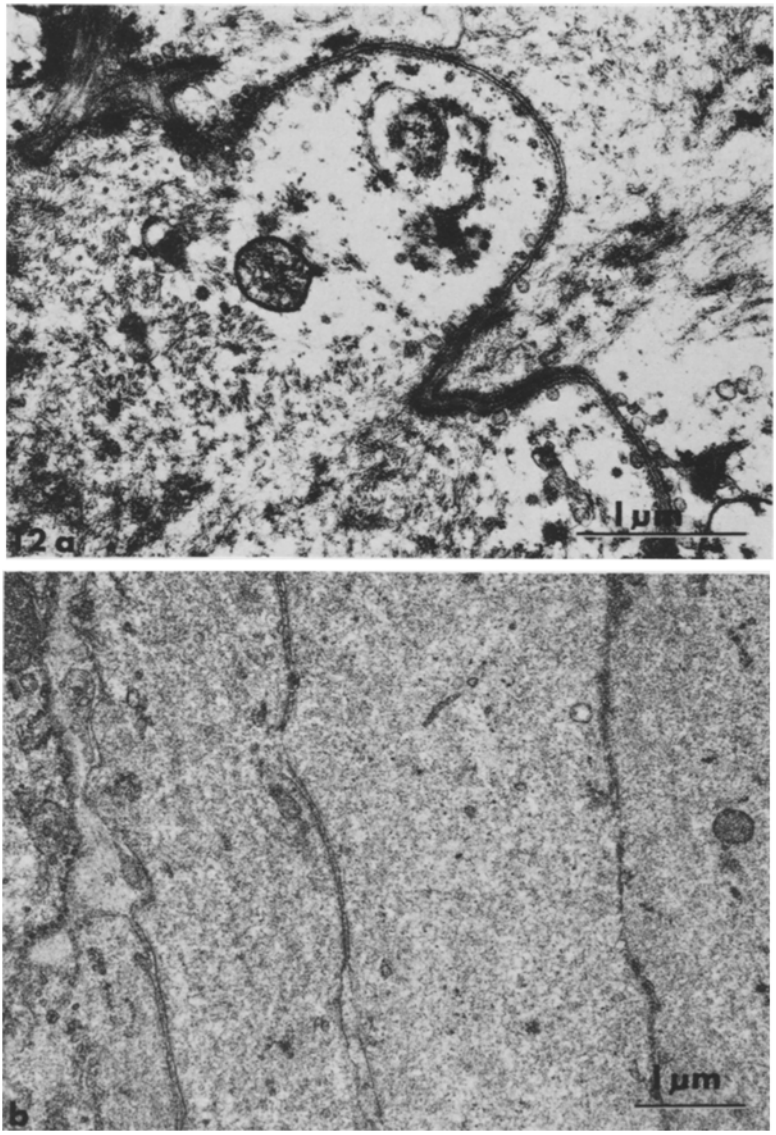


Fig. 12. Rat myometrium. (*a*) Fixed with 1% glutaraldehyde after the cells were swollen in 1/3 hypotonic Krebs-Ringer solution. Note that even at the peg-and-socket type of formation the membranes are still separated from each other. Note also that collagen is well preserved in extracellular space. (*b*) *In vitro* permanganate fixation resulted in swelling of tissue; consequently extracellular space is reduced or absent. Poor fixation of extracellular basement membrane material is also prevalent

## Discussion

In smooth muscle tissues from the gastrointestinal tract and urogenital tract, known to be electrically coupled, nexal structures could not be demonstrated using techniques which gave excellent preservation of other structures. Intra-arterial perfusion of glutaraldehyde resulted in good fixation of dog intestine and stomach, and rat myometrium, as judged by the appearance of cellular structures and membranes. The presence of nexal structures in the longitudinal muscle of the intestine in the dense circular muscle layer, or in any muscle layer of the myometrium or fallopian tube, could not be demonstrated. Their occurrence in the longitudinal muscle of the dog stomach was very infrequent. Nevertheless, nexuses were preserved in the circular muscle layer of the intestine and in the corpus and antrum of the stomach, and in the fibrocytes of the myometrium. These structures were present despite very harsh treatment of intestinal circular muscle: hypertonic solutions, extracellular edema, anoxia, ischemia, etc. Metabolic inhibition which uncouples cells functionally [44] eventually reduced their number, but they were not the most sensitive cell structure to this insult. Furthermore, there was no evidence that greater mechanical stresses on longitudinal compared to circular muscle during fixation which could have affected the preservation of nexuses; in fact, any shortening associated with intra-arterial fixation (which was unusual) or induced by drugs, was accompanied by moderate circular muscle shortening with less evidence of longitudinal muscle shortening. Nexal structures were always found in the circular muscle of the intestine after *in situ* fixation, regardless of whether the cells were contracted or relaxed by drugs, subject to extracellular edema after prolonged perfusion, swollen by permanganate or shrunken by hypertonic solutions [30]. On the other hand, these structures have not been seen in longitudinal muscle, in any of the above conditions or when samples were fixed during relaxation. Finally, the inner dense circular muscle layer was devoid of nexuses, though presumably exposed to stresses similar to those on the main circular layer during fixation.

These findings rule out any explanation of the absence of nexal structures based on the hypothesis that glutaraldehyde fixation is generally inadequate to preserve nexuses, or inadequate except under ideal circumstances, or that permanganate fixation preserves them when glutaraldehyde does not. They also seriously question the opinion that nexuses in general are structurally highly labile depending on the fixative conditions or on stain. Thus our data strongly suggest that nexuses may

not exist in some of the tissues which have been shown to be electrically coupled; if so, their presence is not essential for electrical coupling between smooth muscle cells.

Two possibilities remain which would still allow a necessary role for nexuses in such coupling. One is that nexuses are labile with respect to preparation for EM study in some tissues but not in others. It is difficult to see why circular muscles of the intestine should differ from longitudinal muscle in this regard, and especially difficult to see why the dense circular muscle layer (no nexuses) should differ from the main circular muscle (many nexuses). Similarly, it is difficult to see why uterine smooth muscle and fibrocytes located side by side should differ in this way. However, this explanation may be impossible to exclude. If nexal structures are present only as point contacts, they may be missed in EM thin sections. The likelihood of this oversight decreases as more thin sections are viewed unless the contact areas are too small to occupy the whole thickness of a section and are therefore "invisible" in such preparations. In any case, freeze-fracture studies of longitudinal muscle also failed to find such point contacts, though nexuses were readily found in circular muscle of intestine. Study by freeze fracture should reveal such structures in longitudinal muscle if they are sufficiently frequent to account for coupling and their structures are distinctive. It should also decrease the possibility that they are lost through lability since the dehydration, embedding and staining steps are eliminated and since the typical nexal structure seen by freeze cleavage apparently remains even if the nexus is split [27].

The fact that no nexal and many nexus-like structures are found in longitudinal intestinal and uterine muscle cells swollen when fixed by permanganate is consistent with the hypothesis that permanganate may create nexus-like structures by causing swelling. Structures which had the typical five- or seven-lined membrane junction of nexuses, at typical cell to cell abutments, have never been found after permanganate fixation of longitudinal muscle or the inner dense circular muscle of the intestine, nor in any layer of myometrium. The difference between myometrical cells swollen and then fixed in glutaraldehyde, and those fixed in permanganate, might be due to better or more uniform preservation of basement membrane and collagen by glutaraldehyde. Cells swelling when these are preserved cannot come into close membrane apposition. Examination of published figures fixed in permanganate suggests that poor and variable preservation of these structures has occurred. Cell swelling of intestine after metabolic inhibition by perfusion *in situ*,

which produced extracellular edema, did not produce membrane approximation or "nexus-like" structures. Our results also suggest that the peg-and-socket type of contact [15] is absent in the smooth muscles we studied, but can be produced by procedures which swell the tissue. These will contain real nexal structures only in those tissues which possessed them before swelling occurred.

These morphological studies suggest, but do not prove conclusively, the absence of nexuses in electrically coupled smooth muscles. However, they are sufficient to shift at least part of the burden of proof to any investigator who proposes that nexal contacts in smooth muscle are necessary for cell-to-cell coupling. There are morphological, biochemical and biophysical approaches to further resolution of this problem; further study of freeze-fractures of different muscle layers of the intestine without glutaraldehyde fixation [39], study of cell fractionation of the different muscle layers to yield gap junctions and their characteristic proteins [26, 28], study of space constants in relation to structure using various techniques [1, 8, 18–20, 32, 56, 58], and application of appropriate modelling experiments [38, 53] may help.

If electrical coupling can occur without nexuses, some difficult questions arise. First, what are the structural bases for coupling, and what is the equivalent circuit for current flow between cells? Second, how do we account for finding exponential decrements of electronic currents with distance from the source, and in general the cable-like behavior of these smooth muscle systems [1, 57, 59, 30]?

These and related questions cannot yet be answered in full, but recent studies suggest that electrical coupling in insect heart occurs without low-resistance junctions, and can be explained by capacitative currents [49, 53].

Finally these results should not be interpreted as suggesting that nexal structures not only are not necessary but also are not sufficient for electrical coupling in smooth muscle. Around the circumference of the intestine there is no appreciable phase lag betwen occurrence of slow waves, and coupling in this direction is clearly much better than along the intestine [*see* 30, 50]. Similar results apply to the stomach [*see* 51]. So far no evidence shows that such coupling persists when nexuses in circular muscle are destroyed.

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*Note in Proof.* Genuine nexuses have occasionally been found in myometrium from rats perfused *in situ* with an altered glutaraldehyde fixative. Estrogen-dominated rats were studied as well as pregnant rats before term, but nexuses were found only in the former. Compare Table 5.