ISOLATION AND CHARACTERIZATION OF INVERTEBRATE SMOOTH SEPTATE JUNCTIONS

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SUMMARY

Using modifications of techniques used for the isolation of macula type intercellular junctions (gap junctions and desmosomes) the arthropod smooth septate junction has been isolated from insect midgut tissue. Midguts from cockroaches or mealworms were used and membrane fractions were obtained by sucrose gradient and ultracentrifugation techniques. Preparations with reasonable concentrations of septate junction were obtained and have been studied by thin-section, negative-stain and freeze-fracture electron microscopy. The junctions appeared to be well preserved, although there was evidence that the junction strands were able to slide within the plane of the membrane. Septa were seen to have a cross-striated appearance when viewed after negative staining but their exact structure remained difficult to determine.

Polyacrylamide gel electrophoretic studies demonstrated the reproducibility of the isolation procedure and showed that septa may have a 47 000 molecular weight glycoprotein component. Gel electrophoresis also gave some indication of the intramembrane biochemistry of the smooth septate junction, with proteins of 31 000 and 32 000 molecular weight always occurring in the junction fractions. The junctions were, however, very sensitive to both mechanical and chemical treatments, the septa were destroyed by rough homogenization or by treatment with urea at a concentration as low as 1 M. Freeze-fracture of untreated, isolated junctions demonstrated no differences from junctions in intact tissue, while replicas of urea-treated material were more difficult to interpret as the component parts of the junctions became separated once the septa had been destroyed.

Gap junctions were also obtained and resisted both mechanical and chemical treatments, which destroyed the septate junctions. Their major protein component appeared to have a molecular weight of 36 000. Attempts to isolate pleated septate junctions (from insects, molluscs and annelids) by the same techniques failed, implying a significant difference in the structures of the two types of septate junction.

INTRODUCTION

Techniques for the isolation of gap junctions (Benedetti & Emmelot, 1968; Bloemendal et al. 1972; Culvenor & Evans, 1977; Dunia et al. 1974; Ehrhart & Chauveau, 1977; Evans & Gurd, 1972; Goodenough, 1978; Goodenough & Stockenius, 1972; Henderson, Eibl & Weber, 1979; Hertzberg, Anderson, Friedlander & Gilula, 1982; Kensler & Goodenough, 1980; Kibbelaar & Bloemendal, 1979; Kistler & Bullivant, 1980a,b; Manjunath, Goings & Page, 1982) and desmosomes (Colaco & Evans, 1981; Drochmans et al. 1978; Gorbsky & Steinberg, 1981; Skerrow & Matoltsy, 1974) have been used for some time, but isolation of zonula junctions

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such as the vertebrate tight junction or the invertebrate septate junction has remained difficult. It is evident that these junctions are extremely fragile in an isolated form in comparison with other junction types. This is due at least in part to their structure, which consists of a series of bands or an anastomosing network around cells rather than a relatively solid plaque. This type of structure necessitates the isolation of large pieces of junctional membrane, at least in the early stages of the isolation procedure. Our aim has been to study in greater detail the invertebrate septate junction. Many structural types of septate junction have been described (Green & Bergquist, 1982) but their biochemical composition is unknown and their exact function is still open to discussion. In particular, the chemical structure of the septa has remained enigmatic. To this end we have made crude preparations of isolated arthropod smooth septate junctions using variations of the techniques that are well documented for studies on gap junctions. The septa of the invertebrate septate junction, however, are extremely fragile and further purification by chemical or mechanical means has proved difficult. None the less, we are able to present here a structural study of the arthropod smooth septate junction in an isolated form, which has allowed the use of negative-stain techniques to give better resolution of septal structure. Gap junctions were also obtained in our membrane fractions, along with the septate junctions.

Polyacrylamide gel electrophoretic studies have demonstrated the reproducibility of our isolation technique and given some indication of the biochemical composition of these junctions.

Attempts to isolate the mollusc–arthropod pleated septate junction and the lower invertebrate pleated septate junction by the same techniques have failed, implying a significant difference, at least in the septa, between these two types of junction and the smooth septate junction.

MATERIALS AND METHODS

Arthropod smooth septate junction-rich membrane fractions were obtained from midguts of mealworm larvae (Tenebrio molitor) and adult cockroaches (Nauphoeta cinerea). Most of the experiments were carried out with mealworm larvae according to the procedure below. All larval stages were used except the last stage (prepupal) when larvae become immobile before the molt into the pupal form.

Isolation of junction-rich membrane fractions

A total of 60 larvae at a time were cold-narcotized by placing them in a refrigerator at 4°C for 20 min. The head and the last three abdominal segments were cut off and the intestine was pulled out of the body with fine forceps; this was then rolled from one end with a test tube while pulling out the peritrophic membrane and gut contents from the other. Any Malpighian tubules or hindgut material still attached at this stage were removed and the resulting midgut tubes (0.3–0.35 g) were placed into 30 ml insect saline buffered solution (SBS) made up in 1 mM-NaHCO₃ (final pH 7.7). This solution was at 4°C and all subsequent steps were carried out at 0–4°C.

After dissection of the last midgut they were all blotted dry, chopped into small pieces with a razor blade and placed into 40 ml 1 mM-NaHCO₃, 1 mM-CaCl₂ (pH 7.8) for 20 min with occasional agitation. The intestines were then homogenized in this solution with 10 strokes of a loose-fitting pestle in a Potter homogenizer (Corning glassware). The homogenized material was allowed to stand for a further 10 min with occasional agitation before filtering through two layers of coarse paper filter to remove gut muscle layers and basal laminas. Another 40 ml of 1 mM-NaHCO₃, 1 mM-CaCl₂ was
Isolated smooth septate junctions

washed through the filter to give a final 80 ml of homogenate solution. The homogenate was spun for 15 min, 8500 rev./min (8000 g) in a Beckman JA21 rotor. The resulting pellets were resuspended in 5-4 ml 1 mM-NaHCO₃ (pH 7-8) and 15 ml 81% sucrose was slowly added to give a final 60% sucrose solution. All sucrose concentrations are given as % (w/v) and solutions were made up in 1 mM-NaHCO₃. The sample was layered on six separating sucrose step gradients of 60% (+sample)/54% (broad band)/32% and spun for 90 min 25,000 rev./min (106,500 g max) in a Beckman SW41 Ti rotor, resulting in a membrane-rich fraction at the 32/54 interface. This fraction was diluted in 33 ml 1 M-NaHCO₃ and spun for 20 min at 25,000 rev./min in the same rotor, resuspended by pipette action and respun in the same manner.

Pellets from three lots of the first gradient (180 larvae) were then used for further purification. They were resuspended by pipette action in 7-5 ml 1 mM-NaHCO₃ and 15 ml 81% sucrose was added slowly to give a final concn of 54%. This was layered on six sucrose step gradients of 54% (+sample)/50% (broad band)/42% /36% /32% and spun for 90 min at 25,000 rev./min in the SW41 Ti rotor. The 32/36, 36/42 and 42/50 interfaces were collected and washed by the same method as used after the first and second gradients were used for electron microscopy (EM), polyacrylamide gel electrophoresis (PAGE) and urea-treatment studies.

Mechanical and chemical treatments

Isolations were also made from material dissected into ordinary insect SBS (no NaHCO₃, pH 5-5) or by using bicarbonate solution adjusted to pH 9 with 0-1 M-NaOH.

Samples from the 32/54 fraction (first gradient) and from the 36/42 fraction (second gradient) were treated with 1 M, 2 M or 6 M-urea (1 h or 2 × 1 h) at 4°C, dialysed against 1 mM-NaHCO₃, and repelleted. These pellets were then studied by EM and PAGE.

The 42/50 fraction (second gradient) was rehomogenized for 3 s with a Polytron homogenizer, speed 2, and relayered on a gradient by the same method as used for the second gradient. The resulting 36/42 and 32/36 interfaces were then studied by EM and PAGE.

Electrophoresis

Polyacrylamide slab gel electrophoresis was carried out basically according to the methods of Fairbanks, Steck & Wallach (1971) and Laemmli (1970), using 4% stacking and 12.5% running gels. Membrane pellets were dissolved in sodium dodecyl sulphate (SDS)/mercaptoethanol sample buffer at room temperature for 30 min before running the gels. Protein colouration was by double staining in Coomassie Blue and glycoprotein staining was by the periodic acid/Schiff technique (Fairbanks et al. 1971). Five molecular weight (Mr) markers were used (94,000, 68,000, 42,000, 21,500 and 12,500). Gels were scanned with a Gelman DCD16 densitometer allowing density comparisons between bands in the same gel column.

Electron microscopy

Midgut samples or pellets were fixed in 3% glutaraldehyde in 0.2 M-cacodylate buffer. Samples for thin sectioning were postfixed for 1 h in 1% OsO₄ and stained en bloc for 30 min in 2% uranyl acetate after changing to Michaelis buffer (Bloemendal et al. 1972). They were embedded in Epon/Araldite after dehydration in acetone and sections were double stained according to the method of Reynolds (1963). Fixed samples for freeze-fracture were placed in 30% glycerol in buffer before fracturing in a Balzers apparatus. Negative staining of unfixed pellet material (resuspended in distilled water) was with 1% uranyl acetate and 0/1% ammonium acetate. All micrographs were taken on an Hitachi HU 11 electron microscope.

Pleated septate junctions

Attempts were made to isolate the mollusc/arthropod pleated septate junction from the hindguts of the same two insect species and from gills of the marine mussel Mytilus edulis. Attempts were similarly made to obtain the lower invertebrate pleated septate junction from the intestine of the earthworm Lumbricus terrestris.
RESULTS

The midgut cells of mealworms and cockroaches are joined by extensive smooth septate junctions (SSJs) (Fig. 1), which extend from the very top of the cells lining the midgut to about their midpoint. The membranes of the contributing cells are separated by a gap of about 15 nm, which contains a relatively densely staining intercellular material. In only some places are the septa themselves readily visible in cross-section views. Gap junctions are seen dispersed within the septate junction area, often in a curved region of the junctional membranes (Fig. 1). Freeze-fracture replicas of fixed material show the normal SSJ patterns (see reviews by Noirot-Timothee & Noirot, 1980; Lane & Skaer, 1980) with particles (often fused into short rods) and grooves usually seen on both faces (Fig. 2). In some places, however, there is an almost solid line of particles on one face (either E or P) and grooves on the other as has been described previously for various cases (Noirot-Timothee & Noirot, 1980). Particle rows are unevenly spaced and curved except near the top of cells where they are more closely disposed and parallel to one another.

Isolated junctions

Thin sections. The 32/54 interface of the first separating gradient gave large numbers of clean isolated SSJs along with other membrane types, which were frequently seen as small vesicles, and a small quantity of rough endoplasmic reticulum. Some amorphous material also remained, but the microvilli, mitochondria, nuclear material and the bulk of the rough endoplasmic reticulum were no longer present after the initial gradient. The second gradient gave a richer fraction of SSJs in the 36/42 layer (Figs 3, 5) although junctions were located in all three layers collected, depending on the size of the pieces. Generally, junction pieces in the upper 32/36 layer were very small while those in the lower 42/50 layer were larger but mixed with more impurities than those of the middle layer. Gap junctions were observed in all layers but in greater numbers in the 32/36 layer (Fig. 4). They never formed a substantial proportion of material present.

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Fig. 1. A thin section showing smooth septate and gap junctions between intact midgut cells of a mealworm larva. The gap junctions (arrows) are dispersed within the septate junction area, often in a curved region of the junctional membrane. The adjacent plasma membranes of the septate junctions are about 15 nm apart. \( \times 96 000 \).

Fig. 2. A freeze-fracture replica showing a typical smooth septate junction in intact mealworm larva midgut. Rows of particles and grooves (arrows) are visible on both membrane faces. \( \times 40 500 \).

Fig. 3. A thin section of smooth septate junctions isolated from midguts of mealworm larvae. Septa are seen in cross-section view spanning a 15 nm intermembrane gap and in tangential view. The micrograph is of material collected at the 36%/42% sucrose interface. \( \times 100 000 \).

Fig. 4. A thin-section micrograph showing gap junctions (arrows) isolated from mealworm larva midguts and collected from the 32%/36% sucrose interface. Gap junctions were found in all layers collected but in greater numbers in this lighter fraction. \( \times 88 000 \).
Isolated smooth septate junctions
Figs 5–7
Despite trying a variety of different sucrose gradients we were unable to obtain completely pure junction fractions and in all layers varying amounts of impurities remained, normally in the form of small vacuoles, membrane material and some amorphous material. Nonetheless, in our opinion the predominant repeating structures were the SSJs, especially in the 36/42 fraction (Fig. 5). Some care was necessary in interpreting fractions, as the ability to see junctions easily depended somewhat on the angle of the sections in relation to the layering of the membranes formed during pelleting. In most cases, however, septa were easily seen in cross-section (Fig. 3) or tangential views (Figs 3, 6, 7) due to the lack of background staining from other cellular material. Pegs between the septa, characteristic of the SSJ, were also seen in some tangential views.

The septa were easier to locate in cross-sections of isolated junctions than in intact tissue and the intermembrane space was generally unstained. Where septa were visible the membranes retained their 15 nm spacing (Fig. 3). However, in some places the membranes ran closely together with a very dense-staining material between them, as if the two membranes had slid against one another and the septa had collapsed. When this occurred a structure superficially similar to a gap junction was formed, but close examination revealed that the intermembrane spacing was slightly wider and more electron-dense than for gap junctions. In addition, these 'collapsed septate junction' structures could be of considerable length, exceeding the length of a typical insect gap junction several times over.

Attempts to wash SSJ-rich fractions further even with 1 M-urea caused disruption of the junctions. Treatment with 2 M-urea left few septa intact, and with 6 M-urea resulted in complete loss of septate junctions, though again gap junctions were still intact.

Negative staining. Negative staining of junction fractions resulted in double pieces of membrane, with the intermembrane septal structure quite clearly seen. Low-magnification views often showed septa in similar patterns (Fig. 8) to those seen in freeze-fracture replicas (cf. Fig. 2) or after lanthanum impregnation of intact tissue (Flower & Filshie, 1975; Graf, 1978; Lane & Skaer, 1980; Noirot-Timothée & Noirot, 1980). They were found either in a loose arrangement (Fig. 8) paralleling that typical of the lower regions of the junction or in a closer arrangement (Figs 9, 10) as in the apical part of the junction in the intact midgut. At higher magnifications the intersepal pegs were readily visible (Fig. 9). Many negatively stained junctions had very large numbers of septa compressed together (as, e.g., in Fig. 11), a situation not

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**Fig. 5.** A low-magnification thin-section scanning view of material collected at the 36 %/42 % sucrose interface. The fraction is rich in smooth septate junctions, which form the predominant repeating structure present. At higher magnifications septa would be seen in cross-section and tangential view (cf. Fig. 3). ×29000.

**Figs 6, 7.** Thin sections of material collected at the 36 %/42 % sucrose interface following isolation of smooth septate junctions from mealworm larval midguts. The septa are clearly seen in tangential view, more readily than in intact tissue. This may be due to the lack of background-staining cellular material in these isolated-junction preparations. Fig. 6, ×100000; Fig. 7, ×80000.
Figs 8–9
Isolated smooth septate junctions

Fig. 8. A low-magnification view of negatively stained membrane material isolated from mealworm midgut. In this piece of smooth septate junction, collected at the 36%~/42% sucrose interface, septa are seen outlined by the stain. They run between the membranes in a pattern not unlike that seen by freeze-fracture replication of intact tissue (cf. Fig. 2). In this view septa are in the looser arrangement, more typical of the lower regions of the junction in intact midgut. ×75 000.

Fig. 9. Isolated smooth septate junction negatively stained to reveal the septa in negative contrast. Pegs, characteristic of the smooth septate junction, are seen in rows between the septa (arrows). Septa are in the closer arrangement common to the upper regions of the junction in intact tissue. ×190 000.

Fig. 10. Negatively stained smooth septate junction isolated from mealworm midgut. The septa, 4.5–6 nm wide, appear cross-striated with a 3.5 nm centre-to-centre spacing of the component subunits. In places cross-striations of adjacent septa appear to be in line with one another (arrow). A tricellular junction is seen to the centre right (double arrow). ×122 000. Inset: a higher magnification view of isolated smooth septate junction from mealworm midgut, negatively stained. The septa are seen to be made up of globular subunits that in places are paired (arrowed septum). In most areas, however, no readily discernible pattern is visible. The exact angle at which septa are being viewed may be critical. ×350 000.

Fig. 11. Isolated smooth septate junction from mealworm midgut, negatively stained. The septa in this view are arranged very tightly together as if they have slid within the planes of the membranes to form the closely packed agglomerations seen. Intersetal pegs may still be present (small arrows), but they are difficult to distinguish from the septa themselves. These tightly packed arrangements often consist of short lengths of septa, as in the region indicated by a large arrow. ×243 000.

Fig. 12. A negatively stained preparation of isolated smooth septate junction from mealworm midgut, which has been treated for 1 h with 1 M-urea. Short remnants of septa are seen with their component subunits aggregated into clumps at the ends (arrows). The septa therefore appear to have been destroyed from their ends (cf. Fig. 13). ×140 000.

Fig. 13. Isolated smooth septate junction from mealworm midgut, negatively stained after treatment with 1 M-urea. In this view the septa appear to have been destroyed from their intermembrane centres leaving only small portions visible on the surfaces of the contributing cell membranes (arrow). ×98 000.

Fig. 14. A mealworm midgut gap junction collected at the 32%/36% sucrose interface and negatively stained. The connexons, 6–7.5 nm in diameter, are seen arranged in a regular pattern. Where they appear most clear they are seen to consist of six subunits (circled on Fig. 14, inset). Fig. 14, ×143 000; inset, ×333 000.

Fig. 15. A freeze-fracture replica of smooth septate junction from mealworm midgut collected at the 36%~/42% sucrose interface. The knife fracture method used generally revealed smaller membrane faces than obtained when fracturing whole tissue, due to folding of the membranes during pelleting. The junctional structures are, however, readily apparent with rows of particles and grooves (arrows) visible. ×53 000. Inset: a replica of a smooth septate junction-rich fraction isolated from mealworm midgut and treated with 6 M-urea. Rows of particles (running in the direction of the large arrow) and grooves (small arrow) are seen but it is not evident that these are remnants of septate junction. ×80 000.

Fig. 16. Smooth septate junction material isolated from mealworm midgut. This material was collected at the 42%~/50% sucrose interface, rehomogenized using a Polytron and relayered on a sucrose gradient. The final 36%~/42% fraction has been thin-sectioned to reveal remnants of the junctions. The bulk of the junctions have been destroyed but pieces of septa are visible at higher magnifications. Gap junctions (arrow) remain intact following this treatment. ×58 000.

Fig. 17. Smooth septate junctions isolated from the midguts of mealworm larvae using a medium at pH 9 (adjusted with NaOH). Portions of junction are seen in cross-section and tangential view (arrows) but the bulk of the material has become blistered and the junctions destroyed. ×58 000.
Figs 10–13. For legend see p. 359.
Figs 14-17. For legend see p. 359.
C. R. Green, C. Noirot-Timothée and C. Noirot

commonly observed in whole tissue. In these cases the septa did not appear structurally altered in comparison with preparations showing a wider spacing, but the strands of the junction have apparently moved together to form the closely packed agglomerations seen. The interseptal pegs seemed to be still present (Fig. 11), but it was difficult to distinguish them from the septa themselves. Owing to the packing and some breakage of the membranes into smaller pieces, septa were often seen as a series of short lengths (Fig. 11) rather than the longer meandering structures seen on larger, isolated membrane pieces (Fig. 8) or, again, in freeze-fracture replicas of intact tissue (Fig. 2).

The septa appeared cross-striated (Fig. 10), 4.5–6 nm wide with approximately 3.5 nm centre-to-centre spacing of the striations. In places the striations from adjacent septa appeared to be aligned with one another (Fig. 10). Closer examination revealed a complicated construction in which repeating units were not readily discernible. Frequently, septa appeared to be made up of globular subunits with each septum appearing as a double row of dots. The component subunits were either immediately adjacent to one another, forming pairs (Fig. 10, inset) or stepped alternately to form a zig-zag arrangement. In other places the subunits did not form readily discernible patterns and the angle at which each septum was being viewed may have been a critical factor. Sometimes the subunits seemed to have sidearms or to form chains of small circles, but these were usually seen when septa were compressed tightly together and interpretation was difficult. Tricellular junctions were also seen outlined by the negative stain (Fig. 10).

Negative staining of membrane pieces after treatment with urea gave pictures similar to those of sections, as regards the disappearance of septa. Occasionally remnants of septa were still visible after treatment with 1 M-urea, suggesting that they were being broken up in a series of steps. In some views the septa appeared to have been destroyed from their ends, leaving the occasional short length of septum outlined by the stain. These lengths appeared relatively intact, except near their ends where the globular elements of the septum had formed into clumps (Fig. 12). In the majority of examples found, septa appeared only as faint lines in the negative-stain preparations, as though they had been destroyed from their intermembrane centres, leaving only a small portion visible on the surface of the contributing cell membranes (Fig. 13). However, the bulk of septa had been removed and none was found in negative-stain preparations of junction-rich fractions after treatment with 2 M-urea.

Gap junctions appeared similar to those reported for lanthanum-impregnated tissues (Peracchia, 1973), with the connexons regularly arranged, but only 6–7.5 nm in diameter (Fig. 14). The fine resolution of the negative-stain technique revealed the subunit structure of each connexon, six of these subunits being apparent where they appeared clearest (Fig. 14, inset).

Freeze fracture. Freeze-fracture (by knife microtome) of our pelleted samples generally resulted in only small membrane faces being revealed, due to the folding of unsupported membranes during pelleting. Because of the small size of cleavage faces, characteristic views such as reported in intact tissues (Fig. 2) could not be expected. Nonetheless, the freeze-fracture appearance of isolated junctions (Fig. 15)
Isolated smooth septate junctions

was essentially the same, although particles tended more often to be individual rather than fused into short rods, as is often characteristic of the SSJ (see reviews by Lane & Skaer, 1980; Noirot-Timothée & Noirot, 1980). Their size and structure were still within the range of normal variation for this junction type and grooves were seen in some areas of the junction (Fig. 15).

After treatment with urea the fractured membranes frequently appeared smooth, either without or with only a few dispersed particles. In some places clumps of particles were seen, occasionally as an assembly of parallel rows and grooves (Fig. 15, inset). However, there was some difficulty in interpreting these replicas (see Discussion) and it was not readily evident that the particles observed were of junctional origin.

Isolation procedure variations. If the homogenized tissue sample was washed several times in 1 mM-NaHCO₃ before layering directly onto a sucrose gradient (i.e., using a single-gradient isolation procedure), the final separation was generally not as good as that obtained using two gradients. We found that the homogenized material became more difficult to resuspend after each washing step and the final membrane fractions were not very pure. Isolations attempted after dissecting midguts into SBS containing no NaHCO₃ (pH 5.5) also resulted in bad clumping, even though the subsequent homogenization and washing solutions were the same as those used in the normal isolation procedure. The clumping caused by the low pH dissection medium was often not apparent until several steps later.

Further purification of SSJ fractions was attempted by rehomogenizing material isolated at the 42/50 interface of the second gradient with a Polytron. The resulting homogenate was then reseparated on another similar gradient. This treatment caused the bulk of SSJs to break up, although remnants of septa were visible in a few places and gap junctions remained intact (Fig. 16). Similarly, the use of a higher pH (pH 9, obtained by adding NaOH to the buffer solution during washing steps) resulted in damaged junctions. In most cases the junctional membranes showed a blistered appearance after the second gradient (Fig. 17) and the septa were clearly being disrupted by this pH or by the NaOH that had been added.

Finally, attempts to isolate pleated septate junctions using the methods described yielded clean membrane fractions, but intact septate junctions were never located. Pleated septate junctions (from molluscs, arthropods and earthworms) appeared to remain intact when whole tissue was soaked in the various isolation media used, but were destroyed when the tissue was homogenized, no matter how gentle the homogenization procedure.

Electrophoresis. Fig. 18 shows examples of the results of the SDS/polyacrylamide gel electrophoresis of samples obtained with our isolation procedure. In preparing samples all material appeared to become dissolved in the sample buffer within 5 min. However, gels were run 30–45 min after placing samples into buffer to ensure that all material was well dissolved. All sample material entered the gels and no residue was left in the sample wells or trapped in the stacking gel.

PAGE results demonstrated the reproducibility of the junction isolation method used. Fractions containing large numbers of septate junctions (but also other membranes, impurities and some gap junctions) always gave a similar series of bands on
Fig. 18. A composite plate showing examples of results of polyacrylamide gel electrophoresis. The various fractions isolated from mealworm midgut were solubilized in SDS/mercaptoethanol and the gels were stained with Coomassie Blue. A, 42%/50% sucrose interface; B, 36%/42% sucrose interface (richest septate junction fraction); C, 32%/36% sucrose interface (richest gap junction fraction); D, septate junction-rich fraction following treatment with 1 M-urea; E, septate junction-rich fraction following treatment with 6 M-urea. In all fractions except C a doublet of 31 000/32 000 $M_r$ was predominant with other main bands at 16 000, 28 000, 30 000 and 36 000 always in evidence. A PAS-positive band of 47 000 $M_r$ copurified with the septate junctions (A, B) but was absent from urea-treated samples (D, E) or in samples that had been rehomogenized (not shown). In sample C the 36 000 $M_r$ band appears densest and the 31 000/32 000 $M_r$ doublet is reduced. In sample A, there is additional material present, especially in the higher $M_r$ region, this gel appearing very similar to 32/54 combined fraction gels (not shown). Urea-treated samples (D, E) show reduced density for all bands except the six main bands mentioned above. After treatment with 6 M-urea (E) the 31 000/32 000 $M_r$ doublet is especially pronounced. Molecular weights ($\times 10^{-3}$) are shown on the left.
Isolated smooth septate junctions

with 6 M-urea the same six bands remained but with the 31 000 and 32 000 M, bands especially marked.

In addition to the six bands mentioned above, a doublet of molecular weight approximately 47 000 copurified with the junctions, but was absent from samples that had been treated with urea and somewhat reduced after rehomogenization experiments. It is of note that both the urea treatments and rehomogenization of samples also destroyed the septa of the junctions. This 47 000 M, doublet gave a PAS-positive reaction, suggesting it was a glycoprotein.

DISCUSSION

Successful isolation and purification of intercellular junctions has been achieved for gap junctions of mammalian eye lens fibre (Bloemendaal et al. 1972; Dunia et al. 1974; Goodenough, 1978; Hertzberg et al. 1982; Kibbelaar & Bloemendaal, 1979; Kistler & Bullivant, 1980a, b), liver (Benedetti & Emmelot, 1968; Culvenor & Evans, 1977; Ehrhart & Chauveau, 1977; Evans & Gurd, 1972; Goodenough & Stoeckenius, 1972; Henderson et al. 1979; Hertzberg et al. 1982) and heart (Kensler & Goodenough, 1980; Manjunath et al. 1982), and for desmosomes of mammalian epidermis (Drochmans et al. 1978; Gorbsky & Steinberg, 1981; Skerrow & Matoltsy, 1974) and heart (Colaco & Evans, 1981). A recent abstract by Stevenson, Goodenough & Mooseker (1982) indicates that some progress has also been made towards the isolation of vertebrate tight junctions, but no isolation procedure has yet been published for either this junction type or any of the invertebrate septate junctions. This may not be fortuitous. Tight and septate junctions have a zonular shape, forming a band of spaced strands around each cell, rather than the compact and solid plaque-like structures of the gap junctions and desmosomes. This means that the junction itself is less cohesive and that it is necessary to isolate relatively large pieces of intact plasma membrane in order to retain recognizable portions of the junctions. At the same time, for invertebrate junction studies, it is difficult to find large organs able to provide as big a quantity of starting junctional material as the mammalian liver, for instance.

We have managed to produce membrane fractions rich in invertebrate smooth septate junctions in good quantities considering the weight of starting material used. Our procedure was basically that of Benedetti and coworkers (see references above) for gap junctions, although with some modifications as discussed below.

Invertebrate septate and gap junctions seem easier to obtain in a clean form than vertebrate gap junctions. After the first gradient separation and subsequent washing of the 32/54 fraction the junctions appeared quite clean and numerous. The second gradient was used as an enriching process. In fact, the major problems encountered in the isolation of the SSJ's were due to their fragility. The use of a high pH is normally considered as an aid to homogenization, in producing clean membrane fractions and in reducing clumping of material (DePierre & Karnovsky, 1973). The SSJ's were, however, disrupted by this treatment, although they appeared to be unaffected structurally at lower pH values. The negative-stain technique, for example, necessitated the use of acidic pH solutions with no apparent ill effect. The main problem with
using low pH values during the isolation procedure was a subsequent clumping of material, resulting in poor separation of the junctional membranes from the other cellular material. The septa were also disrupted by extraction with urea at even 1 M concentration, in contrast to the vertebrate gap junctions and desmosomes, which remain essentially intact following treatment with 6 M or even 7 M-urea (Culvenor & Evans, 1977; Drochmans et al. 1978; Finbow, Yancey, Johnson & Revel, 1980; Henderson et al. 1979; Hertzig et al. 1982; Kibbelaar & Bloemendal, 1979; Kistler & Bullivant, 1980b). Furthermore, the junctions were sensitive to rough mechanical treatments, and homogenization attempts with a Polytron homogenizer caused disruption of the junctions even though relatively large pieces of plasma membrane remained intact. This is again in contrast to isolation techniques for the plaque-like gap junctions and desmosomes, in which Polytron type homogenization is used regularly (Bloemendal et al. 1972; Colaco & Evans, 1981; Drochmans et al. 1978; Kensler & Goodenough, 1980; Skerrow & Matoltsy, 1974). This fragility of the junction to both chemical and mechanical treatments severely limited further enrichment of the junctional fractions and varying amounts of non-junctional material remained in our fractions. The junctions often occurred in large quantities within certain regions of the final pellets obtained, but alterations to the separation gradients in attempts to isolate these specific regions proved unsuccessful.

Most of the junctions obtained by our isolation procedure were well preserved as judged by ultrastructural criteria. Larger pieces of membrane studied by negative staining show the septa following a pattern similar to that of particle rows on freeze-fracture replicas of intact tissue. The septa and pegs characteristic of the SSJ appear to remain intact, although it is not possible to know if any part of these structures has been lost during the isolation procedure. The substructure of the septa seen in negative-stain preparations is not unlike that reported by Skaer, Harrison & Lee (1979) and Graf (1978) for the SSJ, Lane & Harrison (1978) for the Limulus septate junction, and Green (1981) for pycnogonid septate junctions following lanthanum impregnation of these junctions. While the negative-stain technique generally gives a better structural resolution for electron microscopic studies, the exact make-up of the septa remains doubtful. They clearly consist of globular subunits, which can form a regularly repeating substructure apparent at lower magnifications. Closer examination showed that the septa consisted predominantly of a double row of subunits, which occurred in some places in pairs and in others to be displaced in relation to one another. In other regions the subunits appeared to delineate small circles or to have an even finer, complex substructure and the interpretation of the images seen was difficult. A tilting-stage microscope might have proved helpful in interpreting these results. It is of note, however, that many of the isolated pieces of membrane viewed after negative staining had large arrays of tightly packed straight septa, a pattern not typical of intact tissue. The strands of the junction had apparently slid along and within the membrane planes to form the tightly packed arrays seen. This is not an unreasonable possibility considering the well-known fluid-mosaic properties of biological membranes. Despite this probable movement and rearrangement of the junction strands in the membranes, the septa themselves appeared unaltered in
comparison with more typical junction pieces, although often shorter in length.

When studied by standard thin-section techniques, the SSJ in situ has dense-staining granular material within the junction and it is often difficult to see septa clearly in cross-section views. In contrast, the intermembrane space of the isolated junctions was clear and the septa were readily visible. Septa were similarly easily located in tangential view and were well delineated, although this may have been due either to a loss of interseptal material, increasing the apparent contrast between the septa and their background, or simply to the fact that there is no background cellular material in these preparations.

Freeze-fracture replicas of pelleted material revealed only small membrane planes, owing to folding of the membranes during pelleting. We used a slicing-knife fracture method and it is possible that free-break fractures would give a better indication of the junctional structures remaining by revealing larger membrane surfaces. After treatment with urea it was difficult to interpret replicas. Once the septa are removed there is no reason to assume junctional particles will remain aligned and they may disperse or become clumped. A clumped pattern was already visible in places on untreated fraction replicas due to the septa moving together within the membrane, so that the intramembrane particle rows became closely aligned. Also, with the removal of septa by urea or mechanical treatment there is of course nothing to hold the two junctional membranes together and they can become widely separated in the final pellets. There is thus no change of face possible during fracturing that would give an indication as to whether groups of particles in a membrane have corresponding counterparts in another. Owing to these factors it is not possible to say definitely whether the particles seen after freeze-fracture of urea-treated samples were junctional or not.

The gap junctions in our preparations appeared intact after all mechanical and chemical treatments. Our interest has been in the septate junction, however, and we have made no further attempts to concentrate the gap junctions obtained. It is of note that the connexons as seen in negative stain have a slightly larger diameter (6–7.5 nm average) than those reported for vertebrate tissues (5–6 nm) (Lane & Skaer, 1980). They are made up of six subunits, as postulated for vertebrate gap junctions and has been previously noted in arthropod tissues on the basis of freeze-fracture and negative-stain studies (Peracchia, 1973). The larger diameter of the connexons corresponds well with their freeze-fracture appearance, in which the particles seen are also larger than those reported for vertebrate tissues (Flower, 1977). Furthermore, the arthropod gap junction is supposedly permeable to molecules up to 3 nm in diameter as opposed to 2 nm for mammalian gap junctions (Schwarzmann et al. 1981). Owing to the nature of the fractions obtained, electrophoretic results need to be treated with some caution. Nonetheless, they do demonstrate the reproducibility of the isolation technique used, since each fraction obtained always had the same principal bands present following PAGE. We never obtained a band that remained after urea treatment that had a molecular weight of either 34 000 or 26 000. It is therefore apparent that either we had insufficient gap junctions in our fractions to give a visible PAGE band of these weights or the arthropod gap junction does not contain these...
more commonly reported protein components of vertebrate gap junctions (Colaco & Evans, 1981; Culver & Evans, 1977; Ehrhart & Chauveau, 1977; Finbow et al. 1980; Goodenough, 1974; Henderson et al. 1979; Hertzberg & Gilula, 1979; Hertzberg et al. 1982; Kensler & Goodenough, 1980; Kibelaar & Bloemendal, 1979; Kistler & Bullivant, 1980a). The latter is quite probable as it is becoming apparent that even gap junctions from different mammalian tissue types have quite different protein components (see, e.g., Hertzberg et al. 1982). The larger physical structure of the arthropod connexons also suggests they might have quite different protein units; in fact, the 36 000\( M_r \) band that appears denser in samples from the lighter fraction obtained (where there was the greatest concentration of gap junctions) is a possible candidate for the major arthropod connexon protein.

The 47 000\( M_r \) glycoprotein doublet that showed concomitant appearance and disappearance with the septa is possibly part of the smooth septate junction. Dallai (1970) reported a deep colouration of the intramembrane material of SSJs when glycol methacrylate sections were treated with chromic acid/phosphotungstic acid according to the method of Rambourg (1971). After treatment with pepsin or Pronase this colouration did not occur, implying the presence of glycoprotein components. However, his periodic acid/thiosemicarbazide (TCH) tests proved negative. Unpublished results (Noirot-Timothée & Noirot), using the latter test, indicate that a positive reaction is obtained after prolonged treatment (48–72 h) with TCH, although thin sections failed to reveal well-defined septa (or pegs); this is in contrast to the pleated septate junction whose septa were delineated even after treatment with TCH for 1 h. These results do, however, suggest that there may be a glycoprotein component of the SSJ and in our experiments the 47 000\( M_r \) doublet was the only band to co-purify with the junctions that gave a positive PAS reaction. We are uncertain about the extremely strong bands of 31 000 and 32 000\( M_r \), that remained following treatment with urea as we have found no obvious repeating structures on freeze-fracture replicas or in thin sections that could correspond to these bands. These bands were very prominent both before and after treatment with urea, which implies that they are major intramembrane components of insect midgut membrane. The SSJ will no doubt prove to be a relatively complicated structure biochemically, since it is formed of a consortium of parts: the intramembrane fraction, the septa and the pegs. Each of these parts may have several protein constituents.

Finally, it is of note that we have been unable to isolate intact pleated septate junctions using the same techniques as those described here. This indicates a large difference between these types of junction, which supposedly have the same function (Noirot-Timothée & Noirot, 1980; Green & Bergquist, 1982). It is not obvious whether the difference between the junctions is due to the chemical sensitivity or a structural weakness of the pleated septate junction. The junctions in situ stay basically intact when tissue is placed in the various solutions used during our isolation procedure. It is only when the tissue is homogenized (no matter how gently) and the membranes are centrifuged that the pleated septate junctions appear to be destroyed. We have not yet attempted freeze-fracturing of pleated septate junction membrane fractions to see if the junctional particles remain evident within the membranes when the septa have been lost.
Isolated smooth septate junctions

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