ISOLATION OF EPIDERMAL DESMOSOMES

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ABSTRACT

A method is reported for the isolation of desmosomes in a high yield and of a purity suitable for biochemical analysis. The procedure utilizes the selective solubilizing action of citric acid-sodium citrate (CASC) buffer, pH 2.6, on the noncornified layers of cow nose epidermis, followed by discontinuous sucrose density gradient centrifugation. Electron microscopy with both thin sections of pellets and unfixed spread preparations reveals that after centrifugation, desmosomes are located mainly at the 55–60% sucrose interface. In the desmosome preparation thus obtained, the characteristic desmosome structure is well preserved, showing the midline, unit membranes, and dense plaques. Furthermore, removal of the epidermal filament bundles by the solubilizing action of CASC buffer has revealed a finely filamentous layer on the cytoplasmic surface of the plaques. The dimensions, location, and appearance of this layer correspond with those of the "connecting component" which has been previously suggested as being responsible for the attachment of epidermal filament bundles to the desmosome.

The desmosome, or macula adherens, has long been recognized as a region of enhanced adhesion between the cell membranes of epithelial cells (2). The characteristic morphology of the desmosome has been well defined by studies on many different types of epithelia (5, 6, 9). The unit membranes of the adjacent cells run parallel in the desmosome. The intercellular space is occupied by material of moderate electron density, which is bisected by a denser central lamina. Presumably, interactions within this material are responsible for the cell-to-cell adhesion at the desmosomal junction.

On the cytoplasmic side of the plasma membrane there is a densely staining plaque. The cytoplasm adjacent to the plaque is characterized by a concentration of filaments which converge on the plaque. In the epidermis, these filaments are seen to be continuous with filament bundles (tonofibrils) stretching a considerable distance into the cytoplasm. It is generally believed that the attachment of such extensible filament bundles to the desmosomal junction provides a mechanism for the transmission of mechanical stress throughout the epidermal tissue, allowing it to respond as a functional whole (9, 14).

In newt epidermis, most of the filaments loop back approximately 400–700 Å away from the plaque (9). This broad zone is occupied by a relatively lightly staining material which may be filamentous in nature. Kelly (9) has suggested that this material, termed "connecting component," serves to anchor the epidermal filaments to the plaque. A comparable but narrower zone between the epidermal filaments and the plaque is seen in desmosomes from other species and tissue types (3, 6, 8).

The elucidation of the molecular interactions responsible for cell-to-cell adhesion and for filament attachment at the desmosome requires the isolation of desmosomes in sufficient quantities and in purity suitable for biochemical analysis. Desmosomes are occasionally seen in preparations

of liver cell plasma membranes obtained by classical techniques (1). However, such methods are not appropriate for use with the desmosome-rich epidermis, as this keratinizing tissue is highly resistant to homogenization at near neutral pH values.

A reagent which is used to extract the major protein component of epidermal filaments, and which rapidly disperses the living epidermal tissue, is citric acid-sodium citrate (CASC) buffer, pH 2.6 (10). Light-microscope observations on the residue from a CASC extraction of cow nose epidermis revealed large numbers of nuclei and membranous fragments. In the electron microscope it was found that these fragments consisted mainly of desmosomes. This observation formed the basis of the preparation method described in this paper.

MATERIALS AND METHODS

Experimental Tissue

Cow nose epidermis was selected as the starting material, as this tissue can be readily obtained in large quantities and is of unusual thickness (about 0.5 mm). Approximately two-thirds of the epidermis consists of living cells, which are joined by numerous desmosomes.

Fresh cow noses were obtained from a local abattoir and washed thoroughly in running water. With an electrokeratotome (Storz Instrument Co., St. Louis, Mo.), a layer 0.2 mm in thickness was removed from the epidermal surface of each nose. This layer contained much stratum corneum and was discarded. A second 0.2 mm layer, consisting of living epidermal cells, was then removed and used in the following preparative procedure.

Homogenization of Tissue

The homogenization medium was freshly prepared 0.1 M citric acid-sodium citrate buffer pH 2.6 (CASC). Epidermal sheets from 10 cow noses were placed in a beaker containing 2 ml of CASC buffer and minced finely with scissors. Subsequently, 100 ml CASC buffer were added and the tissue fragments stirred with a magnetic bar for 30 min at room temperature. During this time the tissue underwent considerable swelling; omission of this step led to incomplete homogenization and low yield of desmosomes. The swollen tissue fragments were homogenized for 4 min at setting 60, with the VirTis "23" homogenizer (VirTis Co., Inc., Gardiner, N.Y.), and the homogenate was filtered through eight layers of gauze to remove the remaining large fragments. The homogenate was then centrifuged at 10,000 *g* for 20 min at 4°C, with rotor no. 296 of the IEC model PR-2 centrifuge, and the supernate was discarded. The pellet was dispersed in 80 ml of CASC buffer by one stroke of the loose pestle of a Dounce-type homogenizer. Centrifugation and resuspension of the pellet was repeated four times, and the final pellet was retained for density gradient centrifugation.

Density Gradient Centrifugation

Discontinuous gradients were prepared in four 12-ml polyallomer tubes (Beckman Instruments Inc., Fullerton, Calif., catalog no. 331374). Solutions of sucrose (density gradient grade, Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N.Y.) in CASC buffer were layered with a Pasteur pipette as follows: 60% (4 ml), 55% (3 ml), 50% (2 ml), 40% (2 ml). The gradients stood for 10 min at room temperature before use. The final pellet was suspended in 2 ml of CASC buffer with 10 strokes of the loose pestle of the Dounce-type homogenizer and 0.5 ml was layered onto each gradient. The tubes were centrifuged at 180,000 *g* for 3 h, in an SW 40 rotor, with a Beckman L 2-65 B preparative ultracentrifuge.

After centrifugation, well-defined bands (Fig. 1) were observed as follows: white bands (I, II) at the 30–40% and 40–50% sucrose interfaces, a pale brown band (III) at the 55–60% sucrose interface, and a large dark brown pellet (IV) at the bottom of the tube. No material was visible between these bands.

Each band was removed with a Pasteur pipette and the suspensions were diluted with CASC buffer to a final concentration of approximately 0.2 M with respect to

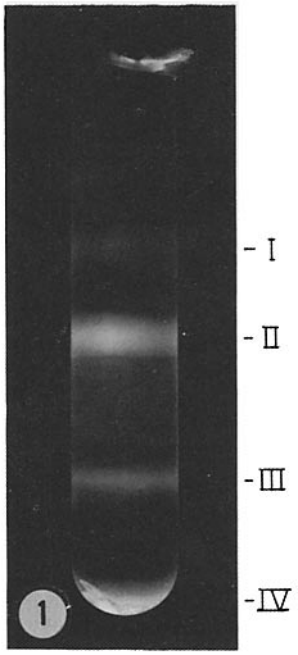


FIGURE 1 Photographs of bands (I–IV) obtained by discontinuous sucrose density gradient centrifugation of a homogenate of cow nose epidermis in CASC buffer pH 2.6. Band III corresponds to the desmosome fraction.

sucrose, giving a volume of about 80 ml. The suspension of material from band III (the desmosome fraction) was centrifuged at 30,000 *g* for 20 min at 4°C, followed by resuspension of the pellet in 80 ml of CASC buffer, pH 2.6. The material was dispersed gently by stirring with a magnetic bar, at low speed, for approximately 5 min. The centrifugation and suspension was repeated three times, and the final suspension stored at 4°C. Lyophilization of the material of band III, after washing with distilled water, yielded approximately 10 mg of desmosomes (dry weight) from 10 g wet weight of epidermal tissue, from 10 noses.

Electron Microscopy

Grids of the unfixed desmosome preparation (band III) were prepared as follows: a drop of suspension (prepared by suitable dilution of the density gradient band III with CASC buffer) was pipetted onto a carbon-coated grid. After 1 min had been allowed for the material to settle onto the grid, excess suspension was removed with a piece of lintless filter paper. The grid was then washed thoroughly with distilled water, and stained with uranyl acetate and lead hydroxide.

Pellets were prepared from the suspensions of bands I, II, and IV, and from the washed desmosome preparation. These pellets were rinsed several times with Veronal-acetate buffer pH 7.2, and subsequently fixed for 1 h in cold 1% osmium tetroxide in Veronal-acetate buffer pH 7.2. After dehydration in an increasing ethanol series and propylene oxide, the hardened pellets were cut into small pieces and embedded in Epon 812. To reduce sampling error and facilitate reagent penetration, the pellets were very thin, and portions from both the center and the edge of each pellet were embedded. Thin sections were cut with a glass knife in the Porter-Blum microtome (Ivan Sorvall Inc., Newtown, Conn.), stained with uranyl acetate and lead hydroxide, and examined in the Philips 300 electron microscope.

The desmosome fraction was also fixed and stained with the following variations of the standard procedure: 1% osmium tetroxide in Veronal-acetate buffer containing 5 mM calcium chloride; 2.5% glutataldehyde in cacodylate buffer, pH 7.2 for 2.5 h at room temperature, both with and without postfixation in cold 1% osmium tetroxide in Veronal-acetate buffer at pH 7.2; the alcian blue-glutaraldehyde followed by lanthanum nitrate-osmium tetroxide procedure described by Shea (17). The preparative procedure was monitored by centrifuging the homogenate at various stages and fixing and embedding the pellets using the standard procedure.

Tissue from the second 0.2-mm slice of epidermis, obtained with the keratotome, was cut with a scalpel blade into small pieces measuring approximately 1 × 1 × 0.2 mm, and prepared for electron microscopy in the same way as the pellets; the alcian blue-lanthanum nitrate method was not used, however, because of poor reagent penetration.

RESULTS

The starting material for the preparation of desmosomes is the 0.2-mm slice obtained with the keratotome from the midportion of the cow nose epidermis. Light microscopy shows this to consist mainly of spinous cells. Other epidermal cell types, such as basal and granular cells, appear in small numbers due to the undulations of the epidermal strata. A few fragments of elongated dermal ridges are also present.

In the electron microscope spinous cells appear separated by relatively wide intercellular spaces. The plasma membranes are highly convoluted and form numerous desmosomes (Fig. 2). A few nexus or gap junctions also occur between adjacent spinous cells. Structural details of the desmosome are shown in Fig. 3 at high magnification. The average width of the space between the outer leaflets is about 350 Å, and the midline is approximately 50-Å wide. The dense plaque, which has smooth edges on both sides, is separated from the 60–80-Å filaments by a zone occupied by material of moderate electron density. A somewhat denser band is often observed which runs across the epidermal filament bundle, immediately adjacent to the moderately electron-dense zone and parallel to the plaque (Fig. 3).

Homogenization of epidermal slices in CASC buffer pH 2.6 disrupts the membranes of the cells and solubilizes most of their contents. Dermal fragments remain grossly intact. Electron micrographs of the homogenate used as the starting material for density-gradient centrifugation show that it contains desmosomes, intact nuclei, small membrane fragments, vesicles, dermal fragments, and cell debris. After density gradient centrifugation (Fig. 1) nuclei, dermis, and cell debris pellet at the bottom of the tube. Pelleted material from bands I and II consists mainly of membrane fragments, vesicles, and some desmosomes.

The desmosome fraction, i.e., material from band III, contains isolated desmosomes and a few membrane fragments and vesicles. The final preparation obtained after the washing procedure consists almost entirely of isolated desmosomes. Contaminants such as membranes and debris are present in small amounts. Representative fields selected from electron micrographs of three separate desmosome preparations are shown in Figs. 4–6. No difference, other than closeness of packing of desmosomes, is observed between different parts of the pellet.

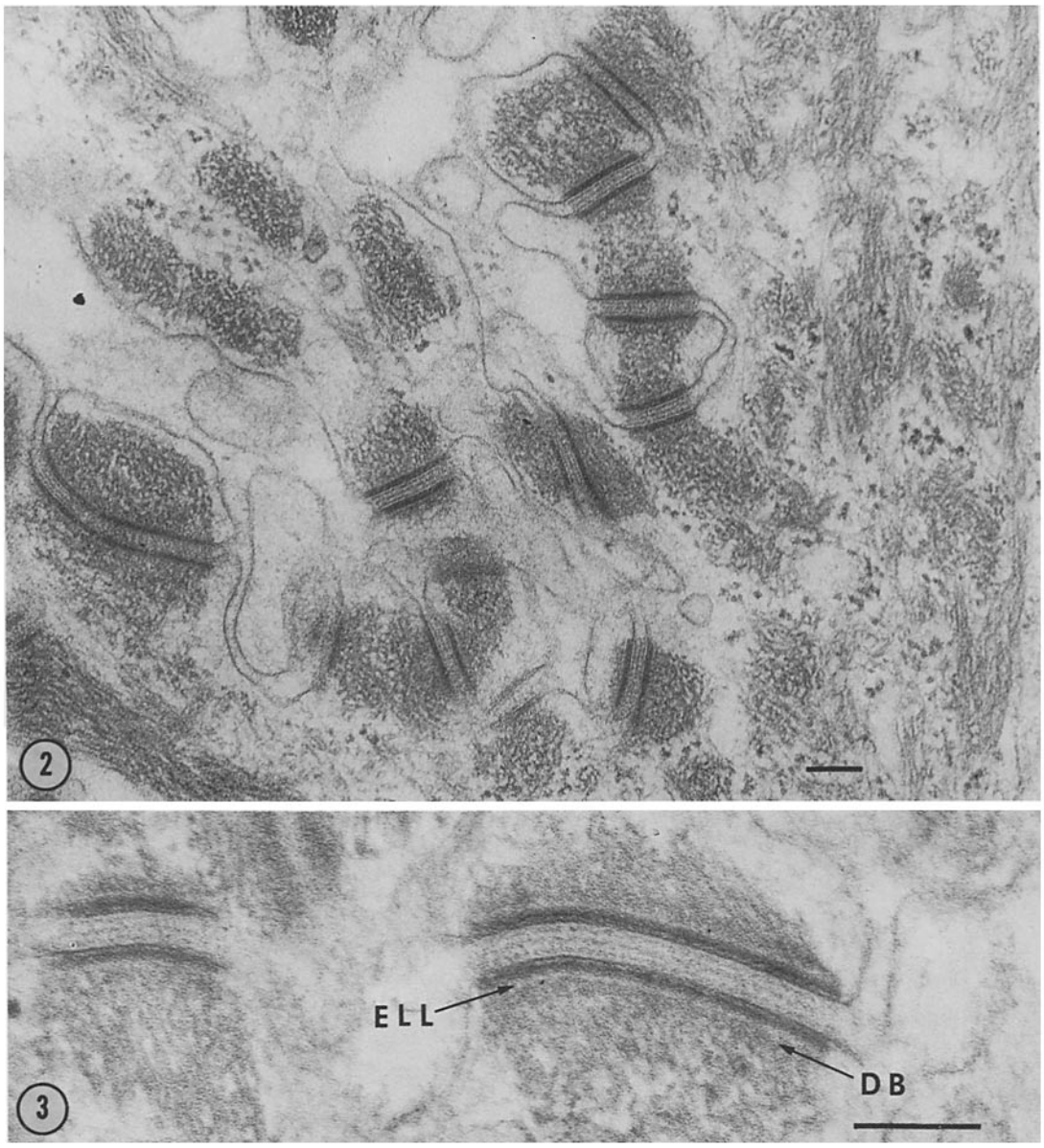
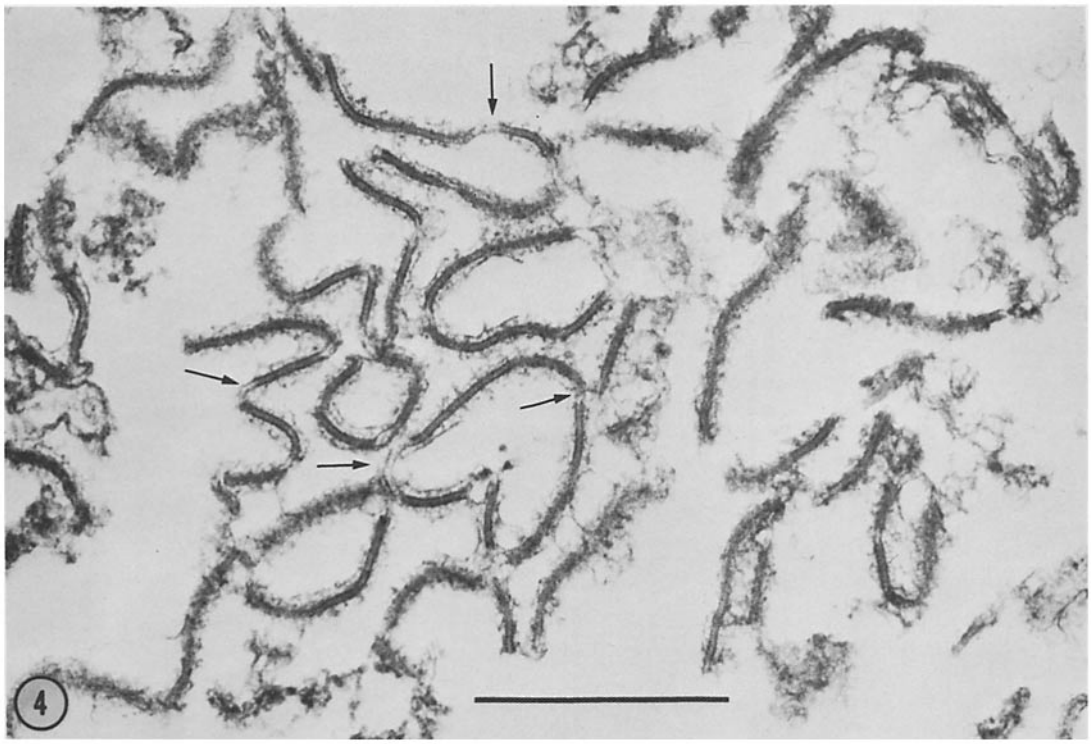


FIGURE 2 Portions of spinous cells of cow nose epidermis showing typical desmosomal junctions at low magnification. Bar 0.1 μm. × 88,000.

FIGURE 3 Desmosomes of spinous cells at high magnification. The midline, and inner and outer leaflets of the plasma membrane, appear well resolved in the area indicated by arrows. Note the electron-lucent layer (*ELL*) next to the plaque and the dense band (*DB*) which runs across the filament bundle. Bar 0.1 μm. × 175,000.

At low magnification (Fig. 4), sectioned isolated desmosomes show variable lengths, appear straight or curved, and have material adhering to their free surfaces. Desmosomes occur singly or connected to one another by short lengths of plasma membrane. When spread on carbon-coated grids (Fig. 6) the isolated desmosomes lie parallel to the surface plane. Such a preparation of flattened desmosomes shows a highly variable size and shape; thus, round, oval, and lobated desmosomes



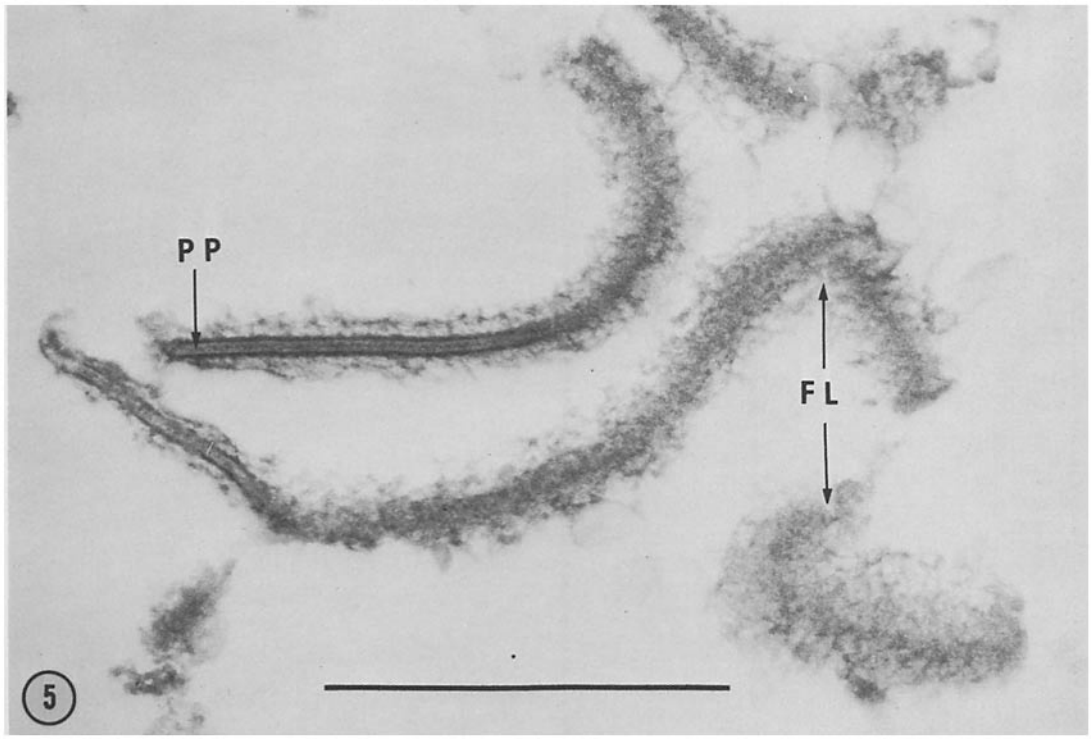
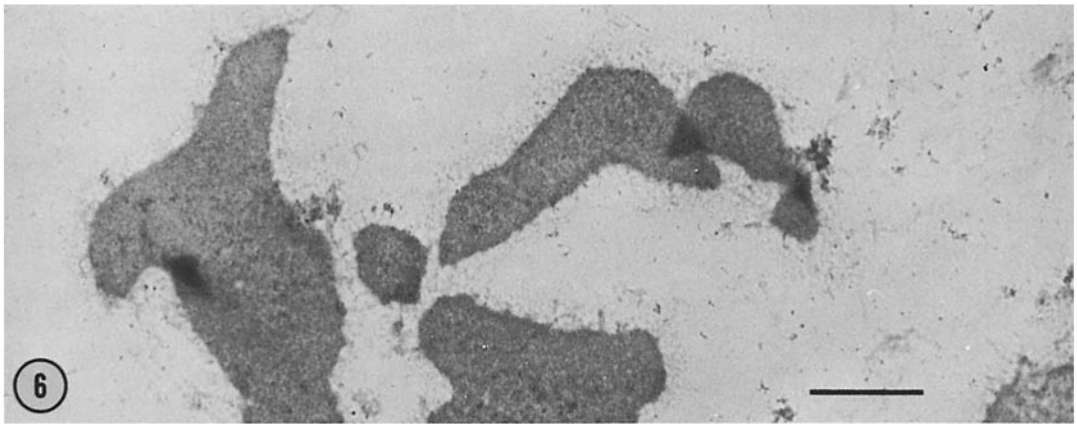


FIGURE 4 Representative field of a desmosome pellet. Note that desmosomes occur singly or connected by short lengths of plasma membranes (arrow) and that material is adhering to their surfaces. Processed according to Shea (17). Bar 1 μm. × 33,800.

FIGURE 5 Isolated desmosomes are shown at high magnification, sectioned perpendicular to the plaque (*PP*) and also through the fuzzy layer (*FL*). Note that the fuzzy layer has a fine filamentous substructure. Processed according to Shea (17). Bar 1 μm. × 54,000.



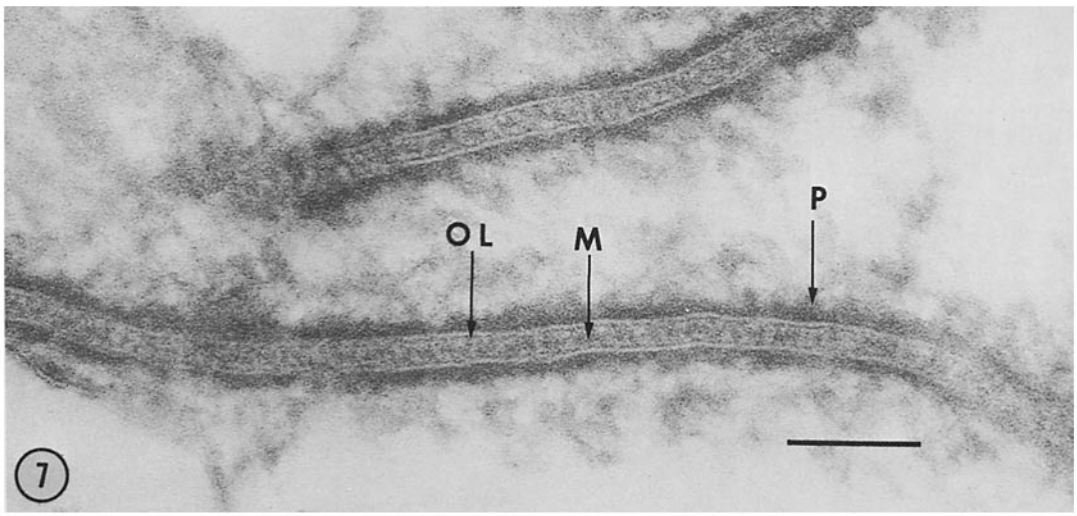


FIGURE 6 Isolated desmosomes, spread on a carbon-coated grid, and positively stained with uranyl acetate and lead hydroxide. Note the variable size and shape, and the fuzzy material surrounding each desmosome. Processed according to Shea (17). Bar 1 μm. × 15,000.

FIGURE 7 Isolated desmosomes, at high magnification, showing a broad midline (*M*), the outer leaflet of the plasma membrane (*OL*), and the dense plaque (*P*). The inner leaflet usually appears fused to the plaque. Note the uneven cytoplasmic surface of the plaque, and its continuity with the fuzzy layer. Processed according to Shea (17). Bar 0.1 μm. × 175,000.

are seen. Most desmosomes appear singly and connections by plasma membrane fragments are not seen. Overlapping areas between adjacent desmosomes are sometimes observed. The edges of each desmosome are slightly curved or rounded; straight edges and sharp angles are rarely present.

At a higher magnification, the dense plaques and midline are clearly seen in favorably oriented cross sections of isolated desmosomes. A layer of material extending from the dense plaques (Fig. 5) is approximately 700 Å in width, and has a "fuzzy" appearance. This layer has a fine filamentous substructure and individual 40–50-Å filaments can sometimes be distinguished. Occasionally, the distal part of the filamentous layer appears more electron dense than the region closer to the plaque. If the plane of section passes through the fuzzy layer, the desmosomes appear as linear or curved structures composed of randomly ordered fine filamentous material (Fig. 5). The structure of the fuzzy layer depends, to some extent, on the fixation and staining procedures

which the desmosomes have undergone. For instance, fixation in osmium tetroxide containing 5 mM calcium chloride gives an extended, finely filamentous layer, whereas glutaraldehyde-osmium tetroxide fixation renders the fuzzy layer more dense and closely packed in appearance. Filamentous material is also observed at the edges of desmosomes spread on carbon-coated grids (Fig. 6). This presumably corresponds to the fine filamentous layer seen in cross sections. The long bundles of 60–80-Å epidermal filaments which attach to desmosomes *in situ* are not seen: they have presumably been solubilized by CASC buffer and removed during the isolation procedure.

At high magnification, the structural components of the isolated desmosomes appear relatively well preserved (Fig. 7). The midplate is seen as a broad, dense line bisecting moderately electron-dense material in the intercellular space. The outer leaflet of the plasma membrane is well resolved, and is the only component of the desmosome to show enhanced contrast when stained with the alcian blue-lanthanum nitrate method. The inner leaflet is usually fused with the plaque. The width of the intercellular space (350 Å) is the same as that in the *in situ* desmosomes. In contrast to the smooth surfaces of the desmosomal plaque *in situ*, the plaques of isolated desmosomes have uneven cytoplasmic surfaces. It is noteworthy that the fuzzy layer is continuous with the material of the plaque (Figs. 5 and 7).

DISCUSSION

Most methods available for the isolation of plasma membrane fractions involve modifications of the method of Neville (15) which utilizes homogenization of tissues in ice-cold 1 mM sodium bicarbonate (4). This hypotonic medium causes swelling of cells and facilitates their subsequent disruption, allowing the use of minimal mechanical force. This effect would be particularly advantageous in the isolation of desmosomes from cow nose epidermis. However, experiments in this laboratory using hypotonic media on this tissue have shown that very little swelling takes place, even after prolonged exposure. In hypotonic media and others, such as isotonic sucrose, homogenization of even a small proportion of epidermis requires the use of drastic procedures such as extrusion through the French pressure cell. After such treatment, only a few fragmented desmosomes are present in the homogenate. Epidermal cells, separated by treatment with 10 mM EDTA at pH 7.4 for 24 h at 37°C, are equally difficult to disrupt and also reveal little swelling in hypotonic media. The resistance of the living part of epidermal tissue to osmotic swelling and to shearing forces can be attributed, at least in part, to the presence of numerous filament bundles in the cytoplasm and to the filament-desmosome attachments. These structures form a stabilizing "cytoskeletal system" which enables both epidermal tissue and individual cells to undergo reversible deformation rather than disruption (9, 13, 14). Thus the preparation of desmosomes from a plasma membrane fraction obtained from cow nose epidermis by classical means seems impractical.

An alternative approach for desmosome isolation is to disrupt the cytoskeletal system by the use of reagents which are known to solubilize epidermal filaments. Such reagents cause rapid swelling and eventual solubilization of most of the epidermal tissue, obviating the problem of homogenization. Prolonged extraction of cow nose stratum corneum with 0.1 N NaOH is the procedure that has been employed for solubilization of the filament matrix complex in the horny cell and isolation of the remaining thickened cell membranes (11). However, it has been noted in this laboratory that this procedure is inadequate for the isolation of desmosomes from the noncornified part of the epidermis, as desmosomes rapidly and completely dissolve in 0.1 N NaOH. It has been shown also that other reagents, such as 3 M urea, rapidly solubilize the desmosomal plaque (16), and so the even higher concentrations (6–8 M) required to solubilize filaments would not be suitable for use in desmosome isolation. CASC buffer, pH 2.6, is a favorable reagent as it is more selective in its action than alkali or urea (10). The nonkeratinized part of the epidermis swells extensively in this buffer, and filaments and mitochondria are rapidly solubilized, whereas nuclei and desmosomes remain morphologically intact. Solubilization of disrupted plasma membranes appears to be a relatively slow process. Even after 30 min in CASC buffer, a few small membrane fragments can be seen with well-preserved trilaminar structure, and long strings of desmosomes often are connected by short lengths of plasma membranes (Fig. 4).

Extraction of the epidermis as described results in a relatively high yield of desmosomes and a

reasonably pure preparation. The structure of the desmosome is well preserved, indicating that its major components are not lost during the preparative procedure. However, the finely filamentous layer on the cytoplasmic side of each plaque in the isolated desmosome is a component which is not observed, as such, *in situ*. It is not likely that this layer represents remnants of epidermal filament bundles, as these are rapidly solubilized by CASC buffer during the preparative procedure. Moreover, the fine filaments have a diameter of 40–50 Å compared with the 60–80 Å diameter of epidermal filaments. It is also unlikely that the filamentous layer represents an adherent extraneous material, as it is of constant appearance and cannot be removed by shearing forces or by intensive washing over a wide pH range. These observations and the fact that the filamentous layer is continuous with the condensed part of the plaque lead to the conclusion that it is a more diffuse region of the plaque material. This would have been rendered visible, and possibly more pronounced, by the removal of 60–80-Å cytoplasmic filaments during the isolation procedure.

The substructure of the desmosomal plaque *in situ* has been studied in epithelia other than the cow nose epidermis and the presence of filaments finer than the cytoplasmic filaments is also noted. Such filaments have been observed in the plaque in both thin sections of embedded tissues and freeze-cleavage replicas (13). In the fascia adherens of cardiac muscle cells, which is related to desmosomes, the plaque consists of a condensation of fine filaments termed by McNutt the "filamentous mat" (7, 12).

Correlation of the observations made in this study with *in situ* studies conducted by other investigators thus suggests that the desmosomal plaque most probably contains condensed fibrous material fraying into fine filaments at the cytoplasmic surface. It is of interest that in a desmosome *in situ*, the filamentous layer would occupy the moderately electron-dense zone and would overlap with the epidermal filament bundles in the region where a denser band is seen (3, 6, 8). Thus it is possible that the filamentous layer fulfills the function of a "connecting component," such as that proposed by Kelly (9). The properties of the filamentous layer so far observed are compatible with its suggested function. It is firmly stabilized and chemically resistant. It has adhesive properties, shown by the tendency of membrane fragments and vesicles to adhere to the outer surface. At neutral pH, isolated desmosomes readily aggregate side-to-side, and also adhere strongly to glass, metal, and plastic surfaces. A better understanding of the nature of desmosomal components and the mechanism of filament-plaque attachment can be expected only after determination of the chemical composition of desmosomes. Work along these lines will be reported in a subsequent publication.

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