# STRUCTURE AND BIOCHEMICAL COMPOSITION OF DESMOSOMES AND TONOFILAMENTS ISOLATED FROM CALF MUZZLE EPIDERMIS

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## ABSTRACT

Complexes of plasma membrane segments with desmosomes and attached tonofilaments were separated from the stratum spinosum cells of calf muzzle by means of moderately alkaline buffers of low ionic strength and mechanical homogenization. These structures were further fractionated by the use of various treatments including sonication, sucrose gradient centrifugation, and extraction with buffers containing high concentrations of salt, urea, citric acid, or detergents. Subfractions enriched in desmosome-tonofilament-complexes and tonofilament fragments were studied in detail. The desmosome structures such as the midline, the trilaminar membrane profile, and the desmosomal plaque appeared well preserved and were notably resistant to the various treatments employed. Fractions containing desmosome-tonofilament complexes were invariably dominated by the nonmembranous proteins of the tonofilaments which appeared as five major polypeptide bands (apparent molecular weights: 48,000; 51,000; 58,000; 60,000; 68,000) present in molar ratios of approx. 2:1:1:2:2. Four of these polypeptide bands showed electrophoretic mobilities similar to those of prekeratin polypeptides from bovine hoof. However, the largest polypeptide (68,000 mol wt) migrated significantly less in polyacrylamide gels than the largest component of the hoof prekeratin (~63,000 mol wt). In addition, a series of minor bands, including carbohydrate-containing proteins, were identified and concluded to represent constituents of the desmosomal membrane. The analysis

† Pierre Drochmans died 26 October 1977. The coauthors have finished the manuscript and dedicate it to his memory.

‡ Deceased.

of protein-bound carbohydrates (total 270 μg/mg phospholipid in desmosome-enriched subfractions) showed the presence of relatively high amounts of glucosamine, mannose, galactose, and sialic acids. These data as well as the lipid composition (e.g., high ratio of cholesterol to phospholipids, relatively high contents of sphingomyelin and gangliosides, and fatty acid pattern) indicate that the desmosomal membrane is complex in protein and lipid composition and has a typical plasma membrane character. The similarity of the desmosome-associated tonofilaments to prekeratin filaments and other forms of intermediate-sized filaments is discussed.

KEY WORDS desmosomes · tonofilaments · composition of desmosomal membrane · prekeratin-epidermis

Among the intercellular junctional differentiations (for review, see reference 60), only gap junctions have been extensively studied in the isolated state (5, 48). A method for isolating epidermal desmosomes based on the selective disruption of the plasma membranes and the solubilization of cytoskeletal elements has been described by Skerrow and Matoltsy (54). Their procedure results in a fraction of desmosomes in which the characteristic structures of the midline, the desmosomal plasma membrane, and the dense plaque are well preserved but tonofilaments are absent, most likely a result of the solubilizing action of the 0.1 M sodium citrate buffer (pH 2.6) used in the initial step. In this report, we propose an alternative procedure for the isolation and purification of desmosomes. The method we present involves the separation of plasma membranes of keratinocytes by hypotonic treatment, followed by homogenization and a series of centrifugations, resulting in an enrichment of membrane-desmosome-tonofilament (MDT) complexes. It is shown that such complexes can be subdivided into fractions enriched in (*a*) desmosomal material, (*b*) interdesmosomal membrane, or (*c*) desmosomal tonofilaments. An analysis of the composition of the desmosome-enriched and the tonofilament fractions is presented. Special emphasis is placed on the comparison of the topologically defined desmosome-attached tonofilaments (e.g., references 14, 30, 50) with the prekeratin filaments of keratinized epidermal layers. Bovine muzzle has been chosen as an especially suitable material as its thick stratum spinosum is rich in desmosomes (40, 41, 44).

## MATERIALS AND METHODS

Fresh calf snouts were received from the slaughterhouse, kept chilled on ice, and used within 1 h after sacrifice of the animals. Unpigmented epidermis was selected to avoid contamination of the homogenates with melanin granules. For isolation of prekeratin, posterior parts of calf and cow hooves were also used (cf. references 20, 62–64).

### *Isolation of Plasma Membranes Including Desmosomes and Tonofilaments (MDT)*

Thin shreds of epidermis were cut from the internostril region of the snout with a scalpel. The uppermost epidermal layer was usually discarded. Sections enriched in stratum spinosum were weighed, placed in distilled water that had been alkalized with a few drops of 0.1 N NaOH to pH 9, 10, or 11, and this was minced with fine scissors. The slight alkalinization has been found very useful to avoid aggregation of the particulate material in the following manipulations (differences of pH in the range indicated did not result in significant changes in the data reported here). Portions of ~1 g of minced epidermal fragments from the stratum spinosum layer were homogenized with an ultra-turrax (model TP 18–10, Janke und Kunkel AG, Ika Werk, Staufen im Breisgau, Germany) in 20 ml of alkalinized water for 1 min at maximum speed. The crude suspension was further homogenized by 20 strokes in a cooled Potter-Elvehjem (type C) homogenizer. The final white homogenate was briefly ("up and down") centrifuged at low speed (~2,000 *g*) in a laboratory centrifuge (International, model PR1, rotor 269, International Equipment Co., Needham Heights, Mass.; or Heraeus-Christ type IKS, standard rotor) to remove pelletable debris and nondissociated cells. All these manipulations were carried out at 0–5°C in a cold room or on ice. The homogenate was then centrifuged at 1,300 *g* for 30 min. The pellet consisted primarily of large membrane segments containing desmosomes with long tonofilaments attached (MDT). In most experiments, the resuspended pellet material was layered on top of a discontinuous density gradient system consisting of four layers of 0.5, 1.0, 1.5, and 2.0 M sucrose (made up in 10 mM Tris-HCl, pH 7.6), and this was centrifuged for 2–3 h at ~50,000 *g* in swinging buckets in an ultracentrifuge (Spinco-Beckman, model L-2, rotor SW 27, Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). A white opalescent layer at the density boundary between 1.195 and 1.255 $g \times cm^{-3}$

contained the MDT material. In some experiments, all media used contained 2 mM 2-mercaptoethanol.

### *Subfraction of MDT*

The MDT fraction was homogenized and sonicated with a Branson Sonifier model S125 (Branson Sonic Power Co., Danbury, Conn.) in 20 mM Tris-HCl buffer (pH 7.4) containing 2 mM mercaptoethanol. KCl was then added to obtain a final concentration of 1.5 M. This suspension was again briefly homogenized and stirred for 1 h in the coldroom. It was then adjusted with supersaturated sucrose solution (same buffer) to 0.7 M sucrose and layered on top of a sucrose gradient ranging from 0.7 to 1.8 M. After centrifugation (WKF-P50K, Weinkauf, Brandau, Germany, rotor 3 × 5 ml; 20,000 *g* for 30 min, then accelerated to 170,000 *g* and run for another 150 min), most of the material was recovered as a pellet and designated "heavy MDT subfraction", whereas variable small amounts of some slightly opaque material ("light MDT subfraction") were collected from levels of the gradient that corresponded to densities ranging from 1.14 to 1.20 $g \times cm^{-3}$. These subfractions were diluted with the same buffer, pelleted by centrifugation for 1 h at 200,000 *g*, resuspended in the same buffer, and pelleted again.

The MDT fraction was resuspended and sonicated in neutralized distilled water (10 cycles of 15 s, each sonication period alternating with an equivalent time of cooling on ice). After a final centrifugation for 30 min at 1,300 *g*, a pellet was obtained which was clearly enriched in isolated desmosomes (fraction D).

MDT fractions, in some experiments also D fractions, were resuspended in distilled water or 10 mM Tris-HCl (pH 7.6) and intensely sonicated. The homogenate was then centrifuged in a laboratory centrifuge for 20 min at ~3,500 *g*, and the pellet containing the desmosomes was separated from the supernate. Tonofilament fragment material was pelleted from the supernate by 1-h centrifugation at 100,000 *g*. In some experiments, the fraction of tonofilament fragments was further purified by sucrose gradient centrifugation in concentrations ranging from 1.0 to 2.2 M (same buffer).

### *Preparation of Total Particulate Material from Stratum Spinosum*

The muzzle epidermis was sliced with a keratotome or a razor blade in a plane parallel to the surface of the skin in such a way that the stratum spinosum and the fully keratinized part of the epidermis were crudely separated. The part which contained the stratum spinosum was homogenized at high speed in a rotating knife device (Fa. E. Buehler, Tübingen, Germany) in 10 mM Tris-HCl buffer (pH 7.4) and centrifuged at 100,000 *g* for 3 h. The pellet obtained was washed in the same buffer and was designated "total particulate fraction."

### *Preparation of Prekeratin from Bovine Snout and Hoof*

Slices of the upper layers from calf snout epidermis or cow's hoof were used. Prekeratin-containing material was extracted either according to the citrate buffer method of Matoltsy (40) or using moderately alkaline (pH 9.0 or 10.2) buffers containing 6 or 8 M urea (cf. references 1, 9, 40, 62–64).

Alternatively, prekeratin was isolated according to the procedure of Steinert and co-workers (62–64), which was modified as described elsewhere (20).

### *Extraction Procedures*

The fractions were extracted by treatment with one of the following procedures: (*a*) Resuspension in buffer containing high salt concentration (10 mM Tris-HCl, 1.5 M KCl, pH 7.4) and stirring in the cold for 2 h or overnight; (*b*) resuspension in 10 mM Tris-HCl (pH 7.4) containing Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.) at a final concentration of 2 mg detergent/mg protein and stirring at room temperature for 1–2 h; (*c*) sequential treatment with the media described under *a* and *b*; (*d*) treatment with 10 mM Tris-HCl buffer containing both high salt concentration (1.5 M KCl) and Triton X-100 (see above) and extraction at room temperature; (*e*) extraction in sodium citrate buffer (pH 2.6; see references 40, 54, 55) using 5 ml buffer/500 μg protein; (*f*) extraction in 100 mM Tris-HCl buffer (pH 10.2) containing 6 M urea for 1–3 h at room temperature under stirring. Alternatively, isolations and extractions as described above were done in media containing 2 mM mercaptoethanol throughout.

### *Chemical Determinations*

Protein, phospholipids, cholesterol, gangliosides, fatty acids, and nucleic acids were determined as described in previous articles (17, 18, 49). Since with most of the fractions studied here, the determinations obtained with the procedure described by Lowry et al. (39) were considerably different from the values obtained by Nesslerization according to Strauch (65), both methods were usually applied in parallel. Protein-bound carbohydrates were determined by gas chromatography (18, 58) and cytochromes by spectrophotometry (25, 49). Cytochrome oxidase activity was determined as described (cf. references 17, 18, 25, 49).

### *Gel Electrophoresis*

Proteins were usually separated by electrophoresis in polyacrylamide slab gels (5, 7.5, and 10% wt/vol) according to Laemmli (34), using the slight modifications described elsewhere (20). Relative amounts of components of different mobilities were estimated from densitometric tracings (cf. reference 22). Gels were also stained with the Periodic-Acid-Schiff (PAS) reaction according to Fairbanks et al. (13). Alternatively, polypeptides were separated in 15% polyacrylamide gels by

the use of a slightly modified version of the procedure described by Thomas and Kornberg (68). Reference proteins used for estimation of apparent molecular weights (69) were: myosin from skeletal muscle of rabbit (mol wt ~220,000), phosphorylase (mol wt 94,000), bovine serum albumin (mol wt 67,000), tubulin from porcine brain (mean mol wt 55,500), ovalbumin (mol wt 43,000), actin from rabbit skeletal muscle (mol wt 42,000), and chymotrypsinogen (mol wt 26,000). Actin and myosin were kindly provided by D. P. Danker (Max-Planck-Institute for Medical Research), and tubulin was a gift from Dr. H. Ponstingl (German Cancer Research Center, Heidelberg). In some experiments, the polypeptides were carboxymethylated (7). For reduction, samples were solubilized in 1 M Tris-HCl (pH 8.6) containing 0.1% EDTA, 0.12 M mercaptoethanol, and 8 M urea and were kept overnight under nitrogen atmosphere. 10 ml of 2.5 M iodoacetic acid dissolved in 8 M urea were added to 100 ml of reduction solution and kept in the dark for 30 min. The acylation reaction was stopped by adding 1.7 ml of mercaptoethanol resulting in a molarity of 0.25 M. The samples were dialyzed overnight against distilled water and prepared for gel electrophoresis.

### *Electron Microscopy*

The pellets of MDT and D fractions were fixed for 3 h at 4°C either in 2.5% distilled glutaraldehyde buffered with 0.1 M sodium phosphate (pH 7.2) or in a mixture of 3% glutaraldehyde-1% paraformaldehyde buffered with 0.1 M sodium cacodylate (pH 7.4). After a short wash with the specific buffer, the pellets were postfixed for 2 h in 1% osmium textroxide dissolved in the specific buffer used in the initial fixation. In some fixations, ions were included in the initial fixation as previously described (19). Gradual dehydration in ethanol solutions was followed by conventional embedding in Epon. Ultrathin sections were stained with lead citrate. In some preparations, the contrast was increased by 1-h impregnation of the final material in a 1% aqueous solution of uranyl acetate in 0.05 M maleate buffer (pH 6.0). The sections were examined in a Siemens Elmiskop I or 101 or in a Philips EM 301. The negatively stained preparations of resuspended pellets were made with 1 or 2% phosphotungstic acid solutions, adjusted to pH 7.2 with NaOH, or with aqueous 1% uranyl acetate or uranyl formiate.

## RESULTS

### *Morphological Observations*

The disruption of the epidermal tissue by swelling in hypotonic solution and mechanical homogenization results in the release of plasma membranes which are produced in such a way that the cell membrane remains associated with the cell membrane of the adjacent cell. The tonofilaments appear to have been torn off in a few microns's distance from the sites of their attachment at the desmosomal plaques. Consequently, electron micrographs of disrupted cells, of isolated plasma membranes, or of the isolated desmosomes show tufts of tonofilaments anchored on either side of the desmosomal plaques of the plasma membrane (Figs. 1–4).

THE MDT FRACTION: The isolated keratinocyte plasma membrane fragments are loaded with desmosomes and tonofilaments (MDT fraction; Figs. 1 and 2) and often have wavy contours reminiscent of the interdigitations of the spinous cell surface membranes observed *in situ* (Fig. 2). This tortuosity of the isolated membranes probably reflects a certain rigidity of the surface membrane resulting from the high density of junctional complexes. In sections of such fractions, the desmosomes rather regularly alternate with interdesmosomal, nondifferentiated intercepts of the plasma membrane. More than half of the total membrane profile length traced in such sections is contained in desmosomes. Sometimes, gap junctions are seen close to desmosomes (e.g., Fig. 1), a situation which is commonly found in epidermal plasma membranes fixed *in situ* (cf. reference 12). In the isolated MDT complexes, the tonofilaments show a looser packing, compared to the state observed after fixation *in situ*, and an enhanced distinctiveness of their substructure, especially in the regions of their anchorage at the desmosomal plate (see below). Among the membrane fragments seen in MDT fractions, a small proportion is obviously derived from upper, more keratinized regions of the epidermis. Such fragments are usually identified by a greater compactness of their tonofilament bundles (as to changes in desmosome and tonofilament structures during late stages of epidermal and wool follicle keratinization; see references 14, 46, 51).

At high magnifications (Fig. 3), the structure of the isolated desmosome has gained in definition compared to the corresponding *in situ* structure. Both plasma membranes of the desmosome are in continuity with the interdesmosomal segments and are separated by an intercellular space of ~30 nm width. The two leaflets, or lamellae, of each membrane delineate an interlamellar space that is relatively electron-transparent, and this trilaminar ("unit membrane") aspect is more often resolved and appears to be more distinct in the desmosomes than in the membranes of the interdesmosomal regions. The outer lamella of each mem-

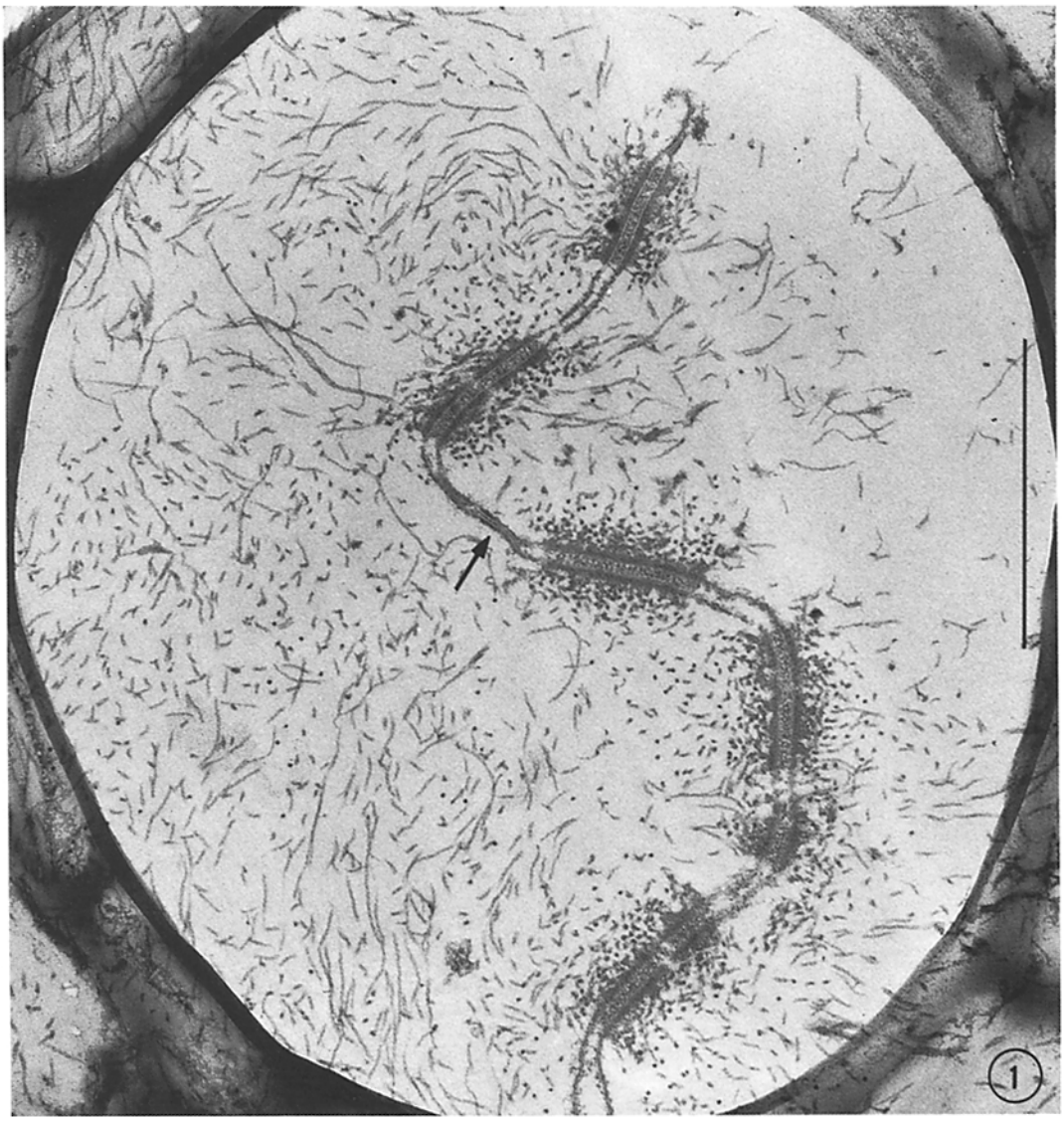

FIGURE 1 Survey electron micrograph showing ultrathin sections through isolated MDT complexes from calf muzzles (prepared on holey films). Note the high proportion of cell surface membrane that is included in the desmosomes and the abundance of tonofilaments. The arrow denotes a gap junction complex. Bar, 1 $\mu$m. × 40,000.

brane is covered by a cell coat which in turn borders on the midline structure, a dense intercellular substance characteristic of the desmosomal junctions. The inner lamella often is slightly thicker than the outer lamella and is in continuity with the inner lamella of the unit membrane of the interdesmosomal membrane segments. Desmosomes of MDT fragments derived from upper layers of the stratum spinosum frequently show a somewhat thickened inner lamella, probably due to the presence of an internal coat. Centripetally from the membrane profile proper, follows the desmosomal plaque which is a dense, 10–15 nm thick line, sometimes separated from the inner lamella by a narrow cleft of reduced stainability. This plaque consists of two or three thin layers of interwoven thin (2.5–3 nm) filaments, similar in size to the "protofilaments" sometimes described

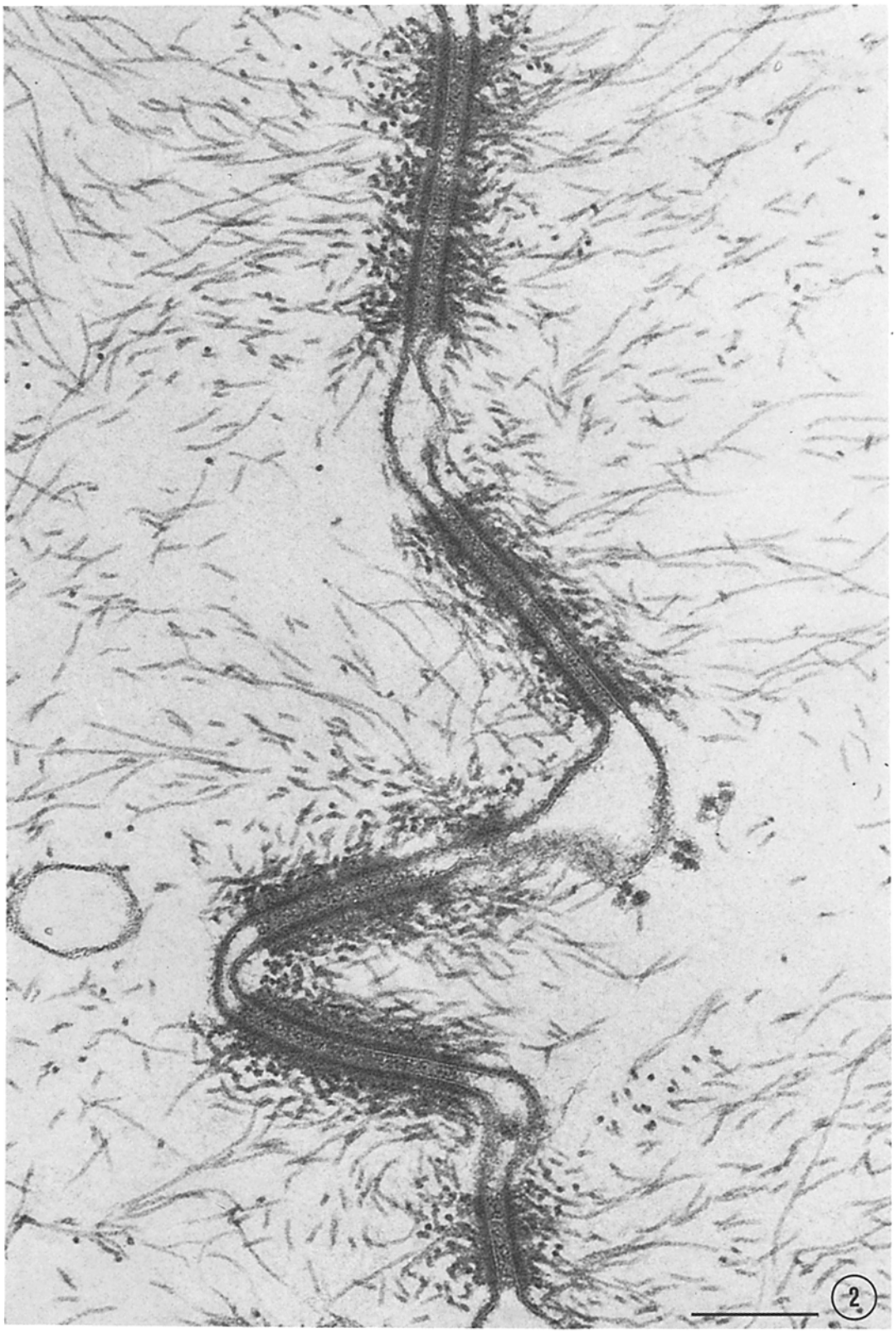


FIGURE 2 Appearance of isolated MDT complexes in ultrathin sections as revealed at higher magnification. Note the occurrence of relatively large units of surface membranes of adjacent stratum spinosum cells with alternating intercepts of desmosomes and interdesmosomal membrane regions. Desmosomal organization and membrane ultrastructure are well preserved. Bar, 0.25 $\mu$m. × 80,000.

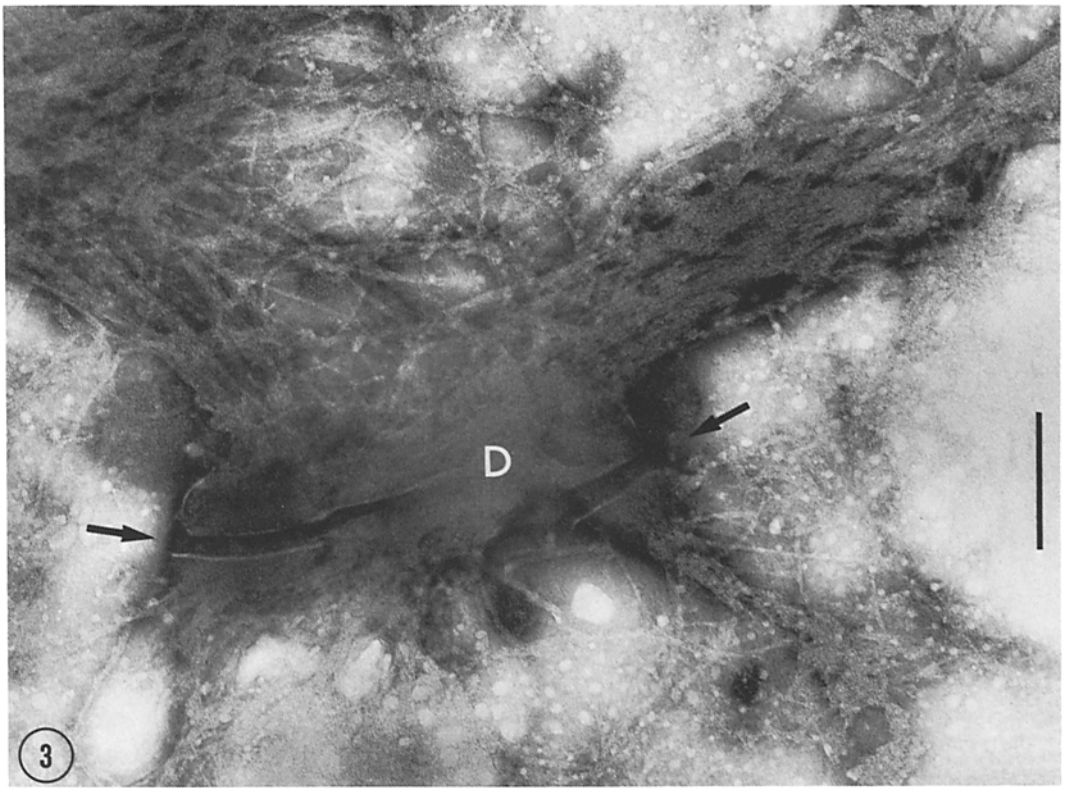


FIGURE 3 Electron micrograph showing a negatively stained preparation of isolated desmosomes (*D*) with their attached bundles of tonofilaments. This particular fraction has been treated with high salt buffer and freezing and thawing. Arrows denote the intercellular space filled with staining solution, uranyl formiate. Bar, 0.2 μm. × 91,000.

as subunits of tonofilaments and prekeratin filaments (8, 10, 21, 26, 40, 41, 43, 44, 54, 64).

In negatively stained preparations of isolated desmosomes (Fig. 3), the large masses of tonofilaments associated per desmosome are directly seen. In such preparations the desmosomes are easily identified when seen in profile (Fig. 3) since the intercellular space of the desmosome is filled with staining material, similar to the descriptions of the isolated synaptic junctional complexes from brain (67). The widths of the negatively stained tonofilaments usually are somewhat smaller (4–7 nm) than those measured in sections, which may be a result of the penetration of stain into the outermost regions of the isolated tonofilaments. Negatively stained preparations as well as cross sections of the tonofilaments of our fractions have confirmed earlier observations made in intact cells that these structures show a hollow core of 2–6 nm inner width (cf. Fig. 5 and references 8, 10, 21, 22, 43, 44, 71, 72).

THE D FRACTION: After sonication of the MDT fraction, followed by differential centrifugation, a relatively pure pellet of isolated desmosomes has been obtained (Fig. 4). Most of the extradesmosomal plasma membrane has been removed from the desmosomes, and the amount of tonofilament material associated with the desmosomes has also been reduced. It appears as if most of the tonofilaments have been sheared off at a distance of ~15 nm from either membrane of the desmosome (Fig. 4*b*). The remaining basal portions of the tonofilaments are less distinct and appear somewhat fuzzy. They have lost in definition of the filament structure, and their appearance suggests that they have partially uncoiled and/or are denatured into their protofilaments. It is obvious, however, from both electron microscopy and biochemical analyses (see below), that the basal portions of the tonofilaments still represent by far the predominant protein structure present in the D-fraction. The only occasional contaminants in

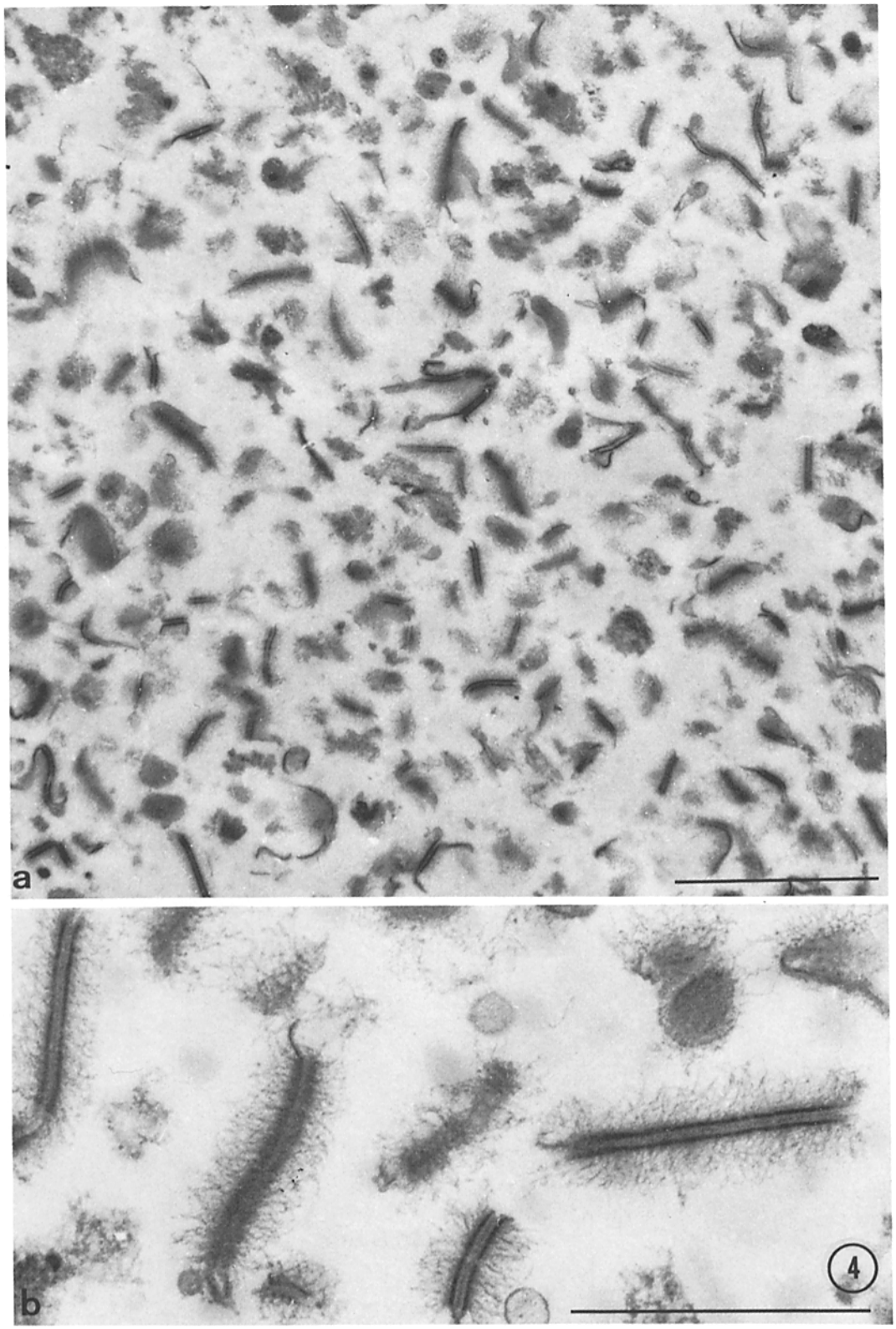


FIGURE 4 Electron micrographs showing a survey (*a*) and details (*b*) of the isolated desmosomal complexes (fraction D) obtained after sonication of the MDT fraction. Note the high purity of the fraction (*a*) and the retention of relatively short (100–150 nm long) residual tonofilaments at the isolated desmosomes (*b*). Bars, (*a*) 2 μm and (*b*) 1 μm. *a*, × 16,000; *b*, × 48,000.

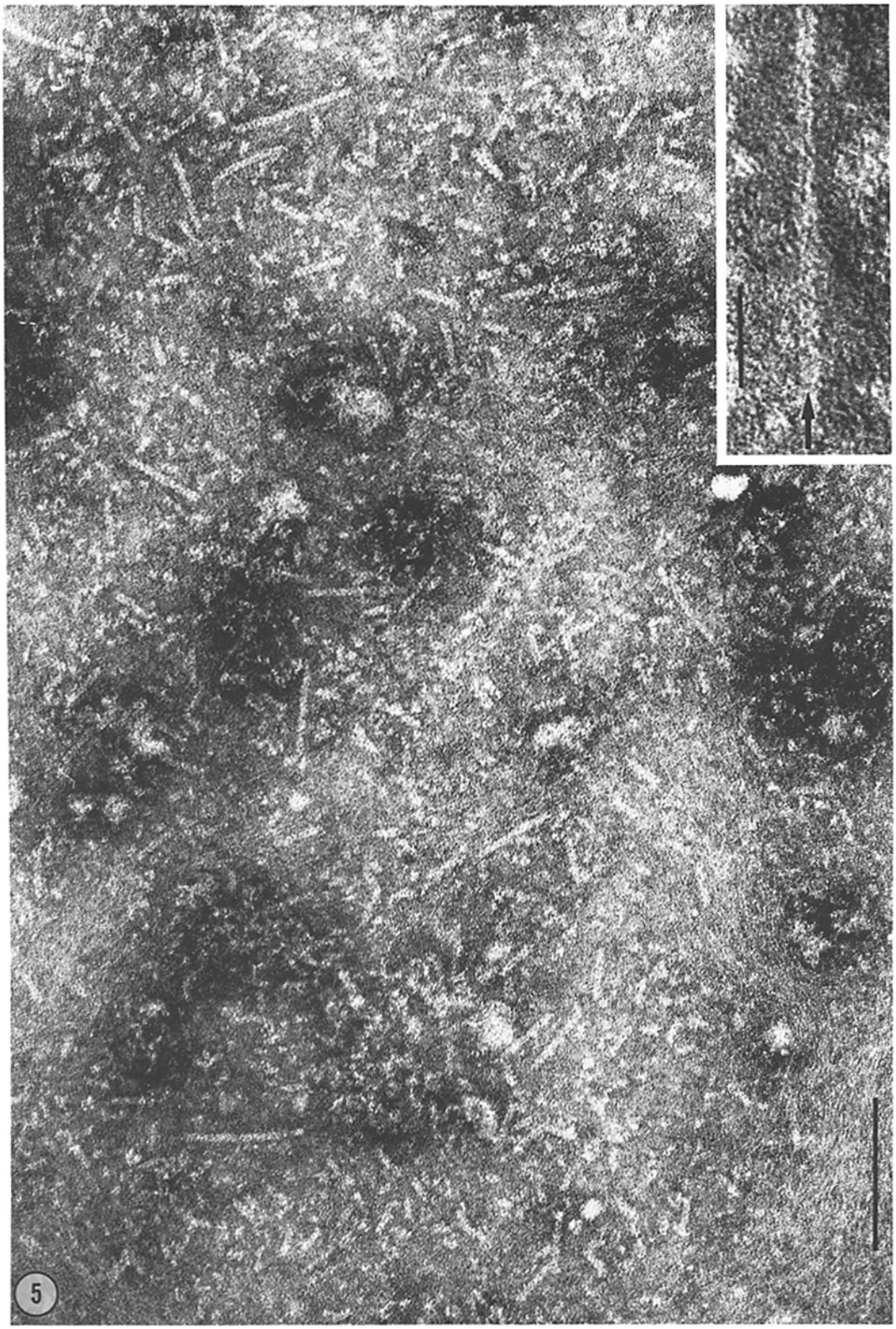


FIGURE 5 Survey electron micrograph of a preparation negatively stained with phosphotungstic acid showing a pure fraction of tonofilament fragments produced by extensive sonication of the MDT fraction, followed by differential centrifugation (see text). Note that the preparation consists of short rod- or disklike fragments of tonofilament width. The insert shows that a central stained core can be identified in some of these rods, indicative of a tubular structure (arrow). Bars, 0.1 $\mu$m and (*insert*) 20 nm. × 240,000; *insert*, × 600,000.

such fractions have been fragments of collagen fibrils which, however, are quantitatively negligible, as has also been demonstrated by coelectrophoresis of authentic collagen in SDS-polyacrylamide gel electrophoresis (22).

STABILITY OF ISOLATED DESMOSOMES: The basic desmosome structure has been found to be resistant to a variety of treatments (procedures *a–f* in Materials and Methods). Freezing and thawing, sonication, and extractions with buffers containing low and high salt concentrations do not significantly change the structure of the isolated desmosome. Treatment with detergents such as Triton X-100 and deoxycholate results in a reduced distinctiveness of the intermembranous material and some effacement of midline structures, which probably reflects the removal of some constitutive membrane glycoproteins. It is interesting to note that the "unit membrane" aspect, i.e., the transparent line bordered by electron-opaque material, is maintained in the detergent-treated preparations from which nearly all lipids have been removed. Treatment of the desmosome-enriched fractions with citrate buffer and moderately alkaline or neutral urea solutions (e.g., procedures *e* and *f* in Materials and Methods) also does not result in significant changes in desmosome morphology.

TONOFILAMENT FRAGMENTS: When MDT fractions are sonicated and then fractionated into velocity splits, fractions of relatively small particles can be obtained that almost exclusively consist of small rod- or disk-like fragments of tonofilaments (Fig. 5). Among the rodlike fragments, small disks or rings with a stain-filled center are also often recognized. In negatively stained preparations, such rodlike fragments sometimes reveal stained cores (Fig. 5, insert), again indicating that their structure is tubular (see above). When MDT fractions are sonicated and separated by gradient centrifugations into heavy and light subfractions, most of the material is recovered in the "heavy subfraction," which consists of tonofilament fragments and desmosome-tonofilament complexes with reduced amounts of interdesmosomal membranes whereas only little material is collected from the light regions of the sucrose gradient. The latter almost exclusively consists of small membrane vesicles and sheets many of which, however, still show attachments with short filamentous fuzzy structures reminiscent of tonofilament residues.

In our hands, extraction of the MDT and D fractions with alkaline buffers containing 6 or 8 M urea or with citrate buffer at pH 2.5 as described by Skerrow and Matoltsy (40, 53–56) has not resulted in a complete separation of the tonofilaments from the desmosome plaques proper.

### *Biochemical Data*

GROSS CHEMICAL COMPOSITION: In concert with the described abundance of tonofilament structures in the desmosome-enriched fractions (MDT and D) are our biochemical findings of a predominance of proteinaceous material in these fractions (Table I). As can be seen from the data of Table I, the membrane portion of the desmosome-tonofilament complexes makes only a minor contribution to the total mass, the only exception being the "light subfraction" obtained from the MDT-fractions. Only in this fraction the wt/wt ratio of phospholipids to proteins is relatively high (0.46), indicating an enrichment of true membranous components. This interpretation is also substantiated by the high contents of cholesterol and glycopeptide-bound carbohydrates in this subfraction: a value of 56 $\mu$g of carbohydrates/mg protein is in the range reported for plasma membrane fractions from other tissues and cells (18).

The low contents of putative RNA (Table I) and the absence of DNA in these fractions indicate its relatively high purity, in concert with our electron microscope findings of the absence of considerable amounts of ribosomes, nuclei, and mitochondria in this fraction (for enzymes and lipid data, see below).

LIPID COMPOSITION: In all the desmosome-enriched fractions, both the high cholesterol contents (molar ratios of cholesterol to phospholipids ranging from 0.80 to 0.95) and the phospholipid pattern (Table II) reflect the plasma membrane character of the desmosomal membrane plaques. A high cholesterol content has also been reported by Skerrow and Matoltsy (55) in their citric acid-treated desmosomal fractions from calf muzzle. Especially characteristic is the relatively high content of sphingomyelin (Table II), which is considerably enriched in the MDT fractions and in desmosomes, in comparison with the total particulate matter. Such an enrichment of sphingomyelin has also been observed in plasma membrane fractions from a variety of other cell types (e.g., references 16, 18, 27, 29, 32, 42, 45). The lipid data shown in Table II also demonstrate the purity of the desmosome-enriched fractions (MDT

TABLE I

*Gross Composition (μg) of Total Particulate Material, the MDT Fraction, the D Fraction, and MDT-Subfractions Isolated from Stratum Spinosum of Calf Snout Epidermis*

| Fraction | Protein* | Phospho-lipids | Cholesterol | Protein-bound carbohydrates | RNA‡ |
|---|---|---|---|---|---|
| Total particulate | 1,000 (690) | 48 | 10 | 6.9 | 157 |
| MDT fraction | 1,000 (535) | 33 | 16 | 8.3 | 51 |
| D fraction | 1,000 (610) | 27 | 11 | 7.3 | 46 |
| MDT, light subfraction | 1,000 (885) | 455 | 220 | 56.0 | 11 |
| MDT, heavy subfraction | 1,000 (595) | 21 | 9 | 7.0 | 66 |
| MDT, fraction, extracted with high salt concentrations and Triton X-100 | 1,000 (645) | ≤2 | Not detected | 6.2 | 34 |

* Values taken from determinations made with the Folin reagent; parallel determinations made with the Nessler reagent are given in brackets.

‡ Values from determinations made with the orcinol reaction; the significance of these data as to true contents of RNA in the fractions is not clear.

TABLE II

*Phospholipid Composition (% of Total Lipid Phosphorus) of Total Particulate Material, MDT and D Fractions Isolated from Stratum Spinosum of Calf Snout*

| | Total particulate fraction | MDT fraction | D fraction |
|---|---|---|---|
| Phosphatidylcholine | 47.0 | 37.6 | 37.8 |
| Phosphatidylethanolamine | 27.4 | 27.5 | 27.0 |
| Sphingomyelin | 8.7 | 19.7 | 22.8 |
| Phosphatidylserine | 4.3 | 4.0 | 3.4 |
| Phosphatidylinositol | 7.4 | 8.3 | 8.4 |
| Cardiolipin (plus some partially separated minor components) | 5.0 | 1.0 | <0.7 |
| Phosphatidic acid | <0.2 | <0.2 | <0.2 |
| Lysophosphatidylcholine | 0.2 | 1.7 | Not detected |

and D) with respect to mitochondrial contaminations (as to observations of especially intimate associations of mitochondria with desmosome-tonofilaments in various epithelia, see references 2, 72). They also document the low degree of lipid degradation during the isolation procedures. Only traces of cholesterol esters and neutral glycerides have been found in the MDT and D fractions (see also 55).

In all fractions examined, the fatty acid pattern of the phospholipids (Table III) is similar to that of the total membrane lipids, which obviously reflects the large relative amounts of phospholipids present in this material (cf. reference 55). The pattern shows a striking predominance for $C_{16}$ and $C_{18}$ fatty acids and, surprisingly, an almost complete absence of longer ones. In the desmosomes, palmitic acid, oleic acid, and linoleic acid occurred most frequently, constituting about two-thirds of the identified fatty acids. The percentage of total unsaturated fatty acids was 50.5% in the most purified desmosome-tonofilament fraction studied (fraction D). This value is in the range of the corresponding figures reported for various other membrane fractions, including plasma membranes, from a diversity of cells (e.g., references 27–29, 32, 59). Moreover, our analyses suggest a tendency toward an increase in the saturated character of the phospholipid-bound fatty acids during the purification from total particulate material via the MDT-fraction and the D-fraction to a figure of 64% saturated fatty acids in the "heavy subfraction," which primarily reflects the relative reduction of linoleic acid, from approx. 33 to 18%).

The desmosome-enriched fractions also contain glycolipids, particularly gangliosides. While the ganglioside contents determined in MDT and D fractions (0.74 and 0.46 nmol lipid-bound sialic acid/mg protein, respectively) are not very impressive on a protein basis, the more adequate expression on a phospholipid basis makes clear that the desmosomal contents of gangliosides (22 and 17 nmol of sialic acid per mg phospholipid in the two desmosome-enriched fractions MDT and D, respectively) are in the same range as those reported for a variety of other plasma membranes (11, 18, 33).

PROTEIN-BOUND CARBOHYDRATES: The carbohydrate contents of the glycopeptides present in MDT and D fractions are relatively low

TABLE III

*Fatty Acids (%) of Total Lipids and Phospholipids in Total Particulate Material, MDT and D Fractions from Stratum Spinosum of Calf Snout*

| | Total Particulate fraction | | MDT fraction | | D fraction | |
|---|---|---|---|---|---|---|
| Major fatty acids identified | Total Lipids | Phospholipids | Total Lipids | Phospholipids | Total Lipids | Phospholipids |
| 14:0 | 4.8 | 4.6 | 4.7 | 4.6 | 5.8 | 4.9 |
| 16:0 | 27.8 | 19.2 | 24.1 | 22.8 | 26.2 | 25.0 |
| 16:1 | 5.1 | 4.0 | 8.7 | 8.0 | 10.5 | 10.1 |
| 18:0 | 10.2 | 10.2 | 11.5 | 12.0 | 12.9 | 12.6 |
| 18:1 | 19.6 | 20.9 | 21.4 | 22.1 | 18.7 | 20.0 |
| 18:2 | 28.8 | 32.7 | 18.6 | 20.1 | 15.8 | 18.4 |
| 18:3 | 2.8 | 2.6 | 2.0 | 2.2 | 1.8 | 2.0 |

TABLE IV

*Contents of Major Protein-Bound Monosaccharides (μg/mg Protein and μg/mg Phospholipid, Respectively) in Total Particulate Material and Desmosome-Tonofilament-Enriched Fractions (MDT and D) from Stratum Spinosum of Calf Snout*

| | Total particulate fraction | MDT fraction | D fraction |
|---|---|---|---|
| Sialic acid | 0.70; 14.5 (2.3)* | 1.10; 33.3 (3.5)* | 0.60; 22.2 (1.9)* |
| Mannose | 1.30; 27.0 | 1.80; 54.5 | 1.45; 53.7 |
| Glucosamine | 1.95; 40.5 | 2.00; 60.6 | 2.20; 81.5 |
| Galactose | 1.50; 31.2 | 1.55; 47.0 | 1.25; 46.3 |
| Glucose‡ | 1.55; 32.2 | 1.65; 50.0 | 1.70; 63.0 |
| Fucose | ≤0.1; ≤2.1 | ≤0.1; ≤3.3 | ≤0.1; ≤3.7 |

* Values in brackets give nm/mg protein.

‡ Mean values given for glucose have been corrected for nonspecific contamination, i.e., the amount of glucose not correlated with total protein as determined from analyses using variable amounts (from 0.25 to 3 mg) of protein and extrapolation to zero concentration.

when expressed on a protein weight basis (Tables I and IV). Since, however, this merely reflects the inclusion of large amounts of nonmembranous, apparently not carbohydrate-linked proteins, namely the tonofilaments, an expression of these determinations on a phospholipid basis seems more sensible. As can be seen from Table IV, both the contents of carbohydrates and their specific pattern, when expressed on a phospholipid weight basis, are similar to the corresponding determinations in plasma membrane fractions from various other tissues and cell types (18). On the other hand, our values are much lower than the content of about 2.8 mg of carbohydrates per mg phospholipid reported in the citric acid-treated and tonofilament-detached calf snout desmosome fractions by Skerrow and Matoltsy (54, 55). This difference might be explained by either the removal of proteins of the one or the other class during the specific isolation procedure or the retention of sucrose in the material. The relatively high contents of sialic acid and galactose (Table IV) again emphasize the plasma membrane character of the desmosomal membrane plaques, although it should be noted that fucose and (N-acetyl-)galactosamine have been observed only in trace amounts. A similarly high content of sialic acid has also been found by Skerrow and Matoltsy (reference 55; their reported value would represent about 19 μg/mg phospholipid). The significance of the glucose consistently found in the analyses remains somewhat questionable (for discussion, see reference 18), even after correction of the data for glucose amounts not correlated with protein mass.

MAJOR POLYPEPTIDES: The proteins present in the various desmosome-tonofilament fractions are characterized by the predominance of five polypeptide bands (Fig. 6) which in SDS-polyacrylamide gel electrophoresis have relative mobilities corresponding to apparent molecular weights of about 68,000 (band 1), 60,000 (band 2), 58,000 (band 3), 51,000 (band 4), and 48,000 (band 5). These components are present in relative molar amounts of approx. 2:1:1:2:2. From our subfractionation experiments, it is obvious

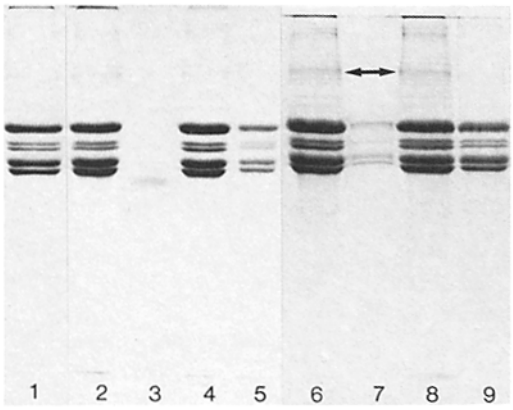


FIGURE 6 Major polypeptides of MDT (slot 1) and D fractions separated as SDS-complexes by electrophoresis in 10% polyacrylamide gels and stained with Coomassie Blue. Slot 1 illustrates the predominance of the five major polypeptides of the tonofilaments. Slot 2 shows the polypeptides of the residual material of the D fraction obtained after treatment with Triton X-100, in comparison with the corresponding patterns of the untreated D fractions (slots 4 and 5). Slot 3 shows ovalbumin as reference. In slots 6–9 the specific residues (slots 6 and 8) and supernatant fractions (slots 7 and 9) are presented, which are obtained after treatment of D fractions with moderately alkaline (pH 10.2) 6 M urea solutions (slots 6 and 7) or sodium citrate buffer (pH 2.6; slots 8 and 9; for details, see Materials and Methods). The major polypeptide bands are numbered 1–5 (from top). Note that components 2 and 3 are present in lesser amounts than components 1, 4, and 5. Only limited amounts of the tonofilament material can be solubilized from D fractions by both acidic and alkaline-urea treatments. In the residues of both extractions, material of lower electrophoretic mobility is obviously somewhat enriched (the double-headed arrow denotes a component of an apparent molecular weight of ~130,000).

that these major polypeptide bands represent the constituents of the tonofilaments. They are resistant to treatments with high and low salt concentrations and with moderately active detergents such as Triton X-100 and 1% deoxycholate (Figs. 6 and 7). In both the "heavy subfraction" from MDT and the tonofilament fragment fraction (Fig. 5), these five bands are the only prominent ones. When the polypeptides of these fractions are compared to those of prekeratin preparations from stratum corneum of bovine hoof, four of the five tonofilament components comigrate with polypeptide bands of prekeratin (Fig. 7). The relative intensities of the bands, however, are different between the desmosome-tonofilaments and the prekeratin (Fig. 7). Most strikingly, however, the polypeptide(s) of the largest minor band of prekeratin migrates much faster (approx. mol wt 63,000) than bovine serum albumin (Fig. 7), in contrast to the largest polypeptide band 1 of desmosome-tonofilaments (approx. mol wt ~68,000). We have noted components comigrating with actin in any of the desmosome-enriched fractions or in the tonofilament fragments. A component that might correspond to polypeptide band 6 of the prekeratin separations of Steinert et al. (62–64) has only sporadically been noted in our gels.

Among the minor components seen by SDS-

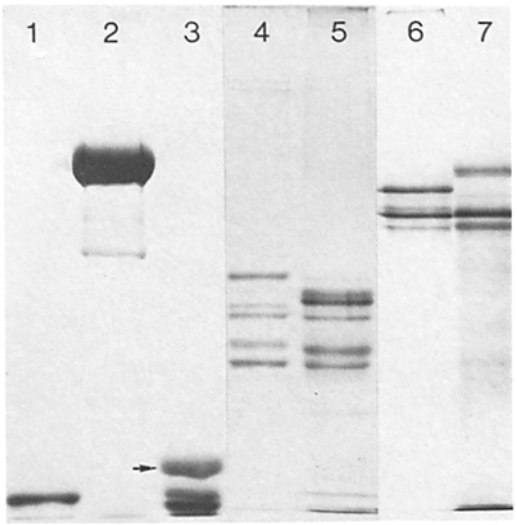


FIGURE 7 Polypeptide patterns of extracted desmosome-tonofilament fractions from calf muzzle (fraction D in slots 3, 4, and 7) and bovine prekeratin (slots 5 and 6) as revealed after complexing with SDS and separation by gel electrophoresis with different concentrations of polyacrylamide: 5% (slots 1–3; references shown are tubulin, slot 1, and myosin from rabbit skeletal muscle, slot 2), 7.5% (slots 4 and 5), and 15% (slots 6 and 7, 15% gels were performed in a different buffer system, for details, see Materials and Methods). Note the absence of considerable amounts of components with electrophoretic mobilities lower than that of band 1 of the tonofilament-polypeptide pattern (component 1 is indicated in slot 3 by the arrow). Note also that the largest polypeptide band of prekeratin migrates significantly faster than bovine serum albumin, in contrast to band 1 of the desmosome-tonofilament fractions. The correspondence of some bands as well as the difference in the largest polypeptide between the tonofilaments and those of prekeratin from stratum corneum is seen in two different gel systems (slots 6 and 7; cf. references 34 and 68; see Materials and Methods).

polyacrylamide gel electrophoresis of MDT and D fractions (but not in tonofilament fragments) are two faint bands of components with relatively high mobilities, corresponding to molecular weights of 10,000–16,000 (Fig. 6), and a series of bands with mobilities lower than that of band 1 of the above described five major tonofilament polypeptides. The approximate apparent molecular weights of these larger components are 78,000, 88,000, 110,000, 130,000 (this band is denoted by the arrow in Fig. 6), 145,000, 210,000, and 230,000. Such bands with electrophoretic mobilities lower than that of band 1, have been barely, if at all, detected in the high salt- and detergent-extracted fractions, not even in gels containing lower concentrations of acrylamide (Fig. 7, slots 1–3). While the major bands 1–5 are completely negative in PAS-stained gels, a PAS-positive though rather faint reaction has been noted in some of the less migrating components, particularly in that with an apparent molecular weight of 130,000. This observation corresponds to the finding of Skerrow and Matoltsy (55) of two major PAS-positive bands of similar mobilities in tonofilament-free, acid-treated desmosome plaques from calf snout and suggests that these components represent true membranous glycoproteins. Usually, some enrichment of the minor, putatively membranous components has been observed in the MDT and D fractions extracted with alkaline buffers containing 6 or 8 M urea or acidic sodium citrate buffer (the residues obtained after such extractions are shown in slots 6 and 8 of Fig. 6).

Contents of cytochromes have also been examined. Significant though relatively small amounts of both *a*-type (0.7 mol/mg protein) and *b*-type (1.6 pmol/mg protein) are found in the total particulate material. However, the fractions MDT and D do not contain appreciable amounts of cytochromes (<0.4 pmol/mg protein for both types of cytochromes), nor do they show detectable cytochrome oxidase activity, indicative of the absence of contaminants of endomembrane and mitochondrial origin.

## DISCUSSION

The isolation procedure described in this article provides an alternative strategy for isolating desmosomal material from bovine muzzle epidermis and avoids the use of acidic solutions as described by Skerrow and Matoltsy (54). The yield of this rather simple method is satisfactory (~20% of total cell protein is recovered in the MDT fraction), and the preservation of desmosomal structure is good. However, in contrast to the method of Skerrow and Matoltsy (54), our procedure results in a fraction of desmosomes still associated with the desmosome-attached tonofilaments. Thereby, this procedure also allows the separation of the desmosome-attached tonofilament from other cytoplasmic filaments.

Many authors seem to consider that desmosome-attached tonofilaments are identical in architecture and composition to "free," cytoplasmic intermediate-sized filaments, including the prekeratin filaments of later stages of epidermal differentiation, both having a prekeratin-like nature (references in Introduction and 9, 23, 35). This assumption, however, has been based on only some structural similarities of these filaments and not on analyses of separated desmosome-associated tonofilaments. Isolated desmosome-attached tonofilaments have the same width (7–11 nm) as prekeratin filaments and other types of intermediate-sized filaments and also often show a hollow core (see references above and 15, 20, 26, 70). Our biochemical results indicate that the desmosome-attached tonofilaments of epidermis, but probably also of other epithelia, consist of five major polypeptide components which occur in a defined stoichiometry. The sizes of four of these tonofilament polypeptides are identical to those of major polypeptides of prekeratin but the largest polypeptide is different in both types of filaments. In addition, the resistance of tonofilaments to a variety of treatments is similar to that of prekeratin filaments. Brysk et al. (4) have reported that a rabbit antibody raised against a major polypeptide (~58,000 mol wt) from rat epidermis reacts with both cytoplasmic prekeratin-containing filaments and desmosome-attached tonofilaments of epidermal cells. Franke et al. (20) have recently demonstrated that guinea pig antibodies against bovine hoof prekeratin not only react with epidermal prekeratin filaments but also specifically decorate desmosome-associated tonofilaments and elaborate cytoplasmic arrays of bundles of 6–11 nm thick filaments present in a diversity of epithelioid cells (cf. reference 47). Thus, it seems now legitimate to conclude that the desmosome-attached tonofilaments represent a special form of filaments that contain prekeratin-like polypeptides. Prekeratin filaments differ from tonofilaments in at least one major polypeptide and in the molar ratios of their polypeptide constituents. In discussing such differences, however, one should keep in mind

the discrepancies reported in the patterns of major polypeptides of prekeratin preparations from different tissues, from different species, and in different laboratories. For example, Skerrow and Matoltsy (references 53, 56; cf. 40) have found three major polypeptides (two of 60,000 and one of ~72,000 mol wt) in epidermal prekeratin from bovine muzzle whereas Baden and co-workers (e.g., references 1, 37, 38) have reported four major bands (apparent molecular weights: 45,000, 47,000, 60,000, and 67,000) in the same material. Steinert and co-workers (62–64) have described, in very detailed studies of prekeratin from bovine muzzle and hoof, the existence of seven polypeptides, present in six bands, with apparent molecular weights of 47,000, 49,000, 54,000, 56,000, 58,000, and 60,000 (two components). Reports of polypeptides present in other preparations of prekeratin or of the corresponding low-sulfur-proteins from keratin have shown further different polypeptide patterns (e.g., references 4, 9, 21, 26, 38; cf. 8, 10). Our prekeratin polypeptide patterns and the apparent molecular sizes are similar to those of Steinert and colleagues (62–64) but show the presence, in the desmosome-associated tonofilaments, of a larger polypeptide of ~68,000 mol wt that seems to replace the largest component observed in the prekeratin from stratum corneum of bovine hoof (63,000 mol wt in our study, 60,000 mol wt in references 62–64). In addition, the studies of Steinert et al. (64) on in vitro assembly have shown that not all the major polypeptides contained in prekeratin are required to form prekeratin-like 7–10-nm filaments (see also reference 37).

A relationship of the desmosome-attached tonofilaments to other classes of intermediate-sized filaments (neurofilaments, the 10-nm filaments of muscle cells, the 6–11-nm filaments present in various cells, particularly in the drug-induced perinuclear caps, etc.) is suggested by the structural similarities, by a similar resistance to a variety of extraction procedures, by the composition by one or several major polypeptides in the 48,000–68,000 mol wt range, and by immunological cross reactions as reported by some authors (e.g., references 3, 6, 15, 20, 24, 36, 47, 52, 57, 61, 66). However, whether such a relationship exists in molecular and/or functional terms is not clear.

The analysis of gap junction complexes isolated from hepatocytes has shown that these represent a paracrystalline arrangement of only one major protein, connexin (for references see Introduction). As to the contents of lipid components in such gap junctions, relatively little is known which perhaps is also a result of the specific isolation procedures that invariably involve the use of detergents. Our data suggest that the lipid composition of desmosomes is complex and shows a typical plasma membrane-type pattern of phospholipids, cholesterol, and gangliosides. Whether or not the observed differences in lipid composition, such as a slightly increased degree of fatty acid saturation, particularly the reduced content of linoleic acid, are important in locally modifying the membrane structure and fluidity remains to be clarified. Freeze-fracture studies of desmosomes have shown clusters of densely packed intramembranous particles not arranged in a paracrystalline form (e.g., references 12, 31, 46). Skerrow and Matoltsy (54, 55) have described 7 major and 17 minor polypeptide bands, including two major PAS-positive bands, in their desmosomal fraction from which the tonofilaments had been completely extracted (this type of preparation presents a clearer pattern of the nontonofilamentous desmosomal polypeptides). Our study confirms their findings and adds to the data indicating that the desmosomal membrane is heterogeneous in protein composition and contains a series of different membrane-bound proteins and glycoproteins. The carbohydrate composition of the desmosomal glycopeptides is also rather complex and includes sialoglycoproteins. Most likely the strongly PAS-positive components described by Skerrow and Matoltsy (54) represent a major portion to the sialoglycoproteins of the desmosome membranes.

It is obvious that the desmosome membrane proteins are associated in a mode that is resistant to rather aggressive dissociation treatments such as high salt concentrations, various nonionic (Triton X-100) and ionic (sodium deoxycholate) detergents, moderate acidic and alkaline solutions, and high concentrations of urea. Whether the desmosomal plaque is involved in this local differentiation of membrane components, perhaps similar to the dark-staining layer associated with the cornified cell membrane envelope described in terminal keratinization of epidermal cells (e.g., reference 23), is under examination.

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