INTERMEDIATE (SKELETIN) FILAMENTS IN HEART PURKINJE FIBERS

A Correlative Morphological and Biochemical Identification with Evidence of a Cytoskeletal Function

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ABSTRACT

Cow Purkinje fibers contain a population of free cytoplasmic filaments which consistently differ in ultrastructural appearance from actin and myosin filaments, irrespective of preparation technique. The fixation and staining techniques, however, influenced the filament diameter, which was found to be 7.4–9.5 nm for filaments in plastic-embedded material, and 7.0 nm in cryo-sectioned material, thus intermediate as compared to actin and myosin filaments. Cross-sectional profiles suggested that the intermediate-sized filaments are composed of four subfilaments.

To provide a basis for further biochemical investigations on the filaments, extraction procedures were carried out to remove other cell organelles. Electron microscopy showed that undulating bundles of intermediate filaments converging towards desmosomes still remained, after the extractions, together with Z-disk material. In spite of the extensive extraction, the shape of the individual cells and the assemblies of cell bundles remained intact. This confirms that the intermediate filaments of cow Purkinje fibers together with desmosomes do in fact have a cytoskeletal function.

On account of (a) the cytoskeletal function of the filaments, (b) the similarities to the smooth muscle "100-Å filament" protein subunit skeletin, and (c) the inadequate and confusing existing terminology, we suggest that the filaments be named "skeletin filaments."

KEY WORDS   heart conduction system · Purkinje fibers · intermediate filaments · cytoskeleton · skeletin · scanning electron microscopy · transmission electron microscopy

In addition to the ordinary contracting myocardium, the mammalian heart contains a system of muscular origin, specialized for impulse initiation and conduction. The Purkinje fibers, the terminating ventricular part of this system, can be distinguished physiologically from ordinary cardiocytes, e.g., by their conduction properties and relative resistance to anoxia (26, 78, 79, 84). On a morphological basis, however, the Purkinje fibers are, in some species (e.g., rabbit and man), difficult to identify (73, 77, 81, 89), while in others (e.g., cow and sheep) the great cell diameter and the high proportion of light cytoplasm, which is

devoid of myofibrils and mitochondria but rich in glycogen and filaments, make such a distinction easy (10, 49, 50, 73, 79, 80, 86).

The richness in glycogen of Purkinje fibers was early noted (46). Specific characteristics of the conducting tissue glycogen have been reported and related to the resistance to anoxia (see reference 78). The filamentous component, on the other hand, is a poorly understood organelle. There is no general agreement concerning its subcellular organization (cf. references 1, 10, 11, 52, 54, 76, 77, 83, 89). The reported diameters of the filaments range from 4 to 10 nm (2, 10, 52, 77-79, 89), and the fine structure is unknown. Biochemically, we have suggested that the filaments are composed of a subunit of 55,000 daltons mol wt (82). Lack of experimental evidence has stimulated speculation on the function of the filaments. The filaments have thus been discussed in relation to the conducting properties (89) and to a proposed embryonal character of the Purkinje fibers (10, 54, 89). Further, the filaments have been considered as having a relation to the organization and binding of glycogen (78, 79), to the synthesis and growth of the myofibrils (77), and to perform a structurally supporting function (52, 79, 89). Also, they have been considered capable of motility (54).

In the present paper, we report an investigation into the fine structure of the cytoplasmic filaments in cow Purkinje fibers. Special attention has been paid to the influence of various ultrastructural preparation techniques on the dimensions of the filaments. To clarify the biochemical and structural identity of the cytoplasmic filaments, we have extracted isolated bundles of Purkinje fibers and, in parallel, examined the effects with biochemical and morphological methods. Evidence is presented that the filaments are composed of a 55,000-dalton subunit and that they perform a cytoskeletal function.

Parts of this work have been presented in abstract form (20, 21).

MATERIALS AND METHODS

False tendons from the ventricles of cow hearts were excised immediately after stunning.

Some false tendons were mounted on glass frames or cork plates in a slightly stretched state and immersed in 2.5% ice-cold glutaraldehyde in isotonic oxygenated Tyrode's solution (pH 7.3-7.4) (60). Specimens were then processed in parallel for conventional plastic sectioning and cryo-ultramicrotomy and analyzed by transmission electron microscopy (see below).

Other false tendons were transferred in a relaxed state to either a relaxing buffer with pyrophosphate (pH 6.8) (23) or a 0.2 M phosphate buffer (pH 7.2), or to Tyrode's solution. Purkinje fiber bundles were mechanically separated from their connective tissue sheaths under the dissecting microscope while still in buffer (80). Selected undamaged cell columns were extracted (see below) and processed for light microscopy (LM), transmission electron microscopy (TEM) and scanning electron microscopy (SEM). Controls were kept in buffer for periods of time corresponding to the extraction time and subsequently examined in the same way. Biochemical characterization by sodium dodecyl sulfate (SDS)-poly-acrylamide gel electrophoresis was carried out in parallel (90).

Extraction of Bundles

Extruded Purkinje fiber bundles were immersed in 0.2% Triton X-100 (72) either in relaxing buffer (23), or in 0.2 M phosphate buffer, or in a low ionic strength solution (60 mM KCl, 20 mM imidazole, 1 mM cysteine, 2 mM EDTA, and 10 mM ATP) (J. V. Small, personal communication) and continuously stirred for 4 h with a change of medium after 2 h. The bundles were then transferred to a high ionic strength solution (0.6 M KCl, 20 mM imidazole, 1 mM cysteine, 2 mM EDTA, and 10 mM ATP). Subsequently, bundles were immersed in the low ionic strength solution. Extraction was performed by alternating solutions for 4 h with change of medium every hour. All procedures were carried out at +4°C.

Light Microscopy of Bundles

Extracted bundles and controls were observed and photographed immersed in buffer in a Leitz Orthoplane microscope with transmitted or incident light.

Transmission Electron Microscopy

CONVENTIONAL PLASTIC SECTIONING

FALSE TENDONS: After fixation in glutaraldehyde (G) for 2 h, the false tendons were cut into 1-mm^3 blocks while rinsing in fresh Tyrode's solution. Some tissue blocks were postfixed in 1% osmium tetroxide (O) in the same buffer (2 h, +4°C). After fixation procedures, the specimens were dehydrated in graded acetone series and either stained en bloc or directly embedded in Vestopal W (Chemische Werke Hüls, Marl, West Germany) without block staining. Block staining was performed at +60°C in ethanol solutions of 1% phospho-tungstic acid (PTA) or 2% uranyl acetate (UAc) for 2 h (43). A brief outline of the fixation and staining procedures follows:

$$\text{tissue} \rightarrow G \underset{\nwarrow}{\overset{\nearrow}{=}} O \overset{\text{UAc}}{\underset{\text{PTA}}{=}} \text{Vestopal W}$$

In this way, six different fixation-staining combinations were obtained, i.e., specimens treated with glutaraldehyde alone (G), glutaraldehyde and osmium tetroxide (GO), glutaraldehyde with UAc block staining (GUAc), or with PTA block staining (GPTA), glutaraldehyde and osmium tetroxide with UAc block staining (GOUAc) or with PTA block staining (GOPTA).

Embedded tissue blocks were oriented for sectioning parallel or perpendicular to the long axes of the false tendons. Survey sections of 1 μm thickness were examined in a light microscope after toluidine blue staining. Thin sections were obtained from selected areas of proper alignment on an LKB Ultrotome I or III equipped with a glass or a diamond knife. The sections were transferred to Formvar-coated one-hole or mesh copper grids or to gold grids. Sections on copper grids were stained with uranyl acetate and lead citrate in a standardized way (69).

EXTRUDED BUNDLES: Controls and extracted bundles were fixed, postfixed, dehydrated, and embedded, as were the false tendons. Block staining was performed on some specimens. Ultrathin cross sections and longitudinal sections of the bundles were mounted on Formvar-coated copper or gold grids. Section staining was performed as for *in situ* fixed bundles (see above).

CRYO-ULTRAMICROTOMY

False tendons were stabilized with 2.5% glutaraldehyde in Tyrode's solution (30 min, +4°C). After stabilization, the Purkinje fiber columns were mechanically extruded (80). Selected undamaged specimens were antifreeze-treated in 10, 20, and 30% glycerol in buffer (10 min in each solution) at room temperature. The columns of the Purkinje fibers were then placed on the top of cleaned copper specimen holders. Excess solution was removed with the aid of a piece of filter paper, and the specimen and its holder were quench frozen in liquid Freon-12 chilled with liquid nitrogen and then transferred to liquid nitrogen for storage until sectioning.

Sectioning was carried out with an LKB Cryo-Kit and 50% dimethyl sulphoxide in water as the trough liquid. The longitudinal axis of the Purkinje fiber bundle was oriented parallel or perpendicular to the knife edge. Hydrated sections were contrasted with ammonium molybdate (2%, pH 7.3) by a technique similar to that used for negative staining of isolated components. Contrasted sections were air dried and stored dry at room temperature (cf. references 67 and 68).

EXAMINATION

Plastic sections and cryo-sections were examined in a Philips EM 300 equipped with an anticontaminating device at 80 kV.

FILAMENT DIAMETER MEASUREMENTS

For filament diameter measurements, randomly selected areas with exactly cross-cut myofibrils at the A-I junction level and adjacent cytoplasmic filaments were photographed at an electron optical magnification of 40,000. Calibration of the magnification was performed with a carbon replica cross-grating (2,160 lines/mm). Size measurements of positively identified actin, myosin, and cytoplasmic filaments were performed with a Zeiss TGZ 3 particle analyzer at a final magnification of 400,000 on three to five cells of each preparation. A total of 100 filaments of each type were measured in each of the fixation-staining combinations (except GPTA, see Results) in each of five cows.

In cryo-preparations, it was not possible to obtain measurements on cross-cut filaments. Instead, the diameters of cytoplasmic filaments were recorded from longitudinal sections. 100 filaments were analyzed by the same procedure as described above.

FILAMENT GROUPING

In micrographs of the peripheral (i.e., adjacent to myofibrils) and central cytoplasm, relatively well-defined groups of cytoplasmic filaments could be identified. The number of filaments in all groups identified in 10 micrographs (five central and five peripheral) from each cow were recorded. Electron optical magnification was 20,000.

Scanning Electron Microscopy

Extruded cell bundles were fixed and postfixed as for TEM. They were then prepared for SEM by the critical point method. After dehydration in an ascending series of ethanol (70, 80, 90, 95, and 100% ethanol) and in 70-30, 40-60, 30-70, and 10-90% ethanol-amyl acetate and 100% amyl acetate (10 min in each solution), the specimens were transferred directly from the amyl acetate to a Polaron Critical Point Drying Apparature E 3,000 (Polaron Instruments, Inc., Line Lexington, Pa.) and dried using liquid carbon dioxide. The bundles were then coated with gold to a thickness of ~25 nm in a vacuum of 10^{-3} Pa during synchronized rotation and tilting. Examination was carried out in a Cambridge Stereoscan S4 scanning electron microscope operated at an accelerating voltage of 20 kV and a beam current of 190 μA.

SDS Gel Electrophoresis

Parts of the isolated Purkinje fiber material were—for each of the extraction steps—taken for biochemical analysis and simultaneously for morphological examinations. Cells and bundles were dissociated in 10 mM phosphate-buffered saline (pH 7.0) with 1% SDS-1% β-mercaptoethanol at +60°C for 5 min and then left overnight at +25°C. 7.5% polyacrylamide gels were prepared according to Weber and Osborn (90). Electrophoresis was performed at a constant current of 8 mA/gel. Gels were stained with Coomassie Brilliant Blue.

By the use of appropriate reference proteins (see reference 82), molecular weights of the protein components were determined.

RESULTS

Internal Structure of In Situ Fixed Material

DISTRIBUTION OF CYTOPLASMIC FILA-
MENTS: In electron micrographs of Purkinje
fibers there was a constant finding of large num-
bers of cytoplasmic filaments. The filaments con-
stituted the greatest part of the electron-transpar-
ent cytoplasm, intermingling with myofibrils, mi-
tochondria, lipofuscin granules, and other cell
organelles (Figs. 1 and 2). The amount of fila-
ments varied in different cells, which could not be
related to the localization of the cell within the
bundle or differences in cell morphology in other
respects.

The filaments were often arranged in bundles
parallel to the myofibrils. Bundle formation was
most constantly found in areas loaded with fila-
ments, while in other areas the filaments were
more randomly dispersed. The number of fila-
ments per bundle was highly variable, averaging
31 ± 21 (mean ± SD, n = 1,500), and was
similar for central and peripheral areas. Bunches
of filaments converged into desmosomes and into
electron-dense material (so-called hemidesmo-
somes) at the plasma membrane facing the sur-
rounding connective tissue (Figs. 3, 4, and 12a).
At the myofibrillar Z-disk level, tufts of filaments
were regularly seen (Fig. 5).

FILAMENT DIAMETER MEASUREMENTS:
The results of diameter measurements on cross-
sectioned, plastic-embedded material are listed in
Table I and illustrated in Fig. 6. The most benefi-
cial preservation for measurements and study of
general morphology was obtained in GOUAc
specimens (cf Fig. 7). In the GPTA specimens, on
the other hand, the filaments could not be mea-
sured with accuracy as they were irregular in
outline and sometimes adhering to each other.
Therefore, values for GPTA filaments are not
reported.

In longitudinally cryo-sectioned and negatively
stained Purkinje fibers, a good resolution of the
structures in myofibrils and in mitochondria was
obtained. Fine cross-periodicities in the A band
and strong M lines were the most apparent details
in the myofibrils (Fig. 8). A distinct difference in
diameter between the myofibrillar actin and
myosin filaments vs. the free cytoplasmic filaments
was confirmed. In very thin cryo-sections the
intermediate-sized filaments appeared as a nega-
tively stained monolayer of isolated filaments (Fig.

9). Measurements on such longitudinal filaments
revealed a diameter of 7.0 ± 0.8 nm (mean ±
SD, n = 100).

FINE STRUCTURE: Cross-sectioned, plas-
tic-embedded filaments exhibited square, round,
or oval profiles (Fig. 10). Some filaments ap-
peared to have a less dense central core while
others were of even granularity. Within a cross-
sectioned filament profile an uneven distribution
of stain was noticed, suggesting subunits. The
definite number of subunits was difficult to deter-
mine. However, Markham rotation of intermedi-
ate filament square profiles enhanced the impres-
sion of four subunits (Fig. 10).

Cryo-sections with filaments negatively stained
showed that the width of the filaments was fairly
uniform. A dense central ribbon parallel to the
lateral margins also suggested the presence of
subfilamentous structures (Fig. 9). Tapering out
into subfilament strands was, however, never
seen. A few filaments exhibited cross-striations at
an interdistance of ~2.5 nm. The length of the
filaments was indeterminate because of the undu-
lating course and superimposition effects. Branch-
ing, side arms, and projections were suggested in
some micrographs but cannot definitely be said to
be present.

### Internal Structure of Extruded Bundles

Purkinje fiber control material was arranged
into cell bundles with the cells adhering tightly to
each other by numerous desmosomes. The regular
arrangement of myofibrils along the inner cell
borders and a central cytoplasm with large
amounts of intermediate filaments was confirmed.
Minute amounts of collagen were adherent to the
periphery of the bundles, occasionally also fibro-
cytes. Morphological features were thus similar to
those of in situ fixed material.

The packing of cells into columns was not
affected by Triton extraction. However, membra-
nous components, i.e., endoplasmic reticulum and
plasma, nuclear, mitochondrial, and Golgi mem-
branes, were solubilized except for the specialized
parts of the plasma membrane. The intermediate
and the myofibrillar filaments were not obviously
affected, though a slight decrease in myosin con-
tent seemed to occur after prolonged immersion
in buffer.

Treatment with low and high ionic strength
solutions resulted in an almost total extraction of
thick and thin filaments. Only Z-disk material,
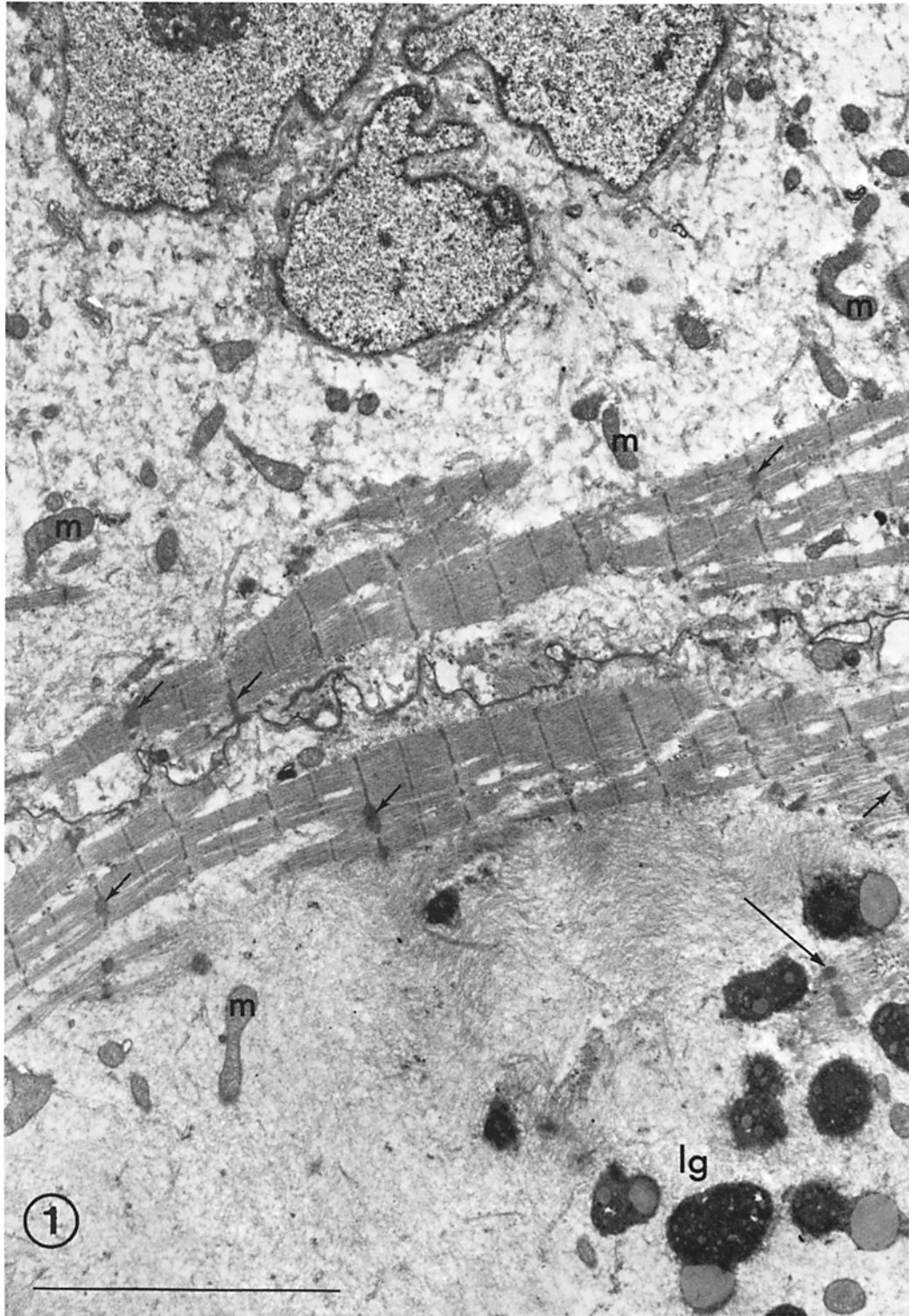
FIGURE 1 Survey micrograph of two longitudinally sectioned Purkinje fibers with peripheral myofibrils containing thickened and irregular Z disks (short arrows). The two cells show a marked difference in intermediate filament content, the upper cell with moderate amounts in addition to several mitochondria (*m*), the lower cell with enormous filament masses following an undulating course. Similar differences in filament loading might explain such histological staining variations as described in reference 58 (Fig. XII: 7, p. 556). Nucleus (upper left), lipofuscin granules (*lg*), and a myofilament-polyribosome complex (see reference 76; long arrow). Bar, 5 μm. × 9,300. (GO)
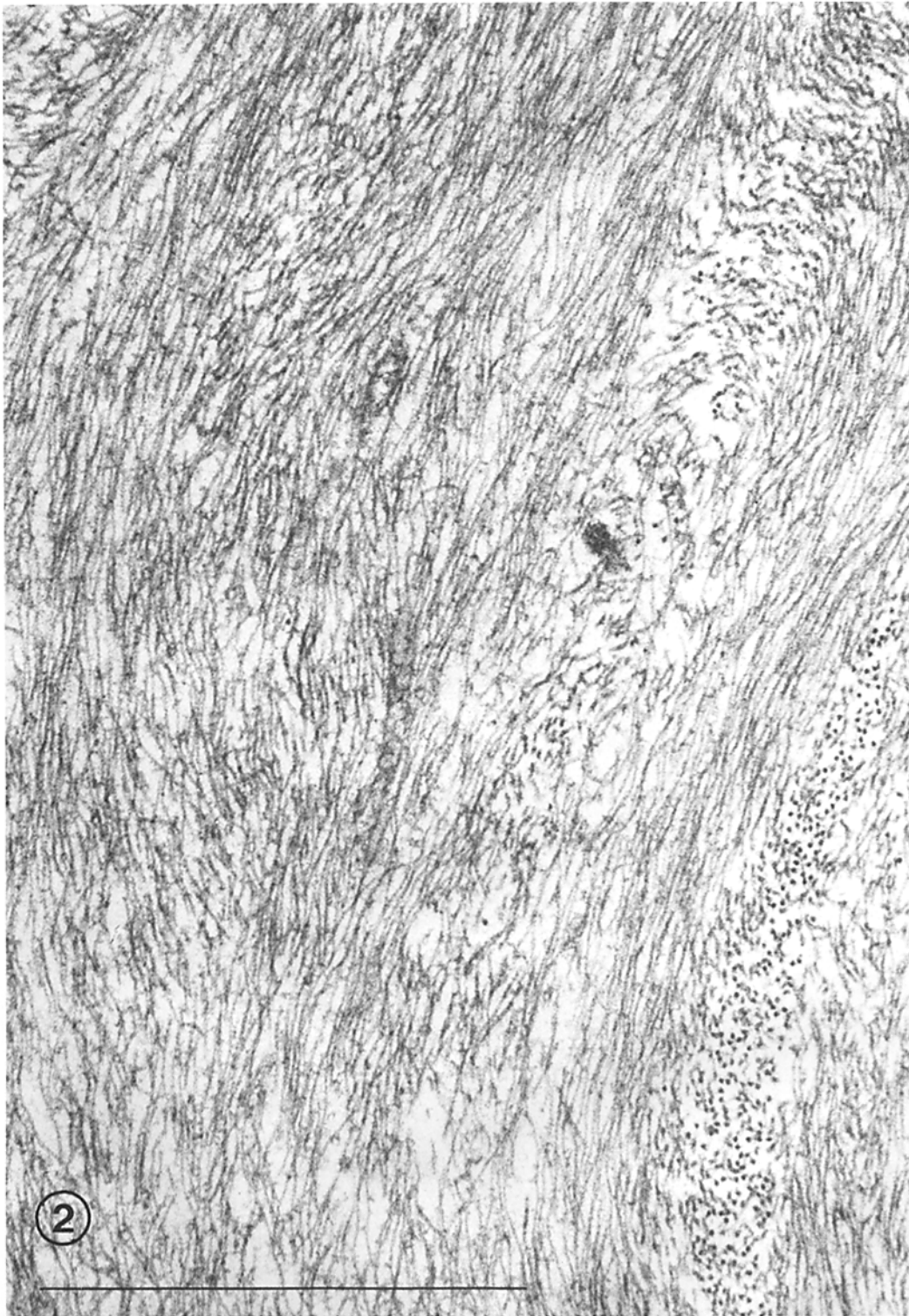
FIGURE 2 Region loaded with intermediate filaments. Both longitudinal and crosscut filaments are seen. Because of the undulating course, their length is indeterminate. Superimposition hampers judgement of whether filaments branch or not. Bar, 1 μm. $\times$ 74,000. (GO)
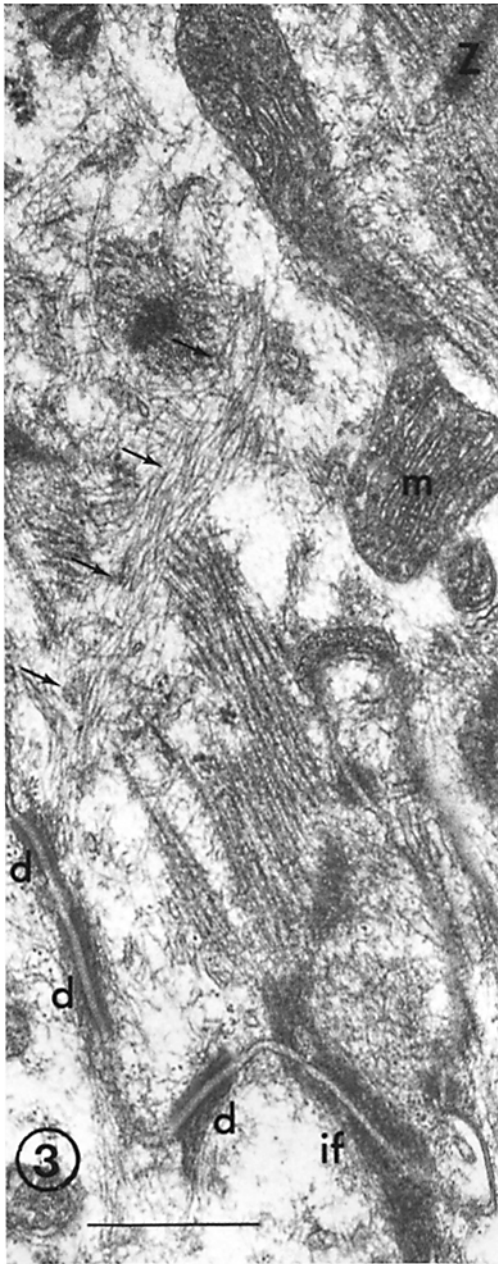
FIGURE 3 Two adjacent Purkinje fibers joined by several desmosomes (d) and an interfibrillary region (if). Bunches of filaments converge into the desmosomes. One bunch (arrows) might possibly interconnect with a myofibrillar Z disk (Z). Mitochondrion (m). Bar, 0.5 μm. × 45,000. (GOUAc)

Surface Topography of Extruded Bundles

LM OBSERVATIONS: Purkinje fiber bundles had a smooth surface with irregular indentations corresponding to cell junctions. Certain subcellular structures, such as nuclei, myofibrils, and lipofuscin granules, could also be detected (Fig. 13).
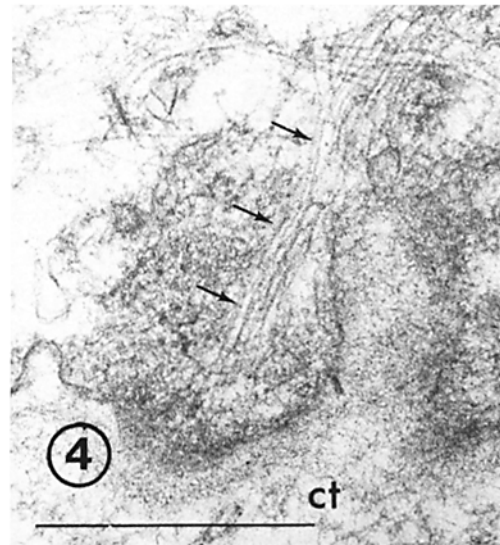
The extraction procedures did not affect the

FIGURE 4 Filament bundle (arrows) converging towards an electron-dense patch — a so-called hemidesmosome — at the cell border facing the surrounding connective tissue sheath (ct) containing collagen fibrils. Bar, 0.5 μm. × 75,000. (GOUAc)
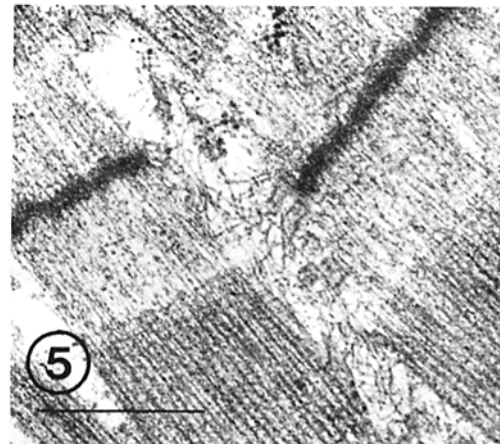

FIGURE 5 Tufts of intermediate filaments in the proximity of adjacent myofibrillar Z disks. Bar, 0.5 μm. × 45,000. (GOUAc)

desmosomes, gap junctions, and nuclear ghosts remained besides the intermediate filaments (Figs. 11 and 12).

TABLE I

Purkinje Fiber Filament Diameters in Relation to Preparation Technique

	A	C	M
G	5.2 ± 0.9	7.4 ± 0.7	10.1 ± 1.4
GUAc	4.8 ± 0.7	7.9 ± 0.7	11.7 ± 1.0
GO	5.5 ± 0.9	8.3 ± 0.7	12.5 ± 1.2
GOUAc	6.3 ± 0.9	8.6 ± 0.9	13.2 ± 1.4
GOPTA	5.9 ± 0.8	9.5 ± 1.1	14.8 ± 1.8

Mean diameters ± SD (nm; n = 500 in each case) of actin (A), cytoplasmic (C), and myosin (M) filaments after glutaraldehyde fixation only (G), glutaraldehyde fixation with uranyl acetate block staining (GUAc), glutaraldehyde fixation with osmium tetroxide postfixation (GO) and uranyl acetate (GOUAc) or phosphotungstic acid (GOPTA) block staining.
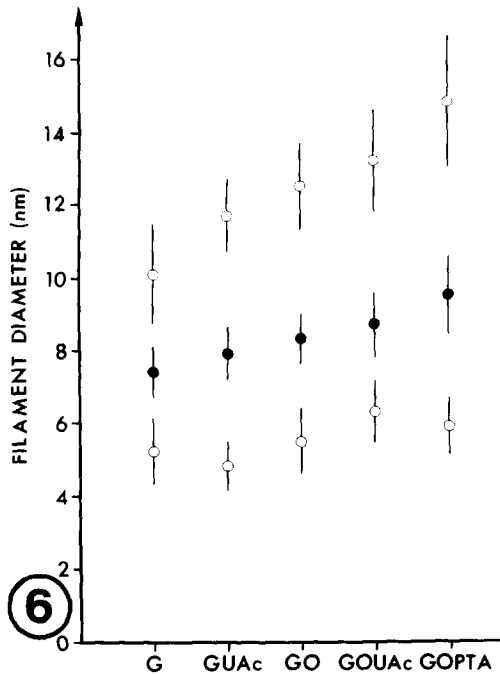

FIGURE 6 Schematic representation of mean diameters ± SD of myosin (O, upper row), intermediate (●), and actin (O, lower row) filaments after different fixation and staining procedures and plastic embedding. n = 500 for each symbol.

shape and assembly of the columns, which remained intact throughout all extractions. Nor could changes in inner structure be observed by means of LM. However, successive extractions seemed to cause a decreased opacity of the bundles.

SEM OBSERVATIONS: Purkinje fiber bundles (up to 15 mm length), extruded from their connective tissue sheaths, consisted of polygonally packed reliefs of different size. These reliefs corresponded to the individual Purkinje cells, and the irregular indentations to the cell borders (Fig. 14a).

After extraction, the three-dimensional arrangement of Purkinje fibers was essentially the same as in untreated bundles. No collapse or shrinkage of the cells or cell bundles occurred in spite of extensive extraction (Fig. 14b).

SDS Gel Electrophoresis

In native Purkinje fibers, myosin heavy chains, α-actinin, a 55,000-dalton protein, actin, myosin light chains, and regulatory proteins were recognized (cf. reference 82). After detergent and salt extractions, the main component was a protein of 55,000 daltons. A minor prominent component had a mol wt of 110,000 daltons (Fig. 15).
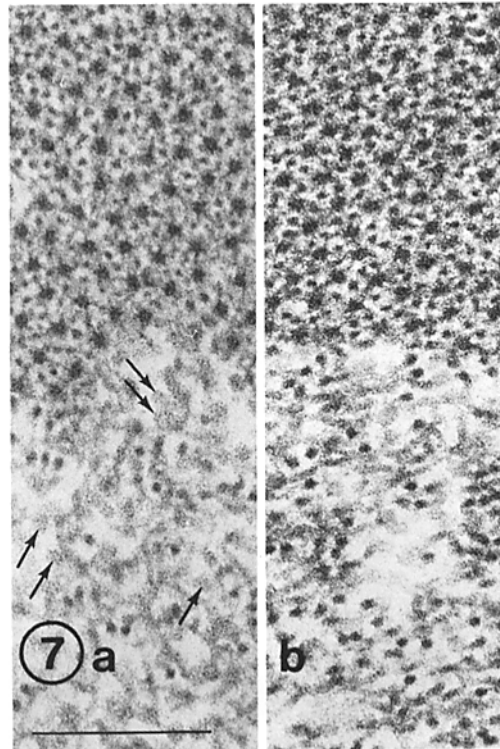

FIGURE 7 Transversely sectioned myofibrils at A-I junction level with hexagonally packed myofilaments and more irregularly oriented adjacent intermediate filaments. Many of the myosin filaments appear hollow. In the GOUAc specimen (b) the contrast is enhanced because of a decreased density of glycogen granules (arrows) as compared to the GO specimen (a). Bar, 0.2 μm. (a) × 120,000 (GO). (b) × 120,000 (GOUAc)
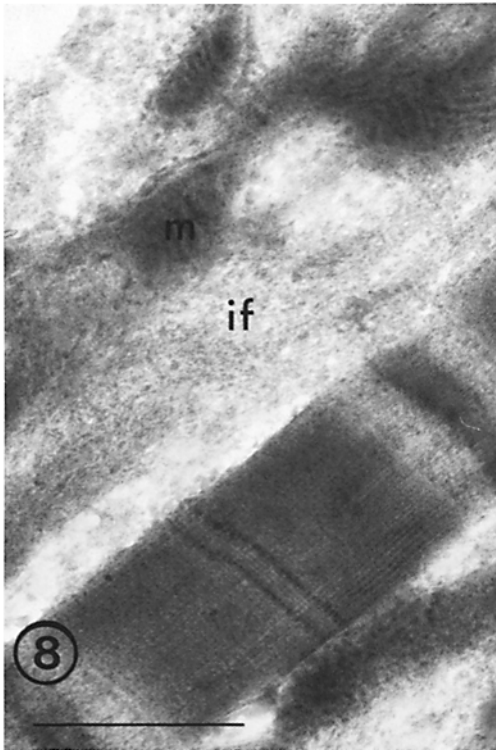
FIGURE 8 Cryo-sectioned and negatively stained Purkinje fibers with longitudinally sectioned myofibril and adjacent intermediate filaments (if). A wealth of fine details is seen in the myofibril. Mitochondrion (m). Bar, 1 μm. × 28,000.

DISCUSSION

Intermediate Filament Structure

Irrespective of preparation, fixation, or staining methods, three subclasses of filaments in cow Purkinje fibers could be distinguished, i.e., actin, myosin, and intermediate filaments. The intermediate filaments comprised a uniform class of filaments as deduced from the measurements carried out. No difference in diameter or fine structure could be seen for filaments anchored to desmosomes or for filaments in the central region of the cell as compared to filaments in the proximity of myofibrils.

The preservation and the diameter of all the filaments were, however, clearly dependent on the different techniques used. A greater filament diameter was seen in block-stained preparations. This was most evident for GOPTA preparations where there probably is a nonspecific interaction between O and PTA, enhancing the size of stained structures (29). In UAc block-stained specimens,

we found the contrast to be greatly enhanced as expected (29, 43). Because of this increased contrast, GOUAc-stained specimens were considered superior for the study of general filament morphology (cf. reference 55).

Regarding the fine structure of the filaments, different cross-sectional profiles were observed in through-focus series. Square profiles might be consistent with four subunits of the filaments. Irregular binding of stain might explain round solid or tubular profiles. Oblique sectioning could explain other profiles observed. Our studies of filaments in cryo-sections — in which the filaments can be expected to be in the most in vivo-like state studied here — also indicate that the filaments are composed of subfilaments.

Biochemical Identity

The content of structural proteins in heart Purkinje fibers has been reported in a previous work. A main protein component of 55,000 daltons was suggested to be derived from the intermediate filaments (82). In the present work, we have, by demembranation and extraction of myofibrillar proteins, obtained a Purkinje fiber residue containing only proteins of 55,000 and 110,000 daltons. As ultrastructural analysis of the residue revealed only intermediate filaments and Z-disk and desmosome material, we concluded that the 55,000-dalton protein constitutes the intermediate filaments. The minor 110,000-dalton component is, we suggest, derived from the Z-disk and the desmosome material. This protein seems likely to be an α-actinin (64, 66, 82). A similar nature for Z disks and desmosomes has been proposed after immunoelectron microscopy studies, by use of antibodies of α-actinin (37, 65, see also reference 40). The protein composition of the Z disk is, however, still a matter of dispute and new proteins are continuously being introduced into the discussion (cf. references 39 and 64).

Cytoskeletal Aspects

A cytoskeletal role of the Purkinje fiber intermediate filaments is confirmed in the present study. In spite of extensive extraction of membranes and myofibrillar proteins, the three-dimensional appearance of individual fibers and columns was the same as in in situ fixed material. Ultrastructurally, the residue is composed merely of Z-disk material and intermediate filaments attached to desmosomes. Signs of a cytoskeletal function were observed also in in situ fixed material, pri-
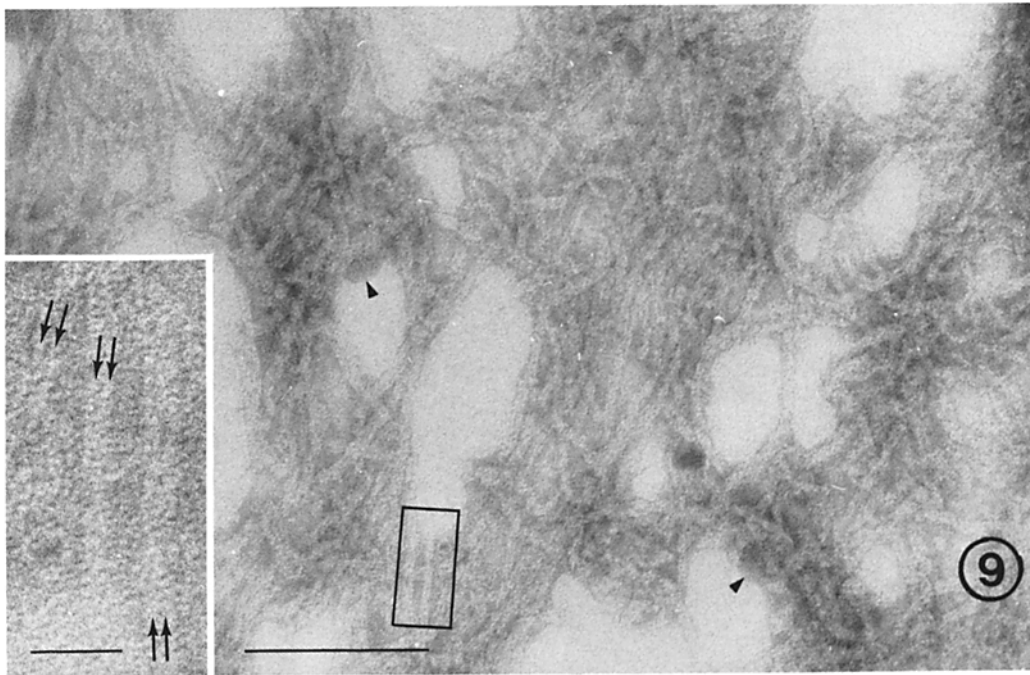
FIGURE 9 Cryo-sectioned intermediate filaments and occasional glycogen granules (arrowheads), negatively stained with ammonium molybdate. Bar, 0.1 μm. × 248,000. *Inset* shows the indicated area in higher magnification. Here the filaments appear to be composed of at least two subfilaments (arrows). Bar, 20 nm. × 600,000.
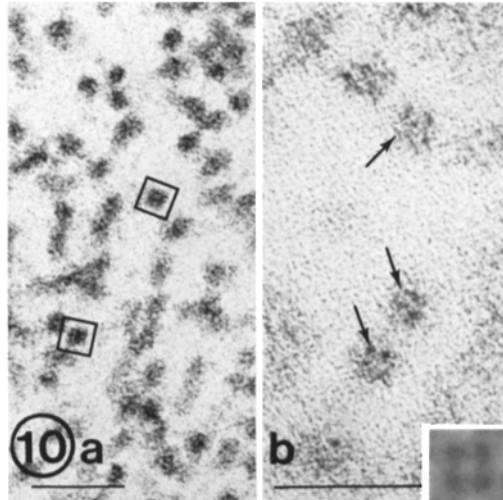

FIGURE 10 Cross-sectional profiles of plastic-embedded intermediate filaments. Some filaments appear to be square (□), others to be round or oval. Most filaments are evenly and densely stained, although, in higher magnification, substructure is suggested (arrows). Square filaments appeared to be composed of four subunits, and a fourfold Markham rotation enhanced this impression (*inset*). (*a*) Bar, 50 nm. × 248,000. (GO). (*b*) Bar, 50 nm. × 400,000. (GOUAc)

marily as filaments attached to desmosomes and hemidesmosomes and filaments running transversely between Z disks. Our results thus substantiate a previous hypothesis based on the ultrastructural demonstration of inter-Z-disk bridge formations and association with intercalated disks (3, 25, 35, 41, 52, 89, see also reference 36).

Why do Purkinje fibers need an extensive cytoskeleton? During heart contraction, the conducting cells are exposed to considerable tension. The integrity of the Purkinje cables is, of course, essential for their conducting properties, and to meet the mechanical stress various specializations should be expected. The intermediate filaments in cow Purkinje fibers are likely to be of great importance in meeting the mechanical strain. Additional supportive structures are probably the numerous desmosomes, the longitudinally arranged myofibrils, and the surrounding connective tissue sheath. The answer to whether there is a direct proportionality between the amounts of desmosomes and intermediate filaments awaits further studies. In species with small amounts of intermediate filaments in the conducting cells (cf. "low differentiation" in references 50 and 86) and/or less well-developed connective tissue
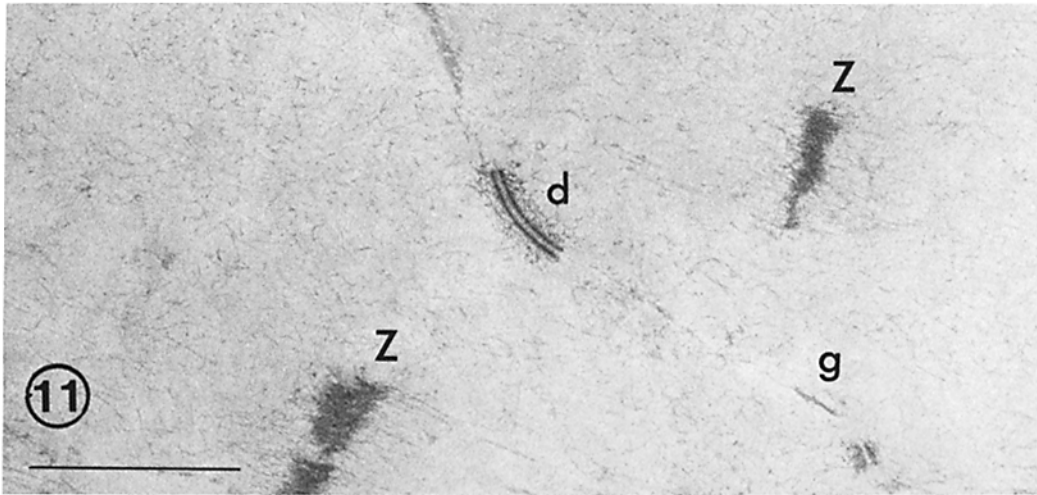
FIGURE 11 Triton- and KCl-treated extruded Purkinje fibers. This survey micrograph reveals residual desmosome (*d*), gap junction (*g*), and Z disks (*Z*), while myofilaments and membranous components have disappeared. Bar, 1 μm. × 28,000. (GO)
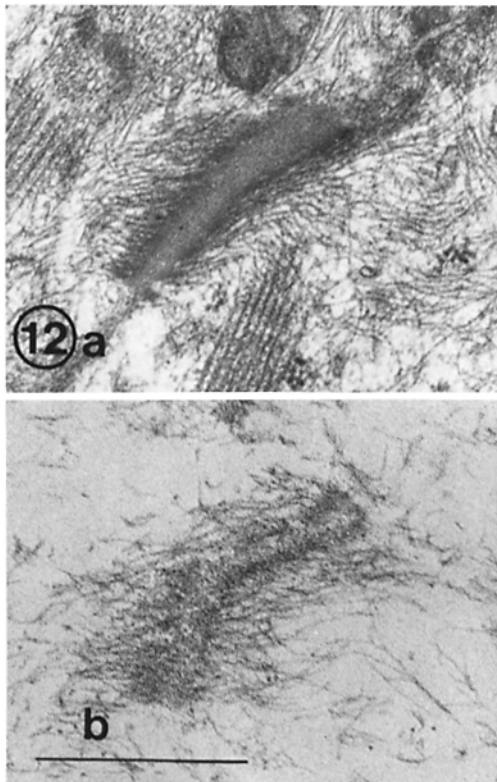

FIGURE 12 Detail of desmosome with inserting filaments. (*a*) GOUAc preparation. (*b*) Triton- and KCl-treated specimen (GO). Bar, 0.5 μm. × 45,000.

sheath, it is suggested that the myofibrils are responsible for a proportionally greater part of the supportive task (cf. reference 83).

The presence of an imposing cytoskeleton in the conducting cells of certain species should, in our opinion, be regarded as a sign of cellular and subcellular differentiation rather than as an embryological remnant (cf. references 7, 52, 54, 89).

Comparison with Other Cell Systems

During recent years, intracytoplasmic nonmyofibrillar filaments have been described in several eukaryotic cells. They have been referred to as "100 Å filaments" (4, 12, 24, 25, 33, 41, 42, 59, 88), "10 nm filaments" (48, 57, 70, 79) or "decefilaments" (17) on account of their approximate diameter. Other authors have found other diameters and consequently named the filaments "9 nm filaments" (93), "80-100 Å filaments" (7), or "110 Å filaments" (87). On account of an intermediate diameter as compared to thick and thin myofilaments, they have also been called "intermediate (-sized) filaments" (5, 8, 30, 31, 55, 56, 77, 85, 92). By comparison with thin (actin) filaments, they have been named "thick filaments" (38). Depending on tissue origin, such terms as "tonofilaments" (e.g. references 28 and 45), "neurofilaments" (e.g. reference 91), "glial filaments" (e.g. reference 91), or "sarcoplasmic filaments" (78) have been designated. Their cross-
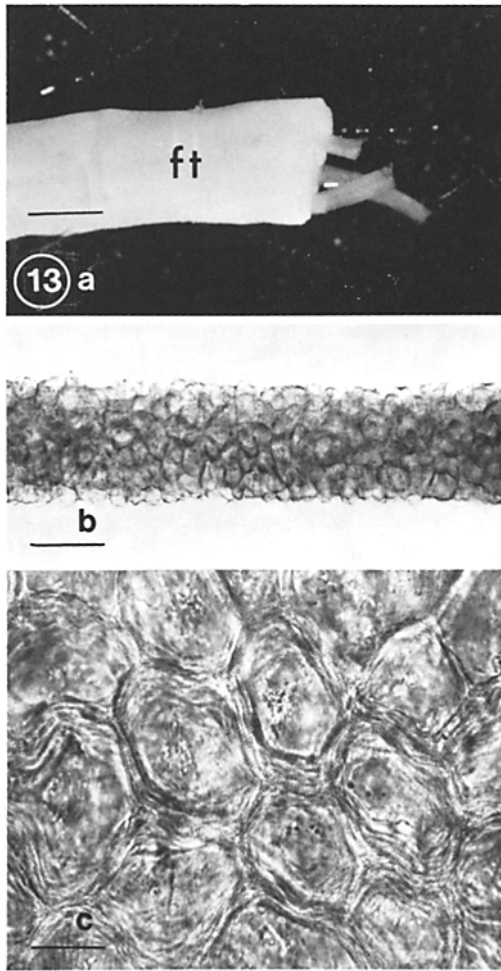
FIGURE 13 The extrusion of Purkinje fiber columns. (a) False tendon (ft) with three partially extruded Purkinje fiber columns (to the right). Bar, 1 mm. × 10. (b) Extruded Purkinje fiber column with irregular indentations corresponding to the Purkinje cell borders. Bar, 0.25 mm. × 40. (c) High magnification of Fig. 13b showing typical bovine Purkinje fibers exhibiting translucent central cytoplasm with lipofuscin granules and peripherally located myofibrils. Bar, 40 μm. × 250.

sectional profile appearance has introduced the terms "round filaments" (44) and "square filaments" (71) and a suggested subcellular origin of the filaments has inspired the term "lentofilaments" (71). A miscellaneous group contains such terms as "beta-filaments" (47), "cytoplasmic filaments" (51, 52, 55, 79, 80, 82), "protofibrils" (54), "fine filaments" (89), or simply "filaments" (1, 27).

In spite of the inconsequent nomenclature, the filaments have often been considered to possess certain properties in common, mainly on the basis of their similar size. Caution must, of course, be used in identifying filaments on the basis of size only (30), and the possibility of biochemical heterogeneity was pointed out early on (55). The elucidation of their biochemistry has progressed slowly, but recently a number of authors have reported a mol wt of ~55,000 daltons for the main protein constituent of several of these filaments—in agreement with our studies on the heart conducting system—e.g., for the smooth muscle 100-Å filament protein skeletin (70, see also reference 12) or desmin (41), for glial fibrillary acidic protein (GFA) (14, 19), peripheral and brain neurofilament protein (14, 18, 34, 93), fibroblast intermediate-sized filament protein (8, 74), and an epidermal tonofilament protein (9, 28).

Similarity with respect to amino acid composition has been reported for GFA, neurofilament protein, and smooth muscle skeletin (70). The amino acid composition of intermediate filament protein of heart conducting tissue (75) is in good agreement with these results. Immunomicroscope investigations have shown cross-reactivity of antibodies to Purkinje fiber 55,000-dalton protein and e.g. smooth muscle, vascular endothelium, and neuroblastoma cells (reference 22 and work to be published). The cross-reactivity of other 55,000-dalton filament proteins has also recently been demonstrated (5, 6, 16, 17, 34, 41, 92). Such cross-reactivity may, however, in some cases depend on contamination (15). Tissue and species variations have been demonstrated in studies using techniques of immunofluorescence (17), immuno-electron microscopy (61), or peptide mapping (16, 17, 92). By immunodiffusion tests, we have recently demonstrated cross-reactivity between but absence of total identity of cow Purkinje fiber intermediate filament protein and murine neuroblastoma neurofilament protein (to be published). A study on chicken, using two-dimensional gel electrophoresis, is consistent with filament protein invariance within this species (32).

The total body of observations has led us to deduce that the intermediate-sized, free cytoplasmic filaments make up a class of similar proteins, in which—as in the myofibrillar proteins—subclasses exist. We favor the term "skeletin" for this protein class (cf. reference 70).
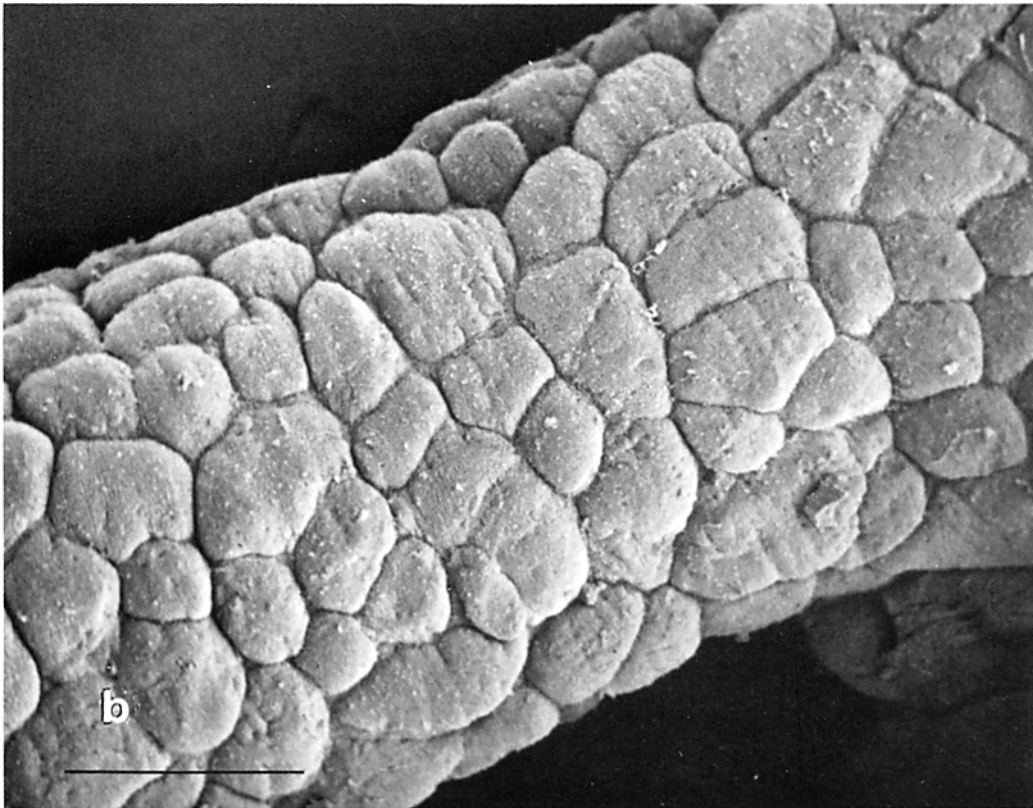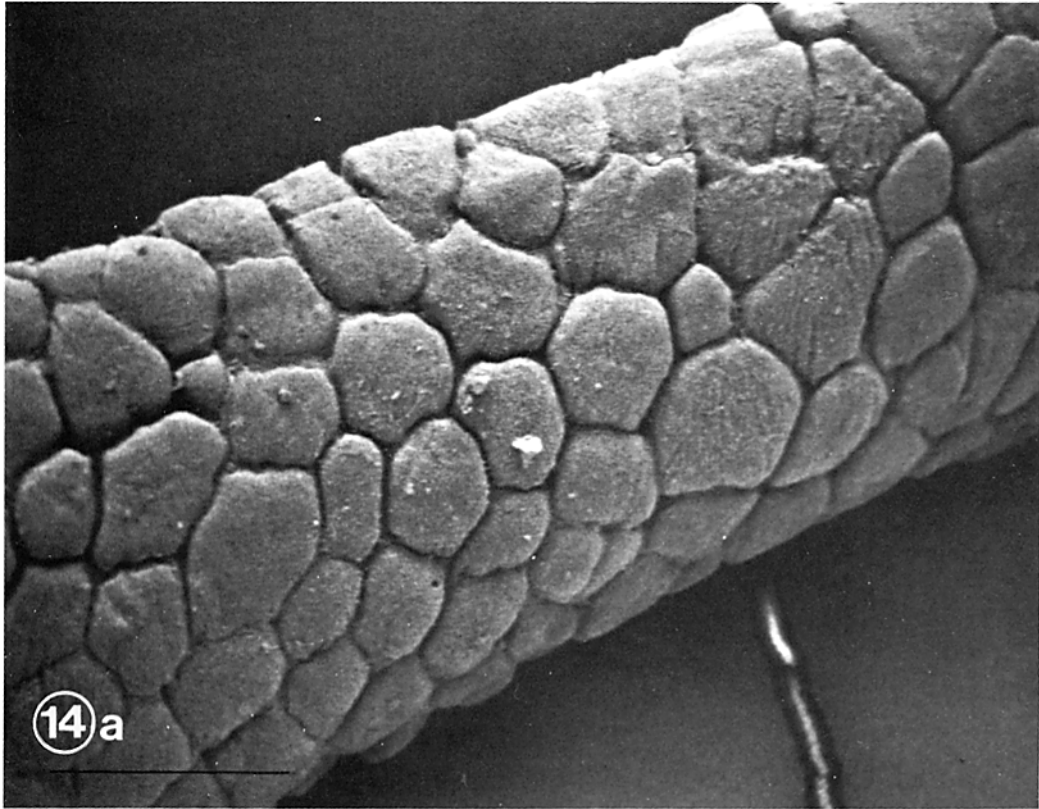
FIGURE 14 Scanning electron micrographs of extruded Purkinje fiber columns. (a) Control bundle with irregular indentations corresponding to the cell borders. Small amounts of adhering connective tissue are seen on the cell surfaces. (b) Triton- and KCl-treated cell column. No shrinkage or collapse of the cells is seen. Bars, 100 μm. × 320.

TABLE II
A Survey of the Reported Diameters of Intermediate-Sized Filaments in Relation to Cell Type and Preparation Technique

Reference	Diameter (nm, mean ± SD)	Cell type	Primary fixation	Post-fixation	Block staining	Section staining	Embedding material
Small & Squire 1972 (71)	7.5 ± 0.8	guinea pig intestinal smooth muscle	2.5% G/c or Ringer or acrolein-D/n.s.	1% O/c or 1% O-1% D/n.s.	—	UAc-PbCi or PP-PbCi or PP-PbCi	Araldite
Wuerker 1970 (91)	7.5 ± 0.7	rat spinal neurons	1% F-1% G/ph	2% O/ph	—	UAc-PbCi	Epon
Ishikawa et al. 1968 (30)	9.1 ± 1.2*	cultured chick myotubes	3% G/c	1% O/c	—	UAc-PbCi	Epon
	9.5 ± 1.5*	cultured chick fibroblasts	3% G/c	1% O/c	—	UAc-PbCi	Epon
Nickerson 1974 (51)	9.5 ± 0.5	guinea pig anterior pituitary chromophobe	3% G/ph	1% O/ph	—	UAc-PbCi	Epon-Araldite
Blose & Chacko 1976 (4)	9.9 ± 1.3	cultured guinea pig vascular endothelium	2% G/c	2% O/c	UAc	PbCi	Epon
Rash et al. 1970 (55)	10.2 ± 1.4*	cultured chick myocytes	(15 different)	O/c or ph	UAc	PbCi or UAc-PbCi	Epon-Araldite or DER 332 or Spurr
Rohrlich 1974 (59)	10.3 ± 1.7	fish, frog, and lizard iridophores	3% G/c or S	1% O/c or S	— or UAc	UAc-PbCi or PbCi	Epon-Araldite
Malech & Lentz 1974 (45)	10.6 ± 3.2	murine epidermal cancer cells	3% G/c	2% O/c	UAc	PbCi	Maraglas
Tumilowicz & Sarkar 1972 (87)	11.0 ± 1.1‡	cultured human breast tumor cells	2.5% G/n.s.	chrome-osmium	UAc	UAc-PbCi	Epon
Wuerker 1970 (91)	11.0 ± 1.0	rat cerebellar and spinal astrocytes	1% F-1% G/ph	2% O/ph	—	UAc-PbCi	Epon
Jimbow & Fitzpatrick 1975 (33)	11.4 ± 2.7	human melanocytes	Karnovsky's	n.s.	n.s.	n.s.	n.s.

Abbreviations: F = formaldehyde, G = glutaraldehyde, O = osmium tetroxide, D = dichromate, ph = phosphate buffer, c = cacodylate buffer, S = Sørensen's buffer, UAc = uranyl acetate, PbCi = lead citrate, PP = potassium permanganate, n.s. = not stated.
* mean and SD calculated from histograms.
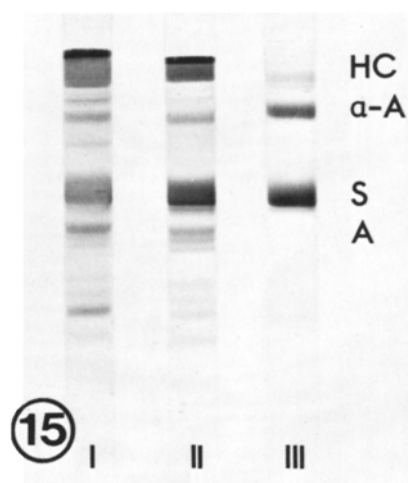‡ SD calculated from histogram.

FIGURE 15 Electrophoretic patterns of isolated Purkinje fiber bundles as revealed by SDS-polyacrylamide gel electrophoresis (7.5% gels). Myosin heavy chains (*HC*), α-actinin (*α-A*), the 55,000-dalton protein (*S*), and actin (*A*). Crude material (gel *I*), material extracted with Triton (gel *II*) and Triton and KCl solutions (gel *III*).

Further studies are needed to clarify the pattern of filament protein variation from ontogenetic and phylogenetic points of view.

The term "100 Å filaments" and those like it emanate from the approximate diameter of the filaments. Quite a few authors have investigated the diameter more meticulously. Diameters of 7.5–11.4 nm have been reported; for a detailed outline, see Table II. In these studies, various conventional plastic-embedding procedures have been adopted. No correlation between the filament size and the staining or fixation methods has been discussed, and retrospective comparison of the reported diameters is hampered by partly incomplete descriptions of the methods used. A tendency to greater filament diameter can, however, be noted in works where block staining has been adopted (see Table II). The in vivo dimensions of the filaments are uncertain. Our value of 7.0 nm in cryo-sectioned material is probably, however, closer to these in vivo dimensions. A similar value (7.5 nm) has been reported for tilted filaments in smooth muscle (71). The various dimensions reported previously and in the present study confirm the inaccuracy of the "100 Å terminology."

A number of authors have found the filaments to possess a less electron-dense central core and have suggested that they are tubular (4, 30, 38, 57, 62, 88, 91), while others have found the filaments to be evenly and densely stained (55, 87). Subfilamentous structures have been reported (48, 63, 70, 71, 87) but, as in our study, no fine structural organization has been established. Definite branching has been observed only in reconstituted filaments (70), and side arms have only exceptionally been reported (91).

Speculations concerning the function of the filaments have been numerous. However, two main functions have been discussed more profoundly, i.e., cellular and intracellular motility (24, 27, 33, 55) and structural support (38, 59). Evidence has, however, often been vague and indirect. In smooth muscle, motile properties have been clearly ruled out, as the contractile machinery still works when the filaments have been removed (70). By the use of additional extraction procedures, strong evidence of a cytoskeletal function has been obtained (70). This work and ours have thus taken account of the early recognized resistance to extraction of the intermediate-sized filaments (13, 55, 56) and have established a cytoskeletal function for the filaments. Also, other biochemical properties have per se been indicative of a supporting function in combination with nonreactivity to heavy meromyosin (24, 31) and to antimyoprotein globulins (see reference 7). Similar conclusions concerning the functional properties of the filaments have been deduced from studies on various cultured cells (7, 8, 42, 85, cf. also reference 53). The evidence for a cytoskeletal function does not, of course, mutually exclude other suggested functions of the filaments, and further investigation on this subject is required (e.g., references 17 and 32).

As it is obvious that methodologic factors have great influence on filament preservation, and that the "100 Å nomenclature" is not absolutely correct, we suggest an alternative name, based on chemical identification and analogous with the myofilament nomenclature. As one can expect further filament types to be included in the skeletin class, "skeletin filaments" could prove to be an appropriate name.

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