INTERMEDIATE-SIZED (SKELETIN) FILAMENTS OF HEART PURKINJE FIBRES

An investigation into their morphology, composition and function

UMÉA 1979
ABSTRACT

INTERMEDIATE-SIZED (SKELETIN) FILAMENTS OF HEART PURKINJE FIBRES.
An investigation into their morphology, composition and function.

Anders Eriksson, Institutes of Anatomy and Forensic Medicine, University of Umeå, Umeå, Sweden

The conducting system of the mammalian heart differs physiologically and morphologically from the myocardium proper. In some species, a main feature of the conducting cells is the presence of large amounts of cytoplasmic non-myofibrillar filaments. The morphological and biochemical structure and the function of these filaments were in this investigation studied by means of light, immunofluorescence and electron microscopy, and biochemical and immunological analyses.

The ventricular conducting cells of the cow exhibited a protein composition distinct from that of the myocardium proper. A main distinguishing component is a 55,000 dalton protein, constituting 50-70% of the total content of structural proteins of the conducting cells. This protein was enriched together with the cytoplasmic filaments after low and high salt extractions, which indicated the identity of the filaments with the 55,000 dalton protein. In spite of extensive extractions of other cell organelles, the filaments maintained the three-dimensional arrangement of cells and cell bundles. This provides strong evidence that the filaments perform a cytoskeletal role, and justifies the name skeletin filaments. The presence of an imposing cytoskeleton can be correlated with the exposure to mechanical strain during the activity of the heart.

The fine structure of the cytoplasmic filaments differed from that of other intracellular filaments, primarily with respect to their intracellular distribution and indefinite length, a smooth outline, a uniform width, and an intermediate diameter as compared with the actin and myosin myofilaments. Fine structure analysis suggested that the filaments are composed of four subfilaments. The subcellular distribution of the filaments, with filament bundles inserting into desmosomes, and tufts of filaments between adjacent myofibrillar Z disks, was further consistent with their performing a cytoskeletal function.

Biochemical analysis of the 55,000 dalton protein - named skeletin - revealed that it is nearly fully polymerized under physiological conditions. This too would be in accordance with a cytoskeletal function.

Purification of the skeletin monomer to homogeneity enabled the production of monospecific antisera in rabbits. Antiskeletin antibodies were shown to cross-react with conducting cells of several species. Antiskeletin could thus serve as a tool for the identification of conducting cells at the light microscopic level. Antiskeletin also cross-reacted with myofibrillar Z disks and intercalated disks, with vascular and intestinal smooth muscle, and with axons. These findings suggest the conservation of skeletin throughout cellular evolution.

The future use of antiskeletin in investigations on the cytoskeleton of malignant cells, on certain disorders of the nervous system, as well as a diagnostic tool in neuromuscular diseases, is suggested.

Key words: Heart conducting system, Purkinje fibres, intermediate filaments, cytoskeleton, structural proteins, skeletin, enzyme histochemistry, electron microscopy, immunomicroscopy, gel electrophoresis

Anders Eriksson, Institute of Forensic Medicine, University of Umeå, Box 6016, S-900 06 Umeå, Sweden
ISBN 91-7174-034-1
INTERMEDIATE-SIZED (SKELETIN) FILAMENTS OF HEART PURKINJE FIBRES

An investigation into their morphology, composition and function

by

Anders Eriksson

Umeå 1979
ABSTRACT

INTERMEDIATE-SIZED (SKELETIN) FILAMENTS OF HEART PURKINJE FIBRES.
An investigation into their morphology, composition and function.

Anders Eriksson, Institutes of Anatomy and Forensic Medicine, University of Umeå, Umeå, Sweden

The conducting system of the mammalian heart differs physiologically and morphologically from the myocardium proper. In some species, a main feature of the conducting cells is the presence of large amounts of cytoplasmic non-myofibrillar filaments. The morphological and biochemical structure and the function of these filaments were in this investigation studied by means of light, immunofluorescence and electron microscopy, and biochemical and immunological analyses.

The ventricular conducting cells of the cow exhibited a protein composition distinct from that of the myocardium proper. A main distinguishing component is a 55,000 dalton protein, constituting 50-70% of the total content of structural proteins of the conducting cells. This protein was enriched together with the cytoplasmic filaments after low and high salt extractions, which indicated the identity of the filaments with the 55,000 dalton protein. In spite of extensive extractions of other cell organelles, the filaments maintained the three-dimensional arrangement of cells and cell bundles. This provides strong evidence that the filaments perform a cytoskeletal role, and justifies the name skeletin filaments.

The presence of an imposing cytoskeleton can be correlated with the exposure to mechanical strain during the activity of the heart.

The fine structure of the cytoplasmic filaments differed from that of other intracellular filaments, primarily with respect to their intracellular distribution and indefinite length, a smooth outline, a uniform width, and an intermediate diameter as compared with the actin and myosin myofilaments. Fine structure analysis suggested that the filaments are composed of four subfilaments. The subcellular distribution of the filaments, with filament bundles inserting into desmosomes, and tufts of filaments between adjacent myofibrillar Z disks, was further consistent with their performing a cytoskeletal function.

Biochemical analysis of the 55,000 dalton protein - named skeletin - revealed that it is nearly fully polymerized under physiological conditions. This too would be in accordance with a cytoskeletal function.

Purification of the skeletin monomer to homogeneity enabled the production of monospecific antisera in rabbits. Antiskeletin antibodies were shown to cross-react with conducting cells of several species. Antiskeletin could thus serve as a tool for the identification of conducting cells at the light microscopic level. Antiskeletin also cross-reacted with myofibrillar Z disks and intercalated disks, with vascular and intestinal smooth muscle, and with axons. These findings suggest the conservation of skeletin throughout cellular evolution.

The future use of antiskeletin in investigations on the cytoskeleton of malignant cells, on certain disorders of the nervous system, as well as a diagnostic tool in neuromuscular diseases, is suggested.

Key words: Heart conducting system, Purkinje fibres, intermediate filaments, cytoskeleton, structural proteins, skeletin, enzyme histochemistry, electron microscopy, immunohistochemistry, gel electrophoresis

Anders Eriksson, Institute of Forensic Medicine, University of Umeå, Box 6016, S-900 06 Umeå, Sweden
ISBN 91-7174-034-1
"Nevertheless, it is somewhat unsettling in this era of analysis of fibers in cells to find that a component that may occupy over 25% of the volume of many kinds of cells is undefined chemically and functionally."

H. Holtzer (1976)
## CONTENTS

Abbreviations ......................................................... 6  
Original papers ....................................................... 7  
Background to the present investigation ......................... 9  
  Purkinje fibre morphology ........................................... 9  
  Intracellular filament systems ..................................... 9  
Aims of the study ...................................................... 13  
Experimental procedures ........................................... 15  
Observations ......................................................... 17  
Discussion of main results .......................................... 19  
  Purkinje fibre biochemistry ........................................ 19  
  Filament morphology ................................................ 19  
  Filament biochemistry .............................................. 20  
  Filament physiology ............................................... 20  
  Filament immunology .............................................. 21  
  Filament terminology .............................................. 22  
  Investigations in progress .......................................... 23  
General summary and conclusions ............................... 25  
Acknowledgements .................................................. 26  
Literature cited ...................................................... 27
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>BHK</td>
<td>baby hamster kidney</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>HMM</td>
<td>heavy meromyosin</td>
</tr>
<tr>
<td>LM</td>
<td>light microscopy</td>
</tr>
<tr>
<td>OM</td>
<td>&quot;ordinary&quot; myocardial cells, designed primarily for contractility</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PF</td>
<td>Purkinje fibres, the ventricular conducting cells of the heart</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>SMA</td>
<td>smooth muscle antibodies</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
</tbody>
</table>
This thesis is based on the following publications and manuscripts, which will be referred to by their Roman numerals:


V. Eriksson, A., Thornell, L.-E. and Stigbrand, T. Skeletin Immunoreactivity in Heart Purkinje Fibres of Several Species. (Submitted for publication).
BACKGROUND TO THE PRESENT INVESTIGATION

PURKINJE FIBRE MORPHOLOGY
For the correct successive contraction of the muscle fibres of the heart as it beats, there is a special cell system which triggers and conducts impulses to the myocytes. This system includes the sinoatrial node, the atrial tracts, the atrioventricular node and bundle, the bundle branches, and the terminal part - the Purkinje fibres - which contact the "ordinary" contracting myocardial cells.

As early as in the middle of the 19th century, the peripheral conducting cells in sheep could be distinguished from ordinary myocardium by their specific morphology (53). This discovery was made by J. E. Purkyne, who lent his name to these cells (usually spelt Purkinje in English). It was no coincidence that the discovery was made in sheep, as the ungulates (e.g. sheep, cow, pig) have conducting cells which are easier to distinguish from ordinary myocardial cells than in most other species. The distinguishing features include a larger cell diameter, a lighter cytoplasm and fewer myofibrils than ordinary myocardial cells. It was demonstrated early on that this light cytoplasm had a high glycogen content (47). Also, the conducting cells are generally surrounded by a connective tissue sheath isolating them from ordinary cardiocytes. In several other species, the distinction of ordinary myocardium and conducting Purkinje fibres is not so clear-cut though there may be some common features (for references, see V). Some authors have therefore restricted the term "Purkinje fibres" to the ventricular conducting cells of ungulates, while others have included the subendocardial termini of the conducting system of all higher vertebrates. By grading them according to the variables mentioned, classification models of the Purkinje fibre morphology have been suggested (49, 65). Three levels of differentiation are then recognized, viz. "good", "intermediate" and "poor". The reason for a great variation in the morphology of conducting cells of different species is not known.

With the introduction of electron microscopy it has become possible to detect not only glycogen particles in the central cytoplasm but also wavy masses of filamentous structures. The presence of large amounts of glycogen has been correlated with the more pronounced resistance to anoxia as compared with the ordinary cardiocytes (see 63), while the function of the filamentous component has been wrapped in obscurity. Many authors who have performed electron microscopic investigations of conducting cells have noticed the filamentous organelle, but at the time that this investigation was commenced, there was no agreement concerning their subcellular localization and fine structure, not to mention their chemical composition, function or immunological properties.

INTRACELLULAR FILAMENT SYSTEMS
By means of electron microscopy, at least three morphologically distinguishable classes of filamentous organelles - apart from the regularly arranged myofilaments of myogenic tissues - have been identified in a wide variety of eukaryotic cells: microtubules, microfilaments and a third class intermediate in size. The characteristics of microtubules and microfilaments are given in order to provide a better understanding of the differences between these components and the intermediate-sized filaments.

Microtubules. Microtubule is the collective name given to a class of subcellular components, defined as cylinders with an outer diameter of about 24 nm, a dense wall 5 nm thick, and a less dense core 14 nm wide. They are uniform in diameter, may be several microns in length and show no evidence of branching. Most often the tubules are straight, occasionally curved and sometimes even helical. The wall of the microtubule seems to be made up of protofilaments 5 nm in diameter, with a globular structure and arranged parallel or helical to the long axis of the tube. The number of protofilaments has been suggested to be 9-14. Although this definition is generally applicable, variations do exist and diameters of 18-34 nm have been reported for microtubules from various cells.

Microtubules have often been observed arranged parallel to the long axis of the cellular extensions, along with a network of stress fibres (microfilament bundles). Also, they have been reported in developing systems where changes in cell shape are occurring and they are generally recognized in a typical 9+2 arrangement in cilia.

The general occurrence of microtubules as outlined
above suggests that they are associated with diverse functions, e.g. chromosome movement during cell division, intracellular particle transport, maintenance of cell form, cellular motility and sensory transduction. Disruption of microtubules, e.g. by low temperature or colchicine, has been valuable in evaluating the functions listed.

The protein constituting the microtubules has been named tubulin and has been biochemically characterized as a heterodimer, with the subunits α-tubulin and β-tubulin of identical molecular weight (54,000). For references and further information, see references 1, 24, 50 and 73.

Microfilaments. Microfilaments are the smallest of the three types, exhibiting a diameter of 4-7 nm and an indeterminable length (see 23, 46). By means of electron microscopy, they have been shown to be a prominent component of the peripheral cytoplasm of many types of mammalian cells. They have also been localized in parallel arrays, so-called microfilament bundles or stress fibres, crossing the cytoplasm (59), and evidence has been presented suggesting that they also exist in a non-filamentous form (18, 23).

With the pioneering works of Ishikawa and coworkers (33), it was recognized that the microfilaments reacted with the myosin subunit heavy meromyosin (HMM), to form so-called arrow-head structures, as did the myofibrillar actin filaments. This suggested an actin nature of the subplasmalemmal microfilaments, a presumption which was corroborated by their diameter and their fibrous appearance and later confirmed by use of other methods, as for example the specific binding of antiaction antibodies (22, 43). Actin has also been identified biochemically as a component of isolated plasma membrane fractions in various cells (see 21, 23). Biochemically, at least three electrophoretic different forms of actin have been recognized, viz. alpha, beta, and gamma actin (23, 25, 72). The α form seems to be confined to muscle cells, while β and γ forms have been localized in all non-muscle cells examined so far. The β and γ forms are also present in cultured muscle cells and may be present in low amounts in mature muscle cells. Interspecies variations exist, though the non-muscle actins (β, γ) seem to be less variable than the muscle actins (25). Also, other differences between muscle and non-muscle actins seem to exist - in muscle cells most of the actin is present in the polymerized form of fibrous actin (F-actin), while in non-muscle cells actin is present in a dynamic state, forming non-filamentous globular actin (G-actin), transitory filaments, and more permanent microfilaments (25). This difference has been correlated with the interference of other cytoplasmic constituents (25) - e.g. meticulous immunochemical analyses have identified myosin, tropomyosin, and α-actinin in the stress fibres (23, 39, 69).

The ultrastructural localization of the microfilament bundles in cell processes and in regions of cell-substrate contact in cultured cells, as well as their interaction with HMM, indicate that they may function as morphologic components of an organized contractile system involved in cellular and intracellular movements. The HMM-binding properties (e.g. 21) as well as the specific disruption of microfilaments by cytochalasin B (e.g. 46) have been valuable tools in the determination of changes in the organizational states of microfilaments accompanying various motile activities. Analogous to the motile properties ascribed to myofibrillar actin, subplasmalemmal microfilaments have been associated with motility of membranes, such as membrane ruffling, pinocytosis, cytokinesis, cytoplasmic streaming, contact-inhibition, and membrane-fluidity (for refs, see 18, 23). Microfilaments have also been discussed in relation to cell motility during wound healing, and in relation to a variety of disease processes as well as to the invasive and motile properties of malignant cells (e.g. 11, 15, 17, 20, 28, 46, 55, 66).

Intermediate filaments. Filaments, intermediate in diameter as compared to the actin and myosin filaments, were first described in presarcomere myoblasts in 1965 (29). These intermediate-sized filaments were then considered as developmental stages of the thick and thin myofilaments. Many other investigators also described free fine filaments in developing muscle at this time. However, it seems as if the intermediate-sized filaments were not recognized as one entity, but instead were confused with thick or thin filaments. In 1968, the intermediate-sized filaments were identified as a separate class of filaments in developing muscle cells, fibroblasts, chondrocytes and chondroblasts, on the basis of their diameter, which was determined as 8-10 nm (32). It was also shown that metaphase-cells, rich in intermediate-sized filaments, were devoid of myosin and actin. The identity of the intermediate-sized filaments as a separate class was corroborated by the later observation that they, in contrast to the thin actin filaments, did not react with the HMM subunit of myosin (33). In these and later works, intermediate-sized filaments were recognized in a number of eukaryotic cells.
However, while considerable energy has been devoted to unravelling the cytoplasmic functions of actin filaments and microtubules, the intermediate-sized filaments have escaped such a detailed analysis. Initially, this was partially due to a belief that the intermediate-sized filaments were either degradation or disassembly products of microtubules, as the anti-mitotic drug colchicine caused both a disappearance of cytoplasmic microtubules and a concomitant increase in intermediate-sized filaments (see 23). Also, the filaments were found to be largely insoluble and to lack specific structural and chemical markers (e.g. 6). In consequence, although something was known about the structure of these filaments, little was known about their biochemistry and even less of their cellular function. Moreover, it was not clear whether these filaments from different tissue sources were related chemically, immunologically, or functionally.
AIMS OF THE STUDY

The principal aims were to answer the following questions:

What are the structural and biochemical features of the intermediate-sized filaments of the heart conducting cells?

In what way are the intermediate-sized filaments organized within the cells and what is (are) their function(s)?

Are they related to morphologically similar filaments in other cell types and cells from various species?
EXPERIMENTAL PROCEDURES

Further description of the methods used can be found in the original papers, which are referred to by their Roman numerals in each section.

MATERIALS

Heart Purkinje fibres (I–V). Hearts from cow, sheep, pig, cat, rat, guinea-pig and hen were obtained immediately after or within 2 h after sacrifice. Human hearts were obtained within 10 h after death. Moderator bands, false tendons, and ventricular septal and/or free wall myocardium were either immediately fixed in a slightly stretched state in 2.5% glutaraldehyde in Tyrode buffer or rinsed in buffer. Fixed material was processed for light and transmission electron microscopy while unfixed material was processed either for enzyme histochemistry and immunofluorescence microscopy or for isolation procedures (see below). For the latter purpose, Purkinje fibres from cow hearts were mechanically separated from their connective tissue sheaths (64). Isolated bundles and cells were either processed for myosin preparation, for preparation of cytoskeletons, or directly for SDS-PAGE analyses (see below).

Ordinary myocardium (I, V). Ordinary myocardium was obtained from cow hearts immediately after or within 2 h of stunning. Fresh material was fixed for TEM while material obtained later was either processed for immunofluorescence microscopy or rinsed, trimmed and homogenized for extraction procedures and SDS-PAGE.

EXTRACTION AND PURIFICATION PROCEDURES

Demembranation (I–IV). Homogenized Purkinje fibre and ordinary myocardium preparations were washed in 0.2% or 0.5% Triton X-100 and subsequently rewashed in buffer alone.

Preparation of myosins (I). Isolated Purkinje fibre strands and homogenized ordinary myocardium were glycerinated and stored at −38°C for approximately one week. After centrifugation, the material was homogenized in the presence of Triton X-100 and washed in buffer. Myosin extraction was carried out in a 0.6 M KCl solution (61). Extracts were dialyzed against distilled water overnight, yielding floccular precipitates which were collected by centrifugation and processed for SDS-PAGE analyses.

Preparation of cytoskeletons (II, IV). Isolated unfixed Purkinje fibre strands were demembranated in 0.2% Triton X-100 and intensively extracted in low and high ionic strength solutions. Residues were processed for LM, TEM, SEM and SDS-PAGE analyses.

Purification of filament protein (III). Isolated Purkinje fibre strands were homogenized and exposed to intensive extractions in 0.2% Triton X-100 and in low and high ionic strength salt solutions (60). The residue was dissolved in 0.2% SDS and run on Ultrogel AcA gel columns. Eluted fractions were analyzed by SDS-PAGE.

MORPHOLOGIC ANALYSES

Conventional light microscopy (LM) and transmission electron microscopy (TEM) (I, II). Glutaraldehyde fixed specimens of ordinary myocardium, moderator bands, false tendons, isolated intact and extracted Purkinje fibre strands, and homogenized Purkinje fibres and ordinary myocardium, were rinsed in buffer and in most cases postfixed in OsO₄ in buffer for 2 h. After rinsing, the preparations were dehydrated and in some cases during dehydration block stained in ethanol solutions of uranyl acetate and phosphotungstic acid. Embedding was carried out in Vestopal W. Survey semithin and fine sections were cut in a LKB Ultrotome I or III. Survey sections were stained with toluidine blue and fine sections with uranyl acetate and lead citrate.

For cryoultramicrotomy, false tendons were stablized in 2.5% glutaraldehyde in Tyrode buffer for 30 min at +4°C. Subsequently, Purkinje fibre strands were mechanically extruded. After antifreeze-treatment in glycerol, isolated Purkinje fibre specimens were rapidly frozen in liquid Freon-12. Sections were obtained in a LKB Cryo-Kit, contrasted with ammonium molybdate and air dried.

Light microscopic examination was carried out in a Leitz Orthoplane Photomicroscope, electron microscopic examination in a Philips EM 300 equipped
with an anticontamination device.

Enzyme histochemistry (I, IV, V). Sections of whole hearts or interventricular septum and/or moderator bands were rapidly frozen in liquid propane. Serial sections were cut in a cryostat at —20°C. Sections were stained for the demonstration of myofibrillar ATPase at pH 9.4 (52) or after acid preincubation for various periods of time (7). Additional histochemical staining procedures were in some cases used for comparison (see V).

Immunofluorescence microscopy (IV, V). Serial cryostat sections to those prepared for enzyme histochemistry were immersed in chilled acetone, air dried, and incubated in immune or control sera (see below) for 1 h at +37°C. After repeated washes in phosphate buffered saline (PBS), FITC-conjugated goat (or swine) antirabbit globulin was applied for 1 h followed by repeated washed in PBS.

Sections were viewed in a Leitz Orthoplane Photomicroscope using epifluorescent optics.

Scanning electron microscopy (SEM) (II, IV). Extruded cell bundles (controls and extracted) were fixed and postfixed as for TEM and then processed for SEM by the critical point drying method. Examination was performed in a Cambridge Stereoscan S4.

BIOCHEMICAL AND IMMUNOLOGICAL ANALYSES

SDS-PAGE analyses (I—III). Protein samples were dissolved in 10 mM PBS containing 1% sodium dodecyl sulphate (SDS) and 1% mercaptoethanol. Dissolved protein was applied on to polyacrylamide gels (70) using several different acrylamide concentrations.

Reference proteins for molecular weight determinations were cytochrome c (11,700), myoglobin (17,800), ovalbumin (43,000), bovine serum albumin (68,000), transferrin (88,000) and immunoglobulin G (160,000).

Gel chromatography (III). Gel chromatography was performed with Ultrogel AcA-44 (LKB).

Amino acid analyses (III). Amino acid analyses were performed in a Beckman 120C Automatic Amino Acid Analyzer equipped with an Infotronics Integrator CRS-100A.

Isoelectric focusing (III). Isoelectric focusing was performed with LKB:s Multiphor for 2.5 h at a final voltage of 300 V and a final current of 1.9 mA.

Preparation of antisera (III-V). The purified SDS-55,000 dalton protein complex was injected subcutaneously into rabbits in the presence of incomplete Freund's adjuvant. After booster doses, serum was collected and the immunoglobulin fraction was concentrated. Nonimmune sera were used as controls.

Immunodiffusion tests (IV). Double diffusion tests were carried out with 1% agarose in 0.1M sodium phosphate buffer at pH 7.4 in the presence of 0.1% SDS.

Quantitative immunoelectrophoresis (III). Rocket immunoelectrophoreses were performed in 1% agarose in Tris-Barbitone buffer at pH 8.6 and 2V/cm.
OBSERVATIONS

Illustration and further description of the results obtained can be found in the original papers, which are referred to by their Roman numerals in each section.

MORPHOLOGY OF IN SITU FIXED MATERIAL

Distribution of intermediate-sized filaments (I, II). The prominent feature of the Purkinje fibres was the large number of intermediate-sized filaments (see below), which filled out most of the cytoplasm, intermingling with other cell organelles. The filament content varied between different cells, a finding that could not be explained. Filaments were often grouped, forming bundles which ran along the long axis of the cell, and often converged into desmosomes. Small tufts of filaments were seen at myofibrillar Z disk level. Peripherally located myofibrils opposed the cell borders of other Purkinje fibres.

In ordinary myocardial cells the well known myofibrillar pattern was recognized. A special search for intermediate-sized filaments resulted in observations of such filaments in spaces between Z disks and in the proximity of intercalated disks.

Filament diameter and fine structure (II). Irrespective of preparation, fixation or staining methods, three filament classes were recognized, viz. actin (diam 4.8-6.3 nm), myosin (diam 10.1-14.8 nm) and intermediate filaments (diam 7.4-9.5 nm). In cryosectioned material, the diameter of the intermediate filaments was 7.0 nm.

Cross-sectioned intermediate-sized filaments exhibited various profiles which sometimes were square and in higher magnification gave the impression of four subunits. This impression was consistent with the occasional observation of a less dense central filament core in cross-sections and a dense central ribbon in longitudinally cryosectioned material, with filament monolayers negatively stained. The length of the filaments could not be determined due to superimposition effects and the undulating course of the filaments.

Myofibrillar ATPase activity (I, V). The ATPase activity in Purkinje fibres was found to be stronger than that of ordinary myocardium. This was the case at all incubations tested except for prolonged acid preincubation when both PF and OM activities were extinguished. In addition, Purkinje fibre ATPase activity was more acid-stable than that of ordinary contracting myofibres.

MORPHOLOGY OF EXTRUDED MATERIAL

Morphology of crude homogenates (I). The Purkinje fibre pellets were composed exclusively of typical Purkinje fibres with negligible amounts of connective tissue. The fine structure of the Purkinje fibres was similar to that of in situ fixed material.

The ordinary myocardium preparations were mainly composed of isolated myofibrils. Occasional contaminating conducting cells were observed.

Internal structure of extruded bundles (II). Controls exhibited a light and electron microscopic morphology similar to that of in situ fixed material. Only minute amounts of connective tissue were attached to the surface of the cells.

Triton extracted material was devoid of membranous structures, except for the specialized parts of the plasma membrane, such as desmosomes, gap junctions and intercalated disks, which remained.

Salt extracted Purkinje fibres contained only intermediate filaments, Z disk material and specialized regions of the plasma membrane.

Surface topography of extruded bundles (II). Purkinje fibres showed up as tightly packed polygons separated by indentations corresponding to cell interfaces. Connective tissue traces were hardly detectable by means of LM and SEM. Detergent and salt extractions did not markedly affect the three-dimensional arrangement of the cells or the cell bundles in spite of the profound changes in internal structure.

PROTEIN COMPOSITION OF CRUDE HOMOGENATES (I, II)

In ordinary myocardium preparations, the well known pattern of myofibrillar proteins was recorded by SDS-PAGE. Thus myosin heavy chains, α-actinin, actin, troponin, tropomyosin and myosin light chains were recognized. In Purkinje fibre preparations es-
essentially the same proteins were present but their relative amounts were different. The most prominent band corresponded to a protein with a molecular weight of about 55,000 daltons which constituted approximately 50% of the stained polypeptides in the gels, as estimated by weighing the peaks from scan curves.

**PROTEIN COMPOSITION OF EXTRACTED MATERIAL (I, II)**

Triton extraction had a negligible effect on the band pattern. After high and low salt extractions, however, the bands were reduced to two major bands of 55,000 and 110,000 daltons, respectively. These two bands were thus significantly concentrated as compared with other components.

OM myosin showed a gel pattern similar to that recorded previously by other authors (56,71), i.e. bands at 200,000, 42,000, 27,000 and 20,000 daltons.

PF myosin was composed of 200,000 and 23,000 dalton components while the complementary residue showed bands at 110,000, 55,000 and 42,000 daltons.

**ISOLATION AND CHARACTERIZATION OF THE INTERMEDIATE FILAMENT PROTEIN, SKELETIN**

**Gel chromatography (III).** Chromatography of isolated homogenized Purkinje fibres, concentrated in intermediate filaments by salt extraction, yielded three peaks as analyzed by absorbance spectrophotometry. One of these corresponded to the 55,000 dalton intermediate filament protein as revealed by SDS-PAGE.

**Solubility properties (III).** The solubility of skeletin was dependent on pH, ionic strength, and the presence of detergents or agents splitting hydrogen bonds. It was obvious that at neutral pH and at physiological ionic strength skeletin was fairly insoluble.

**Molecular weight (I–III).** After calibration against reference proteins, the molecular weight of skeletin was determined as 55,000 daltons. No subunits could be identified, not even at heavier loadings than that shown in III:fig 2.

**Amino acid composition (III).** Strikingly, approximately 50% of the skeletin molecule was shown to be composed of four amino acids, viz. glutamic acid, aspartic acid, alanine and leucine.

**Isoelectric point (III).** The isoelectric point was determined as 6.35.

**CHARACTERIZATION OF ANTISERA**

**Immunodiffusion tests (IV).** Purified skeletin as well as crude Purkinje fibre preparation formed a single precipitation line with immune serum and a complete fusion reaction was observed. No precipitates were formed with control sera.

**Indirect immunofluorescence microscopy (IV, V).** Strong fluorescence was observed in the central regions of the cow Purkinje fibre cytoplasm, while the peripheral regions of the cells were not fluorescent. Myofibrils were very slightly stained in cross-sections; in longitudinal sections it was shown that the fluorescence was confined to the Z and intercalated disk regions. Control sera showed no fluorescence.

**SKELETIN IMMUNOREACTIVE MATERIAL IN CONDUCTING CELLS OF OTHER SPECIES (V)**

Strong fluorescence was observed in the conducting cells of sheep, pig and hen. In man and cat a markedly weaker fluorescence was noticed. Still, the conducting cells were easily distinguished from ordinary myocardial cells. In the rat and guinea-pig, differences in fluorescence were hard to detect even in regions where conducting cells were probably present.

**SKELETIN IMMUNOREACTIVE MATERIAL IN OTHER CELL TYPES**

**Ordinary myocardial cells (V).** In longitudinal sections, fluorescence was confined to the Z and intercalated disk regions.

**Vessels and nerves (IV, V).** In smaller vessels, reaction products were detectable in the endothelial layers, whereas in larger vessels, strong fluorescence was also noticed in the smooth muscular layers of the vessel wall. Small nerves were regularly observed in the moderator band sections. Such nerves showed only faint cross immunofluorescence.
DISCUSSION OF MAIN RESULTS

Further references can be found in the original papers, which are referred to by their Roman numerals in each section.

PURKINJE FIBRE BIOCHEMISTRY (I)

In the search for a biochemical substrate for the morphological differences between ordinary and specialized myocardium, the investigation should start with a species having pronounced morphological differences between the two tissues. Also, it is necessary to have a reliable isolation procedure for the conducting cells at one's disposal. These requirements are met by the cow's heart (63), which has been used in these initial studies, where significant differences between ordinary contracting myocardium (OM) and Purkinje fibres (PF) were shown with respect to the content of structural proteins.

The low molecular weight components in Purkinje fibre preparations were quite different from the well-known pattern of ordinary myocardium. As these components were co-purified when myosin was selectively extracted, it is suggested that they are closely related to the myosin molecule, and most probably correspond to the myosin light chains. This is consistent with the demonstration of differences in the PF and OM myofibrillar ATPase activities, activities which are known to be reflected in the different myosin light chain patterns.

The prominent feature of the Purkinje fibres was, however, a previously not described 55,000 dalton molecular weight protein, constituting approximately 50% of the mass of PF structural proteins. If, however, the relative stainabilities (60) of the structural proteins are taken into account, this value can be corrected to 65-70%. This figure should be compared with the preliminary stereologic finding that the filaments occupy about 75% of the cytoplasm (Eriksson and Thornell, manuscript in preparation). The 55,000 dalton protein was concentrated in the residue after myosin extraction and further concentrated after extensive extraction in high and low salt solutions. By electron microscopy, this concentrated 55,000 protein fraction was shown to consist almost exclusively of intermediate-sized cytoplasmic filaments. Small amounts of a 110,000 dalton protein probably corresponded to residual Z disks and desmosomes. It is suggested that the presence of a 55,000 dalton protein band in the OM preparations represents a combination of similar filaments in ordinary myocytes and contaminating Purkinje fibres.

FILAMENT MORPHOLOGY (II)

Three subclasses of filaments were recognized in the Purkinje fibres irrespective of the methods of preparation used. These were thin actin and thick myosin myofilaments, and a third class, intermediate in diameter and more randomly dispersed in the cytoplasm. The intermediate-sized filaments constituted a morphologically uniform class. However, the morphology of the filaments was apparently influenced by the preparation technique, and an increase in filament diameter was observed in block stained specimens. Depending on the technique used, diameters ranged between 7.0 and 9.5 nm. In previous investigations on the diameter of intermediate-sized filaments, diameters of 7.5-11.4 nm have been reported. In these studies, no correlation of filament diameter with staining or fixation methods was discussed, and retrospective evaluation of the importance of preparation technique is hindered by incomplete descriptions of the methods used. Taken together, the investigations suggest, however, a close correlation between preparation technique and diameter. Still, the in vivo diameter of the filaments is uncertain.

Since the submission of paper II, another two papers on intermediate filament diameter have been published (5,48). Neither of these discusses the correlation of filament diameter with the method of preparation. One paper (48) examines the diameter of "thick filaments" in rat (10.2±0.4 nm) and human (9.7±1.0 nm) brain endothelium, which fits well into the scheme in paper II (table II). The other investigation (5) concerns the intermediate filaments in cultured chick connective tissue cells, where a diameter of 9.7±2.1 nm was obtained (mean and S.D. calculated from the histogram). In this latter investigation, however, the measurements were carried out on negatively stained unsectioned whole cell mounts, and are thus not directly comparable.

Square profiles sometimes observed in crosscut filaments are consistent with the model of four subfilaments constituting the composite intermediate fila-
Fig 1. Reconstituted filaments formed by the precipitation of 55,000 dalton protein extracted from crude Purkinje fibres with 1M acetic acid. The reconstituted filaments form short, irregularly arranged fragments. SDS-PAGE of such reconstituted filaments reveal a highly purified preparation of the 55,000 dalton protein (Eriksson, Thornell and Stigbrand, manuscript in preparation). × 120,000.

**FILAMENT BIOCHEMISTRY (II, III)**

Extraction procedures led us to conclude that the 55,000 dalton protein of the Purkinje fibres was the biochemical substrate of the intermediate-sized filaments. The molecular weight is close to the molecular weights of proteins from smooth muscle filaments, glial filaments, neurofilaments, fibroblast filaments and an epidermal tonofilament protein (for refs, see II), and within the 10% inaccuracy of the method of estimation (70).

By purification of the 55,000 dalton protein to homogeneity, we have been able to characterize for the first time the protein constituting the intermediate filaments of the specialized conducting tissue of the heart. This specific tissue seems particularly beneficial when characterizing this protein as contaminating proteins are few and well known and the relative content of filament protein is extraordinarily large as compared with other cell types.

The amino acid composition shows — in spite of different isolation methods used — great similarities to the three previously characterized types of intermediate-sized filaments, derived from smooth muscle, neuronal and glial tissue (for refs, see III). The recently published amino acid analysis of the filament protein of cultured BHK-21 cells (62) also reveals pronounced similarities. The similarities to epidermal tonofilament protein (4) are however not of the same magnitude.

The isoelectric point and solubility properties of the filament protein indicate that the protein is nearly fully polymerized under physiological conditions. It dissolves at low and high pH and repolymerization into filaments is induced when pH is adjusted towards 7 (fig 1). These characteristics agree well with the solubility properties of isolated neurofilaments as well as with the early recognized fact that the filaments are almost insoluble in high salt concentrations - a condition which rendered the isolation and solubilization of the filament protein difficult before the introduction of detergents.

**FILAMENT PHYSIOLOGY (II)**

The ability of the intermediate filaments to maintain — with the aid of only Z disks and desmosomes — the three-dimensional appearance of the Purkinje cells and cell bundles, clearly indicates that a major function of the filaments is cytoskeletal. This is consistent with previous hypotheses based on the demonstration of intermediate filaments linking together Z disks.
transversely (12, 68). The need for a well developed cytoskeleton is obvious for the conducting cells of the heart - a disruption of the conducting pathways could severely affect the performance of the heart's work. Other important structures in this respect are evidently desmosomes, myofibrils and the surrounding connective tissue sheath. These structures can probably partially substitute for each other with respect to cytoskeletal power. Thus, there may be an inverse relation between e.g. the amount of intermediate filament and myofibrils and/or between intracellular filaments (myofibrils, intermediate filaments, leptofibrils) and extracellular fibrils (collagen). However, the elucidation of these hypotheses awaits future studies.

While microtubules and microfilaments have been extensively studied from the physiological standpoint, the intermediate filaments have been largely ignored until now. Lack of experimental evidence has limited the discussions on filament function to speculation. As a result, such speculation has been frequent and, for the conducting cells, has been associated with the conducting properties, with the binding of glycogen, with the synthesis of myofibrils, with supporting and motile properties, and with a proposed embryonal character of the conducting cells. For other cell systems, several additional functions have been proposed, however two main functions have been discussed in more detail, viz. the motile and supporting functions. Representatives for these two ideas are the filaments in neurons and muscle, respectively. The evidence presented has however often been indirect and questionable. It may, e.g., be that coexisting actin microfilaments, myosin filaments, and/or microtubules are responsible for the observed motility (31, 54). Strong evidence for a cytoskeletal role of the intermediate filaments was obtained in a study on smooth muscle, where the possibility of a motile function was precluded (60). Evidence for a cytoskeletal role has also been obtained in cultured cells where a nuclear anchoring function has been suggested (44, 58). In very recent studies, additional evidence for a supporting function has been obtained by the demonstration of intermediate filament protein gluing together the myofibrillar Z disks with the triads in skeletal muscle (27, 40).

In certain cultured cells, a close relation between intermediate filaments and polyribosomes has been found, indicating - if not merely accidental - either the accumulation of specific synthesized proteins at the site of their formation or a skeletal support for "free" polyribosomes (13, 45, 58). Consequently, it must be stressed that the cytoskeletal function demonstrated by no means excludes other, additional, functions either in the conducting cells of the heart or in any other cell type.

**FILAMENT IMMUNOLOGY (IV, V)**

Though largely unexplored until the end of 1976, the empty spots in the field of intermediate filament structure and function are now rapidly being erased. In part, this is due to the production of antisera specific for the intermediate filaments. Immunological methods are largely insensitive to the vagaries of molecular weights and proteolysis and could therefore serve as additional tools to biochemical methods for gaining an insight into filament interrelationship and for pinpointing real similarities. However, just as control over the 55,000 dalton protein has proved historically to be fairly tenuous, antisera raised to these antigens may be doubly suspect. Early preparations of intermediate-sized filaments (neurofilaments in 1971 - ref 57; glial fibrillary acidic protein in 1972 - ref 67) have in 1978 been the subject of severe criticism suggesting that the antigen is largely extracted and/or contaminated by the methods of preparation described (for refs, see 10). This gives rise to suspicion concerning the majority of the immunological works performed until 1977, as these mostly have dealt with antigens purified by these methods. However, the isolation of intermediate-sized filament proteins from other tissues as well, has in the past two years induced a flood of papers on the subject of filament immunology (fig 2).

In the present work, it has been possible to show that the antiserum produced against the 55,000 dalton protein localizes over the filament-rich regions in the central cytoplasm of the conducting cells, thus indicating the identity of the protein and the filament. This has later also been confirmed by use of immunoelectron microscopy (9). A single precipitate in immuno-diffusion of antiserum and antigen, with complete fusion of the single precipitate formed between antiserum and crude Purkinje fibre preparations, indicates the monospecificity of the antiserum.

The tendency of the antiserum to localize over conducting cells relative to the surrounding myocardium has been utilized to demonstrate successfully conducting cells in other species as well as the cow, from which the original antigen was obtained. Thus, a convenient and rapid method for the localization of the
FYg 2. Frequency of publications 1972-1978 concerned with immunological investigations on intermediate filaments (abstracts not included). Papers are subdivided according to the type of antiserum used. ■: antisera against neurofilament protein and/or glial fibrillary acidic protein, □: antisera against intermediate filament protein from other cell types, ▼: autoantisera from man and rabbit. (Not all of these papers are included in the list of literature cited. A complete list of the immunological investigations can be obtained from author on special request).

conducting system at a light microscopical level has been obtained. This will facilitate, above all, studies on the evolutionary aspects of the conducting system. In addition, this work also indicates interspecies conservation of the filament protein as the cross-reactivity was pronounced - the degree of cross-reactivity (or more correct, immunofluorescence) could be related rather to the actual content of intermediate filaments (as revealed by electron microscopy) than to phyloge-netic relationship. It must, however, be stressed that immunological cross-reactivity does not prove identity but only that some antigenic sites are shared.

In papers IV and V, cross-reactivity also to other cell types was demonstrated. In ordinary myocardial cells, the antibody is localized over Z disks and intercalated disks. This finding agrees well with the ultrastructural demonstration of intermediate filaments at these sites (I, 12, 68), as well as with other immunomicroscopic investigations using antibodies against smooth muscle intermediate filament protein (36, 41). It is also consistent with the finding in ordinary myocardium preparations run on SDS gels of a faint band at the level of 55,000 daltons (I).

The demonstrated cross-reactivity to smooth muscle and endothelium adjacent to the conducting cells was also expected from the wide biochemical similarities between conducting cell skeleton and smooth muscle skeleton (III) and from the previous demonstration of intermediate filaments in endothelial cells (3, 38). Staining was however only faint in the nerves present in the moderator bands adjacent to the conducting cells. This should not be expected as the biochemical similarities to neurofilament protein are as pronounced as those between the skeletins. It must however be recognized that neurofilaments are numerous only in large myelinated axons and the nerves present in the moderator band are mainly thin unmyelinated ones (34). Thus, the faint reactivity to moderator band nerves may be misleading. In fact, we have in later works demonstrated skeletin-like immunoreactivity of the neurofilaments in cultured neuroblastoma cells (10) and peripheral nerves (8). Immunodiffusion tests indicate that the identity of cow skeletin and murine neurofilament protein is partial (10).

**FILAMENT TERMINOLOGY (II)**

A commonly used term for the intermediate-sized filaments is “100 Å filaments” (or “10 nm filaments” in the era of Système International) due to their approximate diameter. As discussed above, this is however not very close to the probable real diameter of the filaments, but rather represents a value obtained in specifically treated preparations. The term “100 Å filaments” is therefore not a very attractive one. This is also reflected in the literature where a menagerie of designations exist besides the above mentioned, e.g. “9 nm filaments”, “80-100 Å filaments”, “110 Å filaments”, “thick filaments”, “thick microfila-
Fig 3. Cryostat cross-section of muscle from patient suffering from Duchenne muscular dystrophy, treated with antigoliatin by the indirect immunofluorescence method. The fluorescence is confined to the small regenerating myogenic cells, which are known to be lodged with intermediate filaments as judged by electron microscopy. Normal cells are weakly stained (Thornell, Eriksson and Edström, manuscript in preparation). x 300.

Though still not definitely settled, these designations probably all refer to a common class of filaments with similarities with respect to morphology, biochemistry, immunology and possibly also function. With this in mind, we suggest an alternative name based on the main function together with the biochemical and immunological identification of the filaments, analogous to the nomenclature of the myofilaments. Skeletin filaments could prove to be an appropriate name in these respects.

INVESTIGATIONS IN PROGRESS

The present work has shown the suitability of cow heart Purkinje fibres for investigations into the morphology, biochemistry and function of the intermediate filaments. It has also enabled the production of antisera to the filament protein, antisera that may help to elucidate further the presence and nature of intermediate filaments in various cells and tissues. More specifically, one can expect skeletin and antiskeletin to be utilized in the following problems:

Morphologically similar filaments have - as mentioned - been observed in a variety of cell types by means of electron microscopy. A filament monomer of about 55,000 daltons has also been identified in cells of different origin. However, the question of whether all these filaments are related or not, remains unanswered. In spite of a rapidly increasing number of investigations (fig 2) on the relatedness of filaments, present opinion is not coherent. Quotations from two reviews, both from 1978, illustrate this controversy: "...immunological studies... suggest profound and widespread similarities within the ~ 55K class of 10 nm filaments." (19); "Comparison, however, of the immunological properties of 100 Å filaments has revealed very little serological cross-reactivity between filaments isolated from different cell types." (42). Our own opinion, based on further investigations using anti-PF-skeletin, indicates that widespread similarities do exist, although the identity is only partial (10).

Another interesting subject of antiskeletin application is in studies on the cytoskeleton of transformed and malignant cells. It is known that the distribution of intermediate filaments is altered during the growth and locomotion of cells and that it appears to be different in malignant cell types (2, 30, 37). However, the organization and importance of filaments during virus and malignant transformation is not known and could possibly be elucidated by antibody techniques using antiskeletin.

In the treatment of certain malignant conditions (and certain other diseases), antimitotic drugs - e.g. vinblastine, vincristine and colchicine - which interfere with microtubular organization - are used. The relation between microtubules and intermediate filaments has been a matter of extensive debate (see e.g.
23) but is still not fully explored. Further studies on the distribution and behaviour of intermediate filaments during and after the addition of antimitotic drugs may contribute to answering the question of the microtubule-intermediate filament relationship.

In some human malignant diseases (and sometimes “spontaneously” in rabbits (26, 51)), autoimmune sera to intermediate filaments have been described (36, 44). Clinically, autoimmune sera are screened by tests to, among others, smooth muscle. Positive sera, so called smooth muscle antisera (SMA), have previously been considered as antiactin antibodies. This is, however, not always the case, and specificities also to skeletin and tubulin have been demonstrated (35). As smooth muscle contains both actin and skeletin, it is not possible to differentiate between antiactin and antiskeletin activities using this substrate. The substitution of Purkinje fibres for smooth muscle provides a possible way of overcoming this problem. Preliminary results are promising and encourage the use of Purkinje fibre cryosections in routine screening of autoantisera (Eriksson, Linder and Thornell, manuscript in preparation).

In the diagnosis of neuromuscular diseases, muscle biopsies are routinely used. In the light microscopic analysis of these biopsies, it is difficult to distinguish between small atrophying and small regenerating muscle cells, a distinction which is of importance in judging the state of the disease. As the regenerating cells contain large amount of intermediate filaments, antiskeletin immunofluorescence technique enables rapid and safe identification of the regenerating cells (fig 3). Antiskeletin antisera may thus have an application in the diagnosis of neuromuscular diseases.
GENERAL SUMMARY AND CONCLUSIONS

1. The specialized conducting cells of the cow heart have a protein composition distinct from that of contracting myocardium. A main distinguishing component is a 55,000 dalton component, constituting 50-70% of the total structural protein content of the Purkinje fibres.

2. The fine structure of the Purkinje fibre cytoplasmic filaments differs from that of myofibrillar myosin and actin filaments. The filaments are characterized by their diameter (7.0-9.5nm), which is influenced by the preparation procedure but is always intermediate between that of actin and myosin filaments. They exhibit indefinite length, a smooth outline, a fairly uniform width, and seem to be composed of four subfilaments. The filaments characteristically insert into desmosomes, and occur in the proximity of Z and intercalated disks.

3. From extraction experiments it is concluded that one of the functions of the intermediate-sized filaments is cytoskeletal. The presence of an imposing cytoskeleton can be correlated with the regular exposure of the cells to mechanical strain during heart activity. Other functions of the filaments remain obscure for the present.

4. Biochemical analysis of extracted residue (see 3.) shows a concentration of the 55,000 dalton protein (see 1.), indicating the identity of this protein with the intermediate-sized filaments.

5. Biochemical characterization of the 55,000 dalton protein shows an amino acid composition almost identical with that of other intermediate filament proteins, with four predominating amino acids, (glu, asp, ala, leu), constituting about 50% of the molecule. The isoelectric point was determined as 6.4. The protein is hardly soluble at pH 4-6 in the absence of detergent. These findings are consistent with the fact that the protein is polymerized at physiological conditions, which is required for the cytoskeletal function.

6. The identity of the 55,000 dalton protein and the intermediate-sized filaments is confirmed by immunofluorescence studies using monospecific antibodies to the 55,000 dalton component.

7. On account of the cytoskeletal function, the similarities to the smooth muscle “100 Å filament” protein subunit, and the inadequate and confusing existing terminology, we suggest that the protein be named skeletin and the filaments skeletin filaments.

8. The antiskeletin antibodies prepared (see 6.) enable the identification of heart conducting cells in several species. The intensity of cross-reaction can be correlated with the content of skeletin filaments rather than with phylogenetic relationship. The degree of cross-reactivity is correlated with a previous model of “differentiation”. In our opinion, the presence of a well developed cytoskeleton should be regarded as a sign of differentiation rather than as a vestigial remnant.

9. The antiskeletin antibodies allow an immunological comparison of skeletin and other intermediate-sized filament proteins. They also provide an interesting tool for further investigations into the pathological filaments of malignant and certain neurological disorders. Antiskeletin may also serve as a convenient tool for the rapid differentiation of regenerative and degenerative cells in neuromuscular disorders. The characteristics of the intermediate-sized filaments in the conducting cells of the ungulate heart seem to permit the use of cryostat sections of moderator bands as a substrate for the immunological differentiation and characterization of human smooth muscle autoantibodies.
ACKNOWLEDGEMENTS

I wish to thank all those who guided, assisted and supported this work, which was performed at the Institutes of Anatomy and Forensic Medicine in collaboration with the Institute of Pathology and the Department of Physiological Chemistry, University of Umeå. I am particularly indebted to:

Docent Lars-Eric Thornell, my supervisor at the Institute of Anatomy, who introduced me to this field and who kindly placed research facilities at my disposal. His own thorough and extensive knowledge in the field of the heart conducting system as well as in the methodological mysteries of electron microscopy provided the best possible background. I owe him much for his friendship and for applying the intellectual spur judiciously throughout the project, thereby offering the encouragement essential for the pursuit of coherent experimental work.

Docent Torgny Stigbrand at the Department of Physiological Chemistry for his benevolence in performing all kinds of biochemical occultisms, for his stimulating cooperation and valuable criticism, and for discussions on the biochemical and immunological parts of the work.

Ph.D. Vic Small and docent Michael Sjöström for valuable discussions and suggestions.

Professor Milan Valverius at the Institute of Forensic Medicine for benevolance and stimulating cooperation during parallel work in the field of forensic sciences.

Mrs. Eva Carlsson, Mrs. Margareta Enerstedt, Mrs. Kerstin Hjortsberg, Mrs. Birgitta Holmbom, Miss Marléne Lundström, and Mrs. Elisabeth Rubing, for skilful technical assistance with an immeasurable number of sections, gels and procedures.

Mr. Bror Berggren, the very able photographer at the Institute of Anatomy, who has rapidly and skilfully solved all phototechnical problems.

Mrs. Britt-Marie Falk, Miss Britt-Marie Johansson and Mrs. Vanja Åström for typing and retyping and retyping the manuscripts.

FK Per Hörstedt for skilful performance of the scanning electron microscopy.

Dr. Ian Jones and Dr. Harold E. Vickers for linguistic revisions of the manuscripts.

The staff at the Umeå slaughter house, without whose kind support and interest this study would not have been possible.

All other members of the staff at the Institutes of Anatomy, Forensic Medicine, Pathology and Physiological Chemistry who directly or indirectly have been involved in this work.

The Swedish Medical Research Council (12X-3934), the Medical Faculty of Umeå University, Lions Research Foundation and the Muscular Dystrophy Association have provided financial support which is gratefully acknowledged.
LITERATURE CITED

15. French, S. W. Is cholestasis due to microfilament failure? Human Pathol 7 (1976) 243-244.
31. Isenberg, G. and Small, J. V. Filamentous actin, 100 Å filaments and microtubules in neuroblastoma cells.


64. Thornell, L.-E. Morphological characteristics of Purkinje fibre bundles separated from their connective tissue sheath. *J Mol Cell Cardiol* 7 (1975) 191-194.


USA 71 (1974) 4561-4564.


