Demonstration of Early Myocardial Ischaemic Damage

Ali Muhammad Ali Hasan Al-Oqlah

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Dedication.

To my brother **Omar Shotar** for his unfailing and most generous support and his personal commitment throughout the period of my study.

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Abstract.

The scope of the study was to evaluate critically histological methods which would enable myocardial ischaemic damage in the pre-infarction stage to be demonstrated unequivocally in human tissue. Blocks taken routinely at autopsy are fixed in formalin and embedded in paraffin; staining procedures adopted were carried out on such material.

Two main methods of identifying tissue damage were investigated:

(1) Immunohistochemical staining with polyclonal and monoclonal, commercially available antibodies for cardiac myocyte constituent organelles and structural proteins.

(2) Demonstration of lectin binding on the surface and cytoplasm of human myocardium. The cases investigated were collected in the process of routine forensic autopsies; as controls, blocks from hearts from patients dying from recent acute myocardial infarction visible macroscopically were chosen. As negative controls, cardiac blocks from persons dying of non-cardiac conditions were chosen.

The antibodies and the lectins were carefully titrated to obtain optimal results.

There were reproducible changes in the ischaemic zones, but single antibodies or lectins did not produce singly pathognomonic and reproducible results. A battery of stains and lectins would have to be used in serial sections of the same block to prove that an area of suspected ischaemia is indeed showing structural abnormalities.

Declaration.

I declare that, apart from the assistance acknowledged, the research described was performed by myself and this thesis is of my own composition.

CHAPTER 1.

HISTORICAL BACKGROUND TO THE DEVELOPMENT OF CONCEPTS OF MYOCARDIAL ISCHAEMIA AND INFARCTION.

" *...If thou examinest a man for illness in his cardia and he has pain in his arm, in his breast and in one side of his cardia...it is death that threatens him...*"
(Papyrus, 1570 BC; quoted by Sethna, 1973).

1.A. A history of sudden death due to heart disease.

Sudden death must have been a phenomenon that was well known to ancient physicians but little survives in their writings to associate this condition with heart problems. Andreas Vesalius, at complete loggerheads with the established orthodox Aristotelian hypothesis, had the audacity to suggest that the centre of personality and emotion was not the heart but the brain; he was also the first to dissect the heart and to describe the coronary arteries in man in 1543 (Baldry, 1971). Leonardo da Vinci sketched the coronary vessels around the beginning of the 16th century. He identified the thickening of these vessels and related this thickening to diminished blood supply in their territory. These vessels were simply regarded as anatomical curiosities and the association between primary disease of the heart and sudden demise was not made until later in the century.

Leibowitz (1960) refers to the work of Amatus Lucianus (1580) and credits him with the first description of a sudden death from heart disease written up in "*Curationum medicinalium centuria sexta*" in which the "*...death of a reverend abbot from the Isle of Croma*" was stated to have resulted from "*a badly damaged heart...his heart lacinated... rather than from apoplexy*". Petrus Salius Diversus in 1586 in his "*De febre pestilenti Tractatus*" also refers to "*...a sensation of constriction of the heart together with syncope, pallor and perspiration ...*" (Leibowitz, 1960).

In seventeenth century England, William Harvey revolutionised medical thinking when he described the circulation of the blood; he was also the first to identify the importance of the coronaries in relation to myocardial nutrition, to re-describe arterial sclerosis and calcification and to describe the syndrome of angina pectoris by referring to the clinical case in Sir Robert Darcy. The latter gentleman died suddenly and an autopsy carried out by Harvey together with Doctor Argent, then President of the

College of Physicians, and Dr. Gorge, an "*outstanding theologian and preacher*" demonstrated a ruptured left ventricle - most likely to have been the consequence of a myocardial infarct.

During the eighteenth century calcified coronary arteries often featured in the descriptions of post-mortem examinations such as in the works of Theophile Bonet (1700)- "*Sepulchretum sive anatomica practica ex cadaveribus morbo denutis*", by Carolus Drelincourt (1700) also published in Geneva, by Lorenzo Bellini in Florence (1703)-"*De Morbis Pectoris*", and in Leyden by Thebasius (1716) in his "*Dissertatio medica de circulo sanguinis in Corde*". Giovanni Maria Lancisi, physician to Pope Clement Xl in Rome in "*De subitaneis Mortibus*" (1707) and Giovanni Battista Morgagni in Bologna in "*De sedibus et causis morborum per anatomeniam indagatis*"- (1761) also described thickened coronary arteries. In 1768 William Heberden in his address to the Royal College of Physicians of London described in great detail angina pectoris.

Contempories of his John Fothergill (1776) and John Hunter (1794) examined the hearts of patients who died from this condition and identified myocardial scarring. Around the same time, Edward Jenner and Caleb Hilliard Parry also found an association between damaged and thickened coronaries and heart problems. Hunter himself died of a myocardial infarct during an acrimonious meeting of the Hospital Board of St, George's Hospital, London. By the end of this century it became possible for Dr. Samuel Black (1797) to say of angina pectoris that "*the primary and original cause of the disorder is, perhaps, in every instance the ossification of the coronaries...*"(Altschule, 1985).

In 1809 Allan Burns of Glasgow summarised the pathophysiology of coronary heart disease and across the Atlantic John Warren (1812), the first professor of surgery and anatomy at Harvard Medical School, reported on the clinical and autopsy features of coronary artery disease. In the latter part of that century references were being made in publications to the appearances of the heart as "*soft, flabby, flaccid, fragile, easily torn, pale yellow or brown* "; one such descriptions is in the book published by Dr. Charles Williams from the Brompton Hospital in London in 1840, "...*A pale yellowish appearance of the substance of the heart is not at all an unusual accompaniment of other lesions of the organ, especially adhesions of the pericardium and accumulation of fat; but I should be induced to refer this to an altered state of the*

nutrition of the organ, owing perhaps to partial abnormality of the coronary arteries...".

Carl Rokitansky in his monumental "*Manual of Pathological Anatomy*" published in Vienna between 1841 and 46 attributed these colour changes to "... *various fatty conditions of the heart...*". The concept of myocardial fatty degeneration was adopted by British physicians, and also across the Irish Sea in Dublin where Dr. Richard Quain in 1850 expounded the concept of cardiac fatty degeneration and suggested that coronary arterial occlusion led to this. In his classical 75 page paper, which included lithographs of histological sections, he reviewed 83 cases of fatty heart. This condition became a popular and widely known diagnosis, and many hearts exhibiting this condition were demonstrated at the Pathological Society of London which had been founded on the 20th October 1846. Indeed this diagnosis became incorporated in everyday Victorian parlance and in Victorian literature: unfortunately, however, it was not listed as a cause of death by the Registrar General till 50 years later so no accurate epidemiological data of deaths from this 'condition' are available for that period.

In 1870 the German pathologist Rindfleisch suggested the cause for fatty softening of the heart: "*atheromatous degeneration of the coronary arteries with plugging of one of their larger branches by a thrombus may be regarded in every instance as the cause of this dangerous lesion*" (Rindfleisch, 1872).

1.B. The development of the concept of myocardial ischaemia and infarction.

Important advances in our understanding of the patho-physiology of ischaemic heart disease (IHD) were first made in Julius Cohnheim's laboratory in 1872 at the University of Leipzig. Julius Cohnheim, born in 1839, went to Berlin to begin his studies in medicine in 1856 which was the same year that Rudolf Virchow was called to Berlin, from his professorship in Wurzburg, to establish his famous Pathological Institute. In 1860, having passed his examinations for the M.D. degree, he started his thesis under Virchow's guidance in 1861. In the autumn of 1868, still only 29 years old, Cohnheim accepted the chair of pathology at Kiel, and in 1872, Cohnheim moved to Breslau to establish a Pathology Institute, where as with Virchow in Berlin, he was able to attract excellent colleagues and students, many of whom were to become outstanding leaders in their respective fields: Paul Ehrlich, Carl Weigert, William

Henry Welch, Albert Neisser, Ludwig Lichtheim, Ottomar Rosenbach, Carl Julius Salomonsen, Charles Roy and William Councilman (Malkin, 1984).

Cohnheim had studied with Rudolf Virchow and extended his mentor's studies on thrombosis and embolism. It fell to Carl Weigert, another of Virchow's pupils who became Cohnheim's assistant in 1874 to open up in 1880 a new era in pathology. He put forward and established the concept of coagulation necrosis and he greatly improved the histological staining techniques.

Carl Weigert recorded the first complete pathological (autopsy) description of coronary artery disease and myocardial infarction (MI) and identified clearly their cause/effect relationship (Lie, 1978). In his article "*Ueber die pathologischen Gerinnungsvorgange*", Weigert (1880), after speaking of renal infarcts, he stated: "*Still more typical, perhaps, is another kind of infarction which strangely enough has not been at all considered (beachtet): Infarcts of the heart muscle...*" He further continued: "*In atheromatous changes of the coronary arteries, not infrequently thrombotic embolic obstructions form in the branches of the arteries. If the obstruction forms slowly, or at least in such wise that collateral channels exists, but not enough to keep up nutrition, a slow atrophy occurs with destruction of the muscle fibers without injury to the connective tissue. The muscle fibers that disappear are replaced by fibrous connective tissue, and the so-called chronic myocarditis is nothing else than such a process. We cannot speak of a disappearance of muscle fibers by 'compression of an inflammatory exudate, or by, contraction of new-formed connective tissue masses...*" (Dock, 1939).

The Swiss pathologist, Professor Ernst Ziegler (1880), then in Tubingen, later in Freiburg, proposed the term "*myomalacia cordis*", softening of the heart, after the analogy of "*encephlomalacia*", for the pathological changes that resulted from coronary occlusion. He described the evolution of histological changes as the duration of the infarction increased (Fye, 1991).

The British physician Donald MacAlister, who translated Ziegler's text, believed that myomalacia cordis "*is really anaemic necrosis ... it is not a rare affection, and when at all extensive it brings about death by failure or rupture of the heart*" (Ziegler, 1883).

By 1884 Professor Ernst Leyden, director of the First Medical Clinic in Berlin, was able to decipher a fairly complete picture, which he presented under the title "*Ueber die*

Sclerose der Coronararterien und die davon abhaengigen krankheitszustaende" in seventy pages of the 'Zeitschrift fur klinische Medicin" (vol;7, pp.458-486, 539-580). Leyden gave many references to contributions of French clinicians, especially Huchard and his school, and prepared the medical world for Huchard's work: "*Diseases of the Heart and Great Vessels*", which appeared in 1889 (Dock, 1939).

John Lindsay Steven, clinician and pathologist, who had worked with Weigert and his colleague Huber in 1882, gave a series of lectures in 1887 on "*Fibroid Degeneration and Allied Lesions of the Heart, and their Association with Disease of the Coronary Arteries*". Among important conclusions, based on sixteen cases in his own experience, Steven stated "*[T]he influence of a morbid state of the coronary arteries must be taken into account in considering all diseases of the heart, but especially of the myocardium, and no examination of the heart can be regarded as comoplete which does not include a careful investigation of the state of those vessels...*" (Dock, 1939).

These classical observations of the Leipzig group had essentially little, if any, impact on contemporary medical practice, however. Although, in retrospect, the clinical event denoted as acute MI had been occasionally described, it was not clearly distinguished from angina. The pathological features of established MI were sometimes discovered at autopsy when they were indeed found to be associated with coronary occlusion, but the relationship of symptoms to pathological findings remained obscure.

Gradually, the concept of MI from a pathological point of view began to appear but in the clinical literature. William Osler, who had studied with Cohnheim and Weigert, discussed the pathophysiology of IHD and suggested the possibility of short term survival after coronary occlusion in 1889: "*[T]he local disturbances of nutrition caused by the blocking of a terminal branch of a coronary artery produce the condition known as infarct of the heart; or, as it is sometimes called, anaemic necrosis...The danger is not alone at the period of preliminary softening, but time gradually effects a transformation of the softened areas into fibrous tissue, which yield and lead in many cases to aneurysm of the cardiac wall and rupture. In fatty degeneration we know that the most extreme grade may be reached without danger to the integrity of the heart muscle, so long as the process is uniform, but a localised fatty change which follows the gradual obliteration by endarteritis of a branch supplying a limited area of the cardiac wall, seems to precede rupture in a number of cases*" (Osler , 1889).

At Breslau, another event occurred in April 1877 which was to have a great impact on the development of medicine generally, and pathology particularly, was the arrival of William Henry Welch. Originally Welch went to Germany to study with Virchow, but Carl Ludwig, with whom Welch worked during the last half of 1876 and the early part of 1877, persuaded him to go to Breslau to study with Cohnheim instead. In 1889, William Welch on the basis of microscopy and autopsy observation wrote "*[W]ith disease of the coronary arteries the muscle walls, the pericardium, endocardium, and valves may be found intact, but in most cases there are associated muscular lesions, namely, fibrous myocarditis, fatty degeneration, white infarction. In fibrous myocarditis, which is also known as cirrhosis of the heart, and as chronic interstitial myocarditis, there are found in the muscular walls grey translucent or white patches of irregular shape and size, in which the muscle has been replaced by connective tissue. These patches situated in any part of the wall may be complicated with pericarditis or with endocarditis. These patches were commonly thought to be like fibroid patches in other parts of the body, the results of interstitial inflammation. As Hilton Fagge, a British physician and pathologist, has pointed out, this cannot be accounted, for we do not find in them any sign of inflammation, any granulation tissue, any small cell formation. These patches are nearly always associated with disease of the coronary arteries, so that they may be considered the result, not of genuine inflammation, but of the death of localized patches...These so-called fibroid patches are probably due to coagulation necrosis of the muscle fibre, as in fibroids of the uterus. An interesting point is the enormous increase in size, three to four-fold, of the nuclei of the adjacent and normal muscle cells. Whilst we affirm that these patches are not the result of pre-existing inflammation, we do not deny that there may be patches of that origin...The best results were to be had by making frozen sections of the fresh heart wall, treating them with osmic acid and mounting them in acidulated glycerine*" (Welch, 1889).

In 1893, Kolster initiated experimental studies on myocardial ischaemia by ligating a small branch of coronary artery in dogs; he was able to keep some of these animals alive for periods varying from one day to one year and five months and was able to describe such artificially produced cardiac infarcts in a series of 12 dogs and demonstrated that the infarcts heal by a process similar to that now known to occur in man. The infarcts that he produced were not, however, complete infarcts and this was probably because only a small-sized vessel was occluded. With the histological techniques which he used, a satisfactory interpretation of the details of the process of healing was impossible (Mallory et al., 1939).

In 1896, William Osler delivered a series of lectures on angina pectoris in which he recounted the then recent experiments that were shedding light on the pathophysiology of IHD. Although the theme of his lecture series was angina, Osler's commented that "*the effect of plugging of the coronary artery is the production of what is known as an anaemic infarct, a well-recognized pathological condition*" (Osler, 1897).

1.C. Microscopical demonstration of myocardial ischaemic damage.

Walter Baumgarten, the son of Porter's mentor Gustav Baumgarten (professor of physiology at St. Louis Medical College in 1873), collaborated with Porter at Harvard on several animal experiments. Baumgarten in 1899 elaborating on the pathologic-anatomical side of Porter's work, reached the same conclusions and provided an excellent and detailed histological study of experimental infarction in cats and in dogs, and he is accredited with the publication of the most comprehensive and insightful article on myocardial infarction written by an American in the 19th century:

"*The microscopical examination of sections stained with haematoxylin and eosin (H&E) showed that coagulation necrosis was found to be fully developed through the whole area of the infarct as early as twenty-two hours after ligation of the artery (dog, circumflex). At this hour many nuclei can no longer be stained, some show irregularities and fragmentation, and a few are swollen and vacuolated. In the earliest cases examined, the protoplasm had undergone granular degeneration, but in cases three or four days old hyaline degeneration is more frequent. Small vacuoles occur in many muscle cells, usually several in each cell so affected...In sections of pieces of infarct hardened in Flemming's solution (to determine the presence of fatty change), small black granules are found in some of the muscle cells...At the margins of the infarct, and only there, groups of normal muscle fibres are scattered through the necrotic tissue, and sometimes acquired macroscopical size. These interruptions of necrotic area by normal tissue are identical with the infarcts which Kolster produced by the ligation of a branch of the ramus descendens. The condition of the principal blood vessels and the capillaries in the infarcted area varies greatly. Throughout the larger part the capillaries and the vessels contain no blood corpuscles and are difficult to distinguish. At the margins of the infarct they are usually distended with blood, and small extravasations of red blood corpuscles are sometimes present in the surrounding tissue. The vessels near the endocardium frequently share in the obliteration which has overtaken those in the greater part of the infarct. In some cases, however, some of these vessels are distended, and in one or two cases small haemorrhages were*

observed in centre of the infarct near the endocardium. The veins on the epicardial surface are always filled with blood. In very recent cases the connective tissue (CT) in the centre of the infarct does not seem to be involved in the necrosis. Its nuclei retain their power of staining and are neither fragmented nor irregular. In older infarcts (three to five days) these have undergone degeneration".

Baumgarten also noted that in almost all cases the larger vessels, both arteries and veins, are still intact, that is, their cells exhibit no alterations in the protoplasm or in the staining power of the nuclei. The histological changes in infarcts of the dog's heart are identical in character with those in the hearts of the cat, and progress with equal rapidity. Local haemorrhagic infiltration occurred more frequently in the dog than in the cat (Baumgarten, 1899).

1.D. Animal experimentation.

By far the most thorough of the earlier studies on experimental MI was that published by Howard Karsner and John Dwyer in 1916 from the Laboratories of Pathology of the Harvard Medical School. They studied sections from the hearts of thirteen dogs stained with H&E, phosphotungstic acid haematoxylin (PTAH), iron hematoxylin, Mallory's CT stain, Verhoeff, Weigert and Unna elastica stains, Herxheimer Scharlach R method and Nile blue sulphate.

Observations were made serially on such infarcted areas: at about one-half hour after ligation of the vessel, the changes found were: congestion, interstitial oedema, several small haemorrhages, and slight diminution of transverse striation of the primary muscle bundles, which last change, however, is not apparent in the PTAH preparations. "*After twelve hours haemorrhage and oedema are found to be more marked, congestion not being notably increased. The muscle shows numerous irregularly outlined areas of partial or complete hyaline necrosis, in which the nuclei are either shrunken and condensed (pyknosis) or have completely disappeared. In these necrotic or partly necrotic area many polymorphonuclear leukocytes are found between the primary bundles, particularly in relation to the coarser capillaries. In addition a few more mononuclear both large (endothelial cells) and small (lymphocytes) are found. There is no evidence of active phagocytosis. Studied in the PTAH preparations there can be seen swelling of the anisotropic substance with lateral fusion of the fibrils, with the swelling there is disappearance of the light central band, diminution of the isotropic substance, and not infrequently lateral bulging of the*

bundle. The agglomeration of the swollen anisotropic substance produces the hyaline change. Associated with this change there are primary bundles showing much fragmentation of the anisotropic substance without fragmentation of the bundle, so that the bundle appears to be a vesicle containing coarse dark-stained granules. Frequently this change is observed in association with the presence of many leucocytes. Sudan-III fails to show fatty change...

Congestion, oedema, and haemorrhage are still conspicuous after twenty four hours, the hyaline change however being more extensive and associated with relatively smaller areas of non-hyaline muscle. The leucocytes are not notably increased in number but show alterations in shape as if in very active ameboid motion; the hyaline muscle shows considerable fragmentation and in some places the leucocytes appear to have dissolved muscle or to have destroyed it by phagocytic activity. The most notable changes are in the CT, particularly about the blood vessels and under the endocardium. There are numerous fibroblasts, some of which show mitotic figures . One capillary shows a mitotic figure in the endothelial wall. Cells appear which are of the type Anitschkow calls myocytes, and occasional muscle nuclei show similar central clumping of chromatin. Fatty degeneration is found in areas apparently corresponding to the hyaline change. Fragmentation degeneration is present in one of the two animals, but careful examination fails to throw any added light on the sarcolemma question. After forty-eight hours the congestion and oedema are not so marked as earlier, but the haemorrhage is still notable, the extravasated corpuscles showing no change. The hyalinized muscle shows increasing fragmentation and phagocytosis by leucocytes. Fibroblasts are numerous, show occasional mitotic figures and are now grouped about small masses of necrotic muscle. The living muscle in the neighbourhood of the necrotic areas shows the presence of multi nuclei and of budding forms... Fat is present in the necrotic areas...

After three days there are no very important changes except in the increasing demarcation of the necrotic areas. The muscle nuclei show beautifully budding, constricted, dumb-bell, and other confirmations such as one sees in cells of degenerate livers; multiple nuclei are frequent, usually lying end to end, but occasionally side by side. A peculiar granular degeneratin of the nucleus suggests mitosis, but although typical mitotic figures are seen in fibroblasts and capillary endothelium, nothing characteristic is found in the muscle. Fat is found in sharply demarcated areas corresponding to the areas of necrosis...

After five days the line between necrotic and living heart is emphasized by a well-defined zone of fibroblasts, in which active mitosis is found... After six days the general picture remains much the same. Perfectly typical plasma cells are now seen in moderate numbers... No mitotic figures were found in muscle. After eleven days the muscle in the middle of the infarcted area shows the late stage of coaugulation necrosis..., but about the edges the growth of the CT has proceeded remarkably... There are no mitotic figures in the muscle. Fat is found at this stage, not in the necrotic muscle but in the form of globules at the margin of the infarct partly in extracellular positions and partly within the protoplasm of fibroblasts...

After fourteen days the proportionate amount of muscle in the infarcted area has decreased considerably and the hyaline is very much fragmented, this material is less acidophilic than the living muscle and likely to retain the basic stain unless well differentiated... No mitotic figures could be found in the muscle. After eighteen days the infarct is present as a well-defined scar... The tissue of the scar is fairly dense, only moderately vascularized, poor in mitotic figures, containing remnants of hyaline muscle and showing intra-and extra-cellular deposit of haemosiderin granules... There are still present in the scar polymorphonuclear leucocytes, lymphocytes, and a few plasma cells... No fat is found...

Other animals were killed after sixty-one and seventy days respectively. The only important additional changes are condensation of the CT and disappearance of the polymorphonuclear leucocytes. Even after ten weeks there still remain unremoved fragments of hyaline muscle" (Karsner & Dwyer, 1916).

CHAPTER 2.

DEMONSTRATION OF MYOCARDIAL INFARCTION AND ISCHAEMIA.

The first detailed attempt to correlate the age of the infarct with the gross and histological findings was the publication of Levine and Brown's monograph in 1929. Levine and Brown presented analysis of the clinical features of 145 cases of coronary thrombosis and the pathological data of 46 of these from Boston, Massachusetts. Under the light microscope, different histological elements in the same pathological process may be demonstrated within the same infarcted area. "*Oedema and haemorrhage are likely to be the earliest manifestations of the acute insult. Shortly after this, necrosis of muscle fibres takes place and by this time leucocytes appear... All of this takes place during the first few hours and days... and may be present to some degree for several weeks*". Repair by connective tissue (CT) apparently follows a similar pathological sequence as encountered elsewhere in the body. In the centre of the infarct the necrosis will be most prominent, and usually the most marked CT activity takes place when necrosis has been most active. Sections from different areas of the infarction may show all three of the pathological processes: haemorrhage, necrosis and CT proliferation in different proportions. The myocardial tissues outside as well as inside the infarcted area may show fatty degeneration which of course is rather early evidence of damage to the cells. "*It is logical to assume that interference with nutrition takes place to a greater or less degree long before the acute insult from complete blocking of the circulation actually occurs.... In recent infarction the findings of sub acute or chronic changes in close approximation to the acute changes suggests that not all of the injury followed an acute insult, but probably was gradual or even somewhat intermittent in its nature*" (Levine & Brown, 1929).

2.A. Histology of human myocardial infarction.

Mallory et al. (1939) from the Mallory Institute of Pathology, Boston City Hospital, carried out the first large study on human material: it consisted of 72 autopsy cases, the material used in their investigation consisted of patients in whom MI was shown at autopsy and who had been thoroughly investigated clinically, so that conclusive evidence as to the onset, and therefore to the age, of the infarct was available (Mallory et al., 1939). The histological material available consisted of the routine autopsy sections of the heart most of which had been stained with phloxine

methylene blue, a few with H&E. In some PTAH, aniline blue CT stain, iron, and fat stains were also carried out. Histologically, the first result of stoppage of the blood supply to a portion of the myocardium was necrosis of the muscle fibres and, to a lesser extent, of the CT and smaller blood vessels. "*Although probably the necrosis of the muscle starts almost immediately, it is difficult or impossible to find any definite histological evidence of it until five or six hours have elapsed*". Later on other changes become evident: the most striking and generalised of these is that the involved myocardial tissue begins to appear somewhat hyalinised and stains more acidophilic. In H&E, and, to a much more marked extent in phloxine/methylene blue preparations the myocardial fibres appear much brighter red. The striations became somewhat more difficult to visualise, but do not disappear, and it should still be possible to identify in later stages when the muscle fibres have been broken into small fragments and have undergone phagocytosis by histiocytes. Other signs of necrosis occur in single isolated fibres or in small groups of fibres. In these the striations are not identified, the fibres are swollen and contain eosinophilic granules of various sizes, or large, irregular cross-bands. Haemorrhage into infarcts is somewhat variable, it is usually focal rather than diffusely seen throughout the infarct; the erythrocytes are most frequently seen within congested venules and capillaries, with rare extravasation around the muscle fibres.

The amount of fat present in an infarct depends largely on how suddenly the thrombosis or occlusion took place. As a consequence of the necrosis of muscle, the polymorphonuclear leucocytes are attracted and soon start to infiltrate around and into the necrotic muscle. This apparently begins about as soon as signs of necrosis can be identified in the myocardium. "*The first signs of infiltration are seen in infarcts during the first twenty-four hours. The amount of infiltration progressively increases during the first four days. At about forty-eight hours some of the polymorphonuclear leucocytes start to undergo degenerative changes, as evidenced by the loss of sharpness of their outlines and the accumulation of fragments of nuclear debris. By the fifth or sixth day many of the polymorphonuclear leucocytes have become necrotic and thereafter gradually disappear. By the fourteenth day they have practically completely disappeared... From the fourth to the eighteenth day varying numbers of eosinophiles may be found in the areas of infiltration... Newly formed blood capillaries can be found growing into the infarcted area, beginning on about the fourth day... Accompanying these blood vessels, fibroblasts can be seen to grow into the infarcted area...These basophilic fibroblasts have been found in infarcts ranging from 4 to 23 days...The removal of the necrotic muscle is the result*

of phagocytosis by mononuclear cells, probably histiocytes... Active absorption can be demonstrated to be taking place in infarcts as old as 6 weeks. After this time necrotic muscle may still be present, but signs of obstruction are difficult to demonstrate..." (Mallory et al., 1939).

Ian Lodge-Patch, from the Bernhard Baron Institute of Pathology at the London Hospital, in 1951, from a total of 216 cases of recent infarction that came to autopsy, selected 18 cases uncomplicated by rupture based on the availability of a clear history of the onset, along with adequate histological preparations (Lodge-Patch, 1951). The purpose of this study was to re-examine the sequence of histological changes in the myocardium in recent infarction. The findings of the study were then compared with those of Mallory et al. (1939): "*In the cardiac muscle the earliest sign of cellular degeneration is found in the nuclei, some of which become swollen and pale with a crenated nuclear membrane. This appearance was noted at 5 hours Later the nuclei become fragmented and disappear by karyolysis; others undergo pyknosis. At about 6 to 12 hours after coronary thrombosis, the cytoplasm becomes 'glassy', and the striations less prominent, although these may persist indefinitely...*". The inter- and intracellular oedema of the muscle fibres, and the nuclear changes mentioned above were the first to be identified. It was concluded that the age of the infarct can be accurately estimated within a range of 24 hours from its histological appearances during the first week and of wider range at later stages (Lodge-Patch, 1951).

2.B. Experimental studies.

Yokoyama et al. (1955) working in the Department of Pathology, North-Western University Medical School, Chicago, carried out an experimental study on 24 dogs. MIs of predictable location were produced in the left ventricle. The freezing-drying technique was employed as the main one used for the preservation and localisation of water-soluble substances. Blocks of tissue were also fixed in 10% buffered neutral formalin and in chilled absolute acetone. In some instances samples were fixed in Maximow's Zenker-formalin for 3 hours and in chilled alcoholic formalin (10% formalin in absolute alcohol) for 24 hours. The slides were treated in the following manner: Schiff reaction without prior oxidation, α-amylase treatment followed by periodic acid-Schiff reaction, H & E, alkaline phosphatase reaction, unstained section for examination with phase-contrast and polarising microscopes, Heidenhain's CT stain, and micro-incineration for inorganic constituents. Within one

hour they showed that after vascular occlusion a marked reduction in stainable glycogen occurs in the ischaemic area. This change is best demonstrated in frozen-dried tissue. Unfortunately it was also noted that this sequence of events is similar to that occurring in autolysis (Yokoyama et al., 1955).

Kent & Diseker (1955) in an experimental study on 15 dogs carried out histochemical studies on enzymatic concentrations in myocardial cells; Alkaline phosphatase, 5'-Nucleotidase, Succinic Dehydrogenase and Non-specific Esterase were looked for. A readily notable decrease in the glycogen content was detected by them as early as one-half hour after coronary artery ligation. Four hours following the development of ischaemia, the sarcoplasm of the damaged myocytes showed positive PAS reaction after diastase digestion, and its intensity increased with the increasing duration of ischaemic period. Small fat droplets began to appear in the ischaemic myocardium at about 9 hours of ischaemia and the amount of fat progressively increased with the passage of time. "*At 15 hours the succinic dehydrogenase activity in the ischaemic myocardium was decreased and was absent by 24 hours. Non-specific esterase activity was decreased in 18 hours and was largely absent in 24 hours*". Other histochemical reactions were unrewarding (Kent & Diseker, 1955).

Wartman et al. (1956) carried out similar experimental work on 40 dogs investigating the distribution of neutral fat in normal dog heart and in experimental MIs of predictable location. They used frozen sections stained with oil red O. They demonstrated slight and variable increase of neutral fat in ischaemic myocardium one to three hours after occlusion of a coronary artery, which after four to six hours became more obvious and later on they increased steadily during the next 18 hours (Wartman et al., 1956).

Bing et al. (1956) performed experimental coronary arterial embolization by plastic spheres in 23 dogs and evaluated the pathological changes at varying time intervals (between 20 minutes and 6 weeks). The hearts were fixed in neutral formalin and multiple sections of muscle were prepared and stained with H&E. Where this procedure revealed microscopical changes, serial sections were prepared and stained by Mowry's method for Alcian-blue (AB) and McManus' technique for PAS, Orcein-van Gieson (O-VG) and PTAH. Sections of representative areas were also stained for fat (Sudan IV) and PAS. The changes observed in this series are similar to those described in dogs by Karsner & Dwyer in 1916. The first changes, 20 minutes

following embolization, consist of small foci of coagulative necrosis (increased eosinophilia with loss of cross striations, pyknosis or loss of nuclei, on H&E). These necrotic areas showed increase in PAS reaction on AB-PAS, increase in orange colouration with O-VG stain, but no recognizable changes with PTAH or Sudan 1V methods (Bing et al., 1956).

Klionsky (1960) in an experimental MI study on 14 rabbits, examined transverse sections of the entire hearts which had been fused in various fixatives which included absolute alcohol and acetone at 4°C, picric alcohol-formalin, cold 10% phosphate-buffered formalin at pH 7, 10% calcium formol, Romeis' and Carnoy's solutions. Many specimens were quenched in isopentane at -160° C or in propane at -175° C and dehydrated by freeze-drying or freeze-substitution. Smaller fragments from the same hearts were examined with the electron microscope. The staining methods included H&E, Gomori's one-step trichrome, and Masson's trichrome stains. A variety of histochemical methods were also employed: Prussian blue reaction for iron and alizarin red S (McGee-Russell, 1958) technique for calcium were used on formalin-fixed tissue, Von Kossa's method for phosphate (Lillie, 1948) after alcohol fixation. A modified cobaltinitrite method for potassium (Crout & Jennings, 1957), on tissues fixed in alcohol and prepared by freeze-substitution. Aqueous and methanol PAS stains (Mowry & Millican, 1953) were performed following a variety of fixatives. Oil red O and Sudan black B in propylene glycol (Chiffelle & Putt, 1951) were used for the staining of fat. In a few animals, stains for succinic dehydrogenase, TPN diaphorase, DPN diaphorase, sulfhydryl and disulfide groups were also performed. Since changes with the latter enzymes were not demonstrable at 12 hours, these techniques were not systematically investigated.

2.C. Glycogen loss from cardiac myocytes.

It was found that in the ischaemic region, the earliest change detected was the rapid loss of glycogen. In tissue fixed in absolute alcohol and stained with PAS, a sharp zone of demarcation between normal and ischaemic muscle could be detected 5 minutes following coronary artery ligation. "*In arrested hearts, definite, clearly demarcated regions with partial loss of perinuclear glycogen could be demonstrated within one minute of ligation. Loss was complete within 5 minutes*". PAS-positive material which was not digested by diastase was demonstrated in the myocytes in tissues fixed in a variety of ways in the period after the loss of glycogen, and this was consistently absent before 60 to 90 minutes. "*This non-glycogen, PAS-positive*

material was first detected in very slight quantity at the periphery of 60 minute lesions. Thereafter, it was found in tissue fixed in formalin, Carnoy's, or picric alcohol solutions, more diffusely in the cytoplasm of injured cells throughout the damaged area". This material could not be observed in tissues which had been fixed in absolute alcohol or absolute acetone, or in those prepared by freeze-drying or by freeze-substitution in alcohol, until a period of 4 to 5 hours after coronary ligation had to elapse.

Regardless of the method of fixation, the PAS reaction was noted and showed coarse, cytoplasmic, particularly perinuclear glycogen, the basement membranes of capillaries, the sarcolemma, the intercalated discs and the Z bands; in chemically fixed tissue, diastase digestion removed only the coarse, perinuclear, presumably labile or unbound glycogen. The Z bands however, after absolute alcohol or acetone fixation, freeze-drying or freeze-substitution in alcohol or acetone, did not show PAS reaction following diastase digestion. Deposition of iron, calcium and phosphate was first seen as small, widely separated basophilic granules within the cytoplasm of myocardial fibers at the margin of the ischaemic zone. No deposits were noted earlier than 4 hours; deposits were minimal and inconstant at 4 and 5 hours after coronary occlusion, but were consistently present at the border of the ischaemic areas in 6 hours. Stains for potassium demonstrated the same pattern of flight to the periphery of the cell as did glycogen. After 21 hours of ligation, loss of cell potassium was evident by comparing the unstained cells with the heavy staining of potassium in oedema fluid. Fatty degeneration was not observed in 6 hours or less after coronary occlusion. At 12 hours, results were variable. As early as two hours after coronary ligation, the infarct could be detected in H&E-stained sections by means of cytoplasmic eosinophilia of the damaged fibres and by the perivascular infiltration with polymorphonuclear cells (Klionsky, 1960).

Shnitka and Nachlas (1963) in a study on 46 dogs in which MIs were produced, in addition to ordinary histological stains, used the Novelli's method for staining mitochondria (Novelli, 1959) and for the demonstration of succinic dehydrogenase (Succinic dehydrogenase is abundant in heart muscle of all mammalian species, which through the histochemical properties of Nitro-BT) (Nachlas et al., 1957), could be visualised in the mitochondria, at the poles of sarcolemmal nuclei and in A bands of myofibrils. The diphosphopyridine nucleotide (DPN) diaphorase, and cytochrome oxidase showed the same results of succinic dehydrogenase on frozen sections. In this study "*irregular swelling of mitochondria between myofibrils and at*

the poles of sarcolemmal nuclei was noted as early as 4 hours after coronary ligation".

These authors also substantiated the observations of Yokoyama et al. (1955) that patchy loss of myocardial glycogen was present at 1 hour and that by 4 to 6 hours large, confluent glycogen-free islands were formed and this zone of glycogen loss extended for a distance of 3 to 5 mm in general beyond the periphery of the infarcted area. "*Even though glycogen depletion of the myocardium is a very sensitive indicator of myocardial ischaemia, histochemical interpretation are frequently rendered difficult because of uncontrolled losses of glycogen from normal areas as a result of ventricular fibrillation at the time of sacrifice*". Furthermore, it was also noted that glycogen stores disappear rapidly as aresult of postmortem autolysis (Yokoyama et al., 1955; Klionsky, 1960).

2.D. Other micro-structural changes.

In conformity with Wartman et al. (1956), the authors "*observed that small droplets of stainable neutral fat, 1 to 2 mm in diameter, appeared between ischaemic myofibrils, usually opposite A bands of sarcomers, as early as 2 to 4 hours after coronary occlusion. By 6 hours, stainable neutral fat had largely disappeared from the infarct but persisted for as long as two and a half weeks in the form of small droplets between myofibrils in a narrow zone of reversibly damaged ischaemic muscle at the periphery of the infarct*".

Previous reports based on histochemical and biochemical studies (Kent & Diseker, 1955; Jennings et al., 1957) had indicated that succinic dehydrogenase begins to disappear from ischaemic heart muscle after a latent period of 4 to 6 hours, and by 15 hours has declined to 50% of its original specific activity. In contrast, in the study of Shnitka & Nachlas (1963) it was observed that in infarcts of 6 to 24 hours' duration, there was an increase in enzyme activity in the swollen and fragmented A bands of necrotic myofibrils occurred and by 24 hours, the over-all intensity of staining in myofibrils was often 2 to 3 times that of the normal heart muscle. After 24 hours, there was no succinic dehydrogenase activity present in the necrotic heart muscle and the activity of TPN diaphorase was shown to be too low to provide reliable cytochemical results (Shnitka & Nachlas, 1963).

Sommers and Jennings (1964) in an experimental study on 41 dogs, the circumflex coronary arteries of which were occluded temporarily in 27, and permanently in 14 cases, showed striking alterations in the architecture of the affected cells in the transient ischaemia group. "*The most prominent changes were the disruption of the regular myofibrillar pattern and the appearance of prominent contraction bands. Other findings were the prominent separation of fibers, intercellular oedema, and inflammatory cell infiltration within the infarct, and the loss of stainable glycogen and the development of a diffuse amylase-fast PAS positive reaction in the affected cells. These changes were obvious at 60 minutes*". In contrast, these changes were not histologically identifiable in the infarcts resulting from permanent occlusion of the same coronary artery until 2 hours after occlusion had lapsed. Marked histological changes similar to those observed in the transient ischaemia group were noted in only a few cells at the junction of the infarct and normal tissue (Sommers & Jennings, 1964).

2.E. Acid fuchsin staining.

Acid fuchsin which was originally described by Nielsen et al. (1958) is a widely used rosanilin dye chemically related to eosin has been associated for a lengthy period with the demonstration of myocardial ischaemia (Nielsen et al.,1958). Mallory et al. (1939) have described increased acidophilia (increased acidophilic staining with eosin) as one of the first histological changes seen in early myocardial infarction and this affinity for acid stain appears to be the basis for fuchsinophilia in degenerating myocardium. This tinctorial reaction is not specific and will demonstrate any area of acidophilic necrosis. It is also uncertain whether fuchsinophilic degeneration represents the direct result of hypoxia or a less specific end product or other injurious factors; indeed, the nature of the biochemical staining reaction is not understood, but a pH change or an electrolytic shift may give the sarcoplasm its affinity for acid fuchsin. Based on this feature Poley et al. (1964) applied the acid fuchsin staining method to detect early myocardial ischaemia in 35 hearts from human necropsies, comparing the acid fuchsin stain with the H&E stained sections. Positive and negative myocardial controls were processed in conjunction with the experimental groups. The positive control tissues were from five hearts which showed definite areas of infarction grossly microscopically in H&E-stained sections. Early necrosis associated with a neutrophilic infiltrate was identified in each section. The negative control tissues were obtained from hearts of

patients where death was instantaneous and due to trauma; blocks from seven such necropsies were available for the study.

In order to evaluate fuchsinophilic changes under more precise conditions, experimentally induced infarcts in three dogs were also studied; in these the myocardial ischaemic period was of known extent and duration. Each heart was fixed in 10% formalin and was sliced transversely. The slices were totally embedded in paraffin, and acid fuchsin and H&E stains were performed on tissue sections from each block. "*Sections from all five positive control cases showed marked fuchsinophilia in areas of neutrophilic infiltration. Other portions of the sections were less strikingly involved. In five of the seven cases of instant, traumatic death, no fuchsinophilia was present in the sections. The remaining negative control tissue (two cases) revealed a minimal degree of staining with acid fuchsin... Eight of the 12 cases of arteriosclerotic heart disease showed extensive and diffuse fuchsinophilia*". In contrast, the hearts which failed to show severe coronary arteriosclerosis tended to exhibit only minimal or moderate fuchsinophilia. Fuchsinophilia was also identified at the margins of healed infarcts. Marked degree of fuchsinophilia in the distribution of the ligated coronary arteries was shown in the experimentally induced myocardial infarct of three dogs and the degree of fuchsinophilia did not appear to vary with the age of the infarct. With the 30-minute and two-hour infarcts appearances were quite similar. H&E-stained sections from these hearts revealed no histological changes. There was only minimal fuchsinophilia in the septum of the myocardium taken from the control animal. The H&E stained sections of the control heart were entirely within normal histological limits. It was concluded that "*grading the degree of fuchsinophilia is necessary in the appropriate evaluation of an autopsy case...* but this method: "*only marked staining of extensive areas can be equated with early infarction*" (Poley et al., 1964).

Fine et al. (1966) ligated the left coronary artery in 36 rats by tying it proximal to its division. The chest and skin were closed after the ligation. One control animal was operated upon in the same manner as the study group but without arterial ligation, and was sacrificed after completion of the operation; while the other animal was not operated upon. The test rats were sacrificed at intervals varying from 5 minutes to 72 hours following the operation and complete transverse sections of the beating heart were immediately frozen and were stained with Oil red O, cytochrome and monamine oxidase. The dehydrogenases were studied by incubating medium containing 0.9 ml Nitro-Blue Tetrazolium (Nitro-BT) in phosphate buffer pH 7.1, 2

mg of di- or tri-phosphopyridine nucleotide (DPN, TPN). Nitro-BT and 2 mg of reduced di- or tri-phosphopyridine nucleotide (DPNH, TPNH) were used for the respective diaphorases. Unfixed 6 mm cryostat sections were used for the hydrolytic enzymes except for the azo dye method for alkaline and acid phosphatase and non-specific esterase. Post-fixation in acetone at 4°C for 15 minutes was performed for the phosphatases and another post-fixation in formalin vapour at room temperature for 15 minutes to apply the non-specific esterases.

In 26 of the 34 rats evidence of myocardial damage was shown by one or more of the histochemical studies. Three animals died during the operation, therefore they were not included in the study. In five rats the coronary artery ligation was considered unsuccessful because histological or histochemical changes were not demonstrable. "*The earliest changes with the H&E stain occurred in half an hour and consisted of left ventricular dilatation, vascular congestion, and interstitial oedema of a portion of the inter-ventricular septum and left ventricle; the sub-endocardial and sub-epicardial areas were uninvolved. These changes became more accentuated as the interval of ischaemia was prolonged. Polymorphonuclear leukocytic vascular margination and myocardial infiltration occurred at 6 hours and swelling, granularity, and eosinophilia of myocardial fibers were seen at ten hours*" (Fine et al., 1966).

2.F. Histochemistry of myocardial ischaemia.

The histochemical changes identified by Fine et al. (1966) varied in their intensity among the muscle fibres of the ischaemic myocardium with reactions being strongly positive in isolated cardiac fibres in the midst of negatively reacting muscle. "*The muscle immediately adjacent to the endocardium, epicardium, and blood vessels in the infarcted area showed less enzymatic depletion than the remainder of the myocardium supplied by the ligated coronary artery. The earliest alteration, diminution in glycogen and glycolytic enzymes (phosphorylase, branching enzyme, and uridine diphosphate glucose-glycogen transferase [UDPG]) occurred at 5 minutes in the area supplied by the ligated coronary artery. The presence of PAS-positive, diastase-resistant material was noted in the infarcted muscle at one and half hours and increased in amount as the period of ischaemia was prolonged... Irregularly distributed lipid in the ischaemic area was not observed until four hours and increased in amount thereafter. Other enzymes altered early in the process of myocardial ischaemia were found among the dehydrogenases and reductases. The*

earliest depletion, was noted at one and half hours and progressed with prolongation of the ischaemia... Depletion of the dehydrogenases requiring a coenzyme was most striking using DPN in the case of β-hydroxybutyrate, malate, and lactate substrates and TPN in the case of isocitrate substrate. A number of enzymes were found to be of no value in detecting early myocardial damage because of late depletion, inconsistent results, or minimal normal or negative reaction making their depletion difficult to detect.

The phosphate-splitting enzymes (ATP, 5'-nucleotidase, and alkaline phosphatase) were determined at pH 7.5 and 9.2 by the 'lead method'; alkaline and acid phosphatase, by the 'azo dye' method at pH 8.3 and 5.6 respectively. Enzyme activity was greatest at pH 9.2 and very slight and irregularly distributed at pH 7.5; the 5'-nucleotidase being the most reactive of the three at pH 7.5. With adenosine triphosphate as the substrate, enzymatic activity was present in the cardiac muscle and vascular smooth muscle. The capillaries were not positive. The reaction using muscle adenylic acid and b-glycerophosphate substrates was positive in the capillary endothelial cells and minimal or negative in the cardiac muscle... Non-specific esterase, aryl-sulfatase, and monamine oxidase activity was abundant in the cardiac muscle but they were not found useful in detecting early myocardial damage. The non-specific esterase and aryl-sulfatase enzymes were unaltered during the first 18 hours of ischaemia; they were not determined after this. Although monamine oxidase depletion was observed early in the ischaemic area, its significance is limited because of its uneven distribution in unaffected and control myocardium " (Fine et al., 1966).

Morales and Fine (1966) carried out a study on 11 clinically suspected early human cardiac infarcts without gross myocardial changes but with marked arteriosclerotic narrowing of one of the larger coronary arteries or thrombosis. Myocardium obtained 6 to 36 hours after death from areas supplied by the affected artery were quick frozen for enzyme staining, and fixed in formalin for H&E, azure eosin, acid fuchsin and Masson's trichrome staining and Carnoy's fluid for PAS staining with and without prior diastase digestion. Frozen sections were also used for the latter and the flaming red fat stain. Myocardium from two open heart operations were quick frozen and stained for enzyme activity.

Under anoxic conditions, as in infarction, the main source of energy derives from the anaerobic degradation of glycogen. It is logical to assume that the first histochemical

indication of myocardial ischaemia would be glycogen loss from the infarcted area. This finding was consistently demonstrated in experimental MI, within 5 minutes following ligature of the coronary artery (Klionsky, 1960). Consequent to anaerobic degradation of glycogen, there is production of pyruvate and lactate in the anoxic area and accordingly, this may lead to alteration of local pH. This along with an accumulation of anionophilic protein (Shnitka & Nachlas, 1963) could explain the increased acidophilia of anoxic myocardium not seen with the H&E stain until 6 to 12 hours after the onset of ischaemia. Although enzymatic loss from ischaemic human myocardium appears to be more easily interpreted and a better indicator of myocardial damage in autopsy tissue, but, there was also variation in the rate of loss of the various enzymes applied in the infarcted areas. (Morales & Fine, 1966).

Niles and Barnhouse (1967) in an experimental study on 34 rats collected blocks from the ventricular musculature and in addition to H&E stain carried out a modified acid haematein stain for phospholipids on them (Niles et al., 1964) using unfixed cryostat sections. The acid haematein test appears to reflect the physical state of the intra-cellular phospholipid and not its total amount as indicated by the observations after exposure to methanol-chloroform fixative. "*The change in the availability of the phospholipid to the stain which is due to un-masking by hypoxia is reflected by the increase in 'fattiness' as measured by the uptake of benzpyrene*". Physical molecular relationship such as a phospholipid-to-protein bond may be a more critical factor to assess in ischaemic damage, this can be affected by delicate environmental changes with a serious detriment of the cell (Niles & Barnhouse, 1967).

Lie (1968) repeated the acid fuchsin staining technique described by Poley et al. (1964) on the myocardium of 50 autopsy cases which were sub-divided into four groups: one group with definite clinical, biochemical, electrocardiographical and histological evidence of MI. The second group on clinical grounds has sustained an MI but there was no biochemical, electrocardiographical and histological evidence of MI. The third group showed coronary arteriosclerosis but no other evidence of MI, and the fourth group consisted of patients with other diseases which might produce secondary myocardial ischaemia. With this technique the ischaemic myocardium appeared dark red (fuchsinophilia) whereas the non-ischaemic myocardium appeared blue-green. Freshly procured sheep and ox hearts and of patients who had died from sudden traumatic death were used as the negative control tissues.

This study confirmed that the acid fuchsin staining technique is a sensitive method of demonstrating myocardial ischaemic damage in that all proven MIs were positive and 13 of the 30 probable cases were also staining positively. Lie (1968) also showed that the degree of positivity correlated well with the degree of ischaemic damage present. It was suggested that a pH change or an electrolyte shift may be responsible for the increased affinity for acid stains by the damaged myocardium, but why such staining should be specific in ischaemia was not clearly understood. It was particularly noted that in performing the acid fuchsin staining technique, the staining time and the stain concentration needed considerable adjusting and had to be guided by the appearances of the positive and negative control tissues in order to obtain optimal results (Lie, 1968).

Malinin et al. (1968) carried out an experimental study on 25 pigs with ten normal animals as controls. Gradual coronary arterial narrowing was produced by using a constrictor composed of hygroscopic plastic substance (ameriod) encased in a steel cuff to the left circumflex coronary artery 5 millimetres from its ostium. Pigs were chosen because of previous experience in experimental production of MI and the similarity of anatomical distribution of coronary arteries between humans and this species. The resected block of myocardium was immediately divided into portions. Pieces were frozen and other pieces were fixed in Carnoy's and Helly's fluid and in formalin. The stains performed on the latter were H&E, Maximow's haematoxylin-eosin azure 11, iron haematoxylin, and PTAH. Mitochondria were demonstrated by the Altmann-Metzger method, metachromasia by toluidine blue, and fuchsinophilia by the method of Poley et al. (1964).

Other histochemical methods used were:
For inorganic Ions: Iron (Ferrocyanide), Calcium (Von Kossa and Alizarin), Potassium (Sodium Cobaltinitrite).
For lipids: Oil Red O, Sudan Black B, Nile Blue Sulphate.
For nucleic Acids: Methyl Green-pyronin (MGP), Feulgen and Feulgen-oxidized tann-azo technique (FOTA) (Malinin, 1961).
For proteins: Ninhydrin-Schiff, Feulgen-oxidized tannin-azo technique (FOTA).
For carbohydrates: PAS reaction and Bauer's stain for glycogen.
For enzymes: Succinic dehydrogenase, Adenosine triphosphatase, Cytochrome oxidase and peroxidase.

In the H & E stained sections from the 10 control animals cytoplasmic striations and intercalated discs were clearly defined. In the MGP stained sections, nuclei were green, nucleoli, red and the sarcoplasm, dark red. The individual myofibrils were prominent revealing well defined cross-striations. In the sections stained by the FATO technique to show concomitantly protein and DNA, had Feulgen-positive nuclei, a pale perinuclear zone and uniform brown staining protein of the sarcoplasm. Metachromasia was not shown on the Toluidine blue-stained sections. Peroxidase activity was not demonstrated. Succinic dehydrogenase, cytochrome oxidase, and adenosine triphosphatase showed uniform reactions. PAS-positive reaction indicating the presence of glycogen was demonstrated best by the frozen section procedure.

In this study, the most useful stains, besides the H&E, were methyl green-pyronin and FOTA which demonstrated morphological structural aberrations of sarcolysis even in very early ischaemic damage, but their value was much more apparent at the 24 and 48 hour periods after constrictor application. Glycogen depletion though it was detedted in the earlier lesions, it was very difficult to interpret because of the irregular staining associated with muscle contraction. As a result of the rapid depletion of glycogen from the myocardium following death, this feature of irregular staining becomes even more difficult to interpret in specimens removed from animals found dead and as such it is of limited practical value in human pathology. Interpretation of enzyme changes and potassium loss are determined mainly on the comparison of colour intensity and quantity of the reactive granules. Enzyme methods in this series were difficult to interpret without constant referral to paired controls; controls obtained from the right ventriclular wall was not satisfactory because of differences in the muscle architecture. "*Fuchsinophilia provided no useful information in these experiments because of its irregular staining reaction in the controls as well as the test cases*" (Malinin et al., 1968).

2.G. Ultrastructural observations.

The tinctorial changes observed histologically are demonstrable also on ultrastructural studies. Acute ischaemic damage of the myocardium is accompanied by rapid depletion of glycogen granules from the sarcoplasm after 10 minutes, and after 40 minutes of ischaemia, glycogen is virtually absent (Jennings et al., 1965). Nuclear changes are also early and prominent signs of acute hypoxia and have been reported as early as 5 minutes (Caulfield & Klionsky, 1959) and as late as 4 hours

(Bryant et al., 1958); moderate degree of margination of nuclear chromatin was noted after 10 minutes (Jennings et al., 1965).

Mitochondrial changes also occur and usually begin after 10-20 minutes but are indistinct (Caulfield & Klionsky, 1959; Jennings et al., 1965); other prominent changes e.g. loss of cristae, dense intra-mitochondrial granules and some disruption of their membranes are well developed after 40 minutes of ischaemia (Jennings et al., 1965).

Evidence of changes in the components of the sarcoplasmic reticulum may be seen at 35 minutes, in that there is swelling, widening or vacuolization and they do not contain electron dense material (Caulfield & Klionsky, 1959). Associated with these changes is intra-cellular oedema manifested by separation of myofibrils. The contractile elements are the organelles most resistant to ischaemic damage. Decreased myocardial contractility may be reflected by early elongation or relaxation of sarcomeres but the myofibrils usually maintain their structural integrity until the later stages of the ischaemic event. Some authors have described transient contraction bands after a period of 20 minutes (Caulfield & Klionsky, 1959). Changes in the intercalated discs are not usually present in the early hours of ischaemia and it is believed that the early oedematous separation or widening of the intercalated discs is an important mechanism in dilatation of the heart (Poche, 1969).

Acute ischaemic changes, induced by coronary occlusion, appear to be completely reversible up to 15 minutes, while irreversible damage occurs after 30 minutes or more (Jennings et al., 1965). In animal studies, following the production of ischaemia in the posterior papillary muscle of the dog after the occlusion of the circumflex artery, the ischaemic cells were shown to be reversibly injured for the first 15-18 minutes of ischaemia, a few isolated cells or groups of cells died after 20-25 minutes of ischaemia, about 50% of the cells were dead after 40 minutes, and after 60 minutes of ischaemia most of the cell were dead (Jennings et al., 1969). Experimental work on transient coronary ligation in the dog, followed by restoration of blood flow, revealed a more rapid development and extension of abnormalities than those produced by epuivalent periods of ischaemia as a result of permanent vascular occlusion (Herdson et al., 1965).

For obvious reasons there have been few ultrastructural studies of acute ischaemia in the human myocardium. The changes of chronically ischaemic heart are similar to the effects of chronic hypoxia in the rat heart. These abnormalities include swelling of myofibrils, vacuolated appearance of the muscle fibres and separation of the membranes of the intercalated dics (Sulkin & Sulkin, 1965).

A problem made obvious by EM is the apparent lack of demarcation of an ischaemic area in the earliest stages of damage. Many histochemical procedures applied for the demonstration of morphological of ischaemia are based on the delimitation of such zones. In theory, it is expected that there will be a defined territory of damage from the earliest events after the acute episode. In practice, however, it was shown that many cells did survive for some period in the central parts of ischaemic areas. In that not all cells of a the affected tissue are uniformly and simultaneously involved in the pathological process (Herdson et al., 1965; Jennings et al., 1969). Also within individually affected cells, not all organelles are uniformly involved, e.g. normal mitochondria are seen intermingled between damaged mitochondria, and zones of segmental damage may be randomly distributed within the sarcoplasm. Therefore, in the earliest stages of injury it is not possible to map out on a fine structural basis a well defined area of infarction. Ultrastructurally, autolytic changes mimic early ischaemic changes quite closely and include rapid depletion of glycogen, elongation of sarcomeres, clumping of nuclear chromatin and increased numbers of lipid droplets in the sarcoplasm (Bryant et al., 1958; Hibbs & Black, 1963).

Consequently, it is unrealistic to expect to obtain acutely ischaemic human myocardium immediately after death to be of value in the estimation of the duration of ischaemia by ultrastructural studies. Accordingly, deaths due to acute myocardial ischaemia would seem to fall into two categories: first, those who die unattended or outside hospital and with a long postmortem interval; second, those in which the acute attack receives medical attention, possibly from a coronary-care unit close at hand, and with the benefit of vigorous cardiac resuscitative procedures which may themselves induce significant morphological alterations in the myocardium. Such damage will obviously obscure the interpretation of any underlying ischaemic changes (Wolfe et al., 1968; Heggtveit, 1969).

2.H. Lie's stain.

Lie et al. (1971) described a new, non-enzymatic, histochemical technique, the haematoxylin-basic fuchsin-picric acid (HBFP) stain which is applicable to routinely processed formalin-fixed and paraffin-embedded tissue. The technique was performed on experimentally produced myocardial infarcts of 1/2 to 48 hours' duration obtained from dogs. Hearts were also obtained at autopsies of patients with diagnosed or suspected early MIs. These autopsies were performed on un-embalmed bodies within 20 hours of death. Both the animal and the human tissues were formalin-fixed and paraffin-embedded, and sections cut at a thickness of 5-6 μm were stained with H&E, periodic acid-Schiff (PAS) after diastase digestion, and the hematoxylin-basic fuchsin-picric acid (HBFP) stain.

The HBFP technique stained the normal human and dog myocardial tissue in a similar pattern. After decolourisation in picric acid-acetone, the normal myocardium lost its affinity for the basic fuchsin dye and uniform light brown staining by the picric acid was noted. The myofibrils and cross-striations were clearly recognizable, and the nuclei retained the usual purple hue of the counterstain: hematoxylin. The occasionally irregular fuchsin staining of a few myocardial cells seen at the edges of the section was considered to be artifactual and was disregarded.

In HBFP-stained sections, fuchsin staining of the ischaemic myocardium was noted long before any identifiable change in the H&E-stained sections. Positive reactions in these studies were apparent as early as 30 minutes after the ligation of the coronary artery. The portions of the myocardium which were likely to be ischaemic were not decolourized by picric acid-acetone, and retained the brilliant crimson-red colour of basic fuchsin. Normal fibres stained light-brown. The staining was most intense in peri-nuclear portions of the sarcoplasm.

"The number of fuchsin-stained fibres and the intensity of staining of individual cells decreased with longer intervals after the onset of myocardial ischaemia. After 6 hours, when the myocardium showed early but definite features, the HBFP-stained sections remained strongly positive. After 48 hours, when the myocardium appeared frankly necrotic and the polymorphonuclear inflammatory infiltrate was reaching a peak, fuchsin staining had completely disappeared and the dead muscles stained a slightly darker hue of brown than did normal fibres. However, fuchsin-stained cells were frequently seen among fibres that appeared normal in H&E-stained sections.

These positive cells were some distance away from the infarct. The fibroblastic granulation tissue of healing infarcts did not stain with basic fuchsin... In hearts with presumed infarcts of less than 6 to 8 hours' duration, the HBFP technique was clearly positive in the regions suspected to be ischaemic and demonstrated early ischaemia of the myocardium in areas that were normal or not diagnostic with other staining. Additionally, the HBFP stain demonstrated the presence of ischaemic fibres surrounding acute MIs, these fibres often appear normal in H&E-stained sections" (Lie et al., 1971).

The pattern of HBFP staining of both normal and ischaemic myocardium were reproducible in serial sections of the same block of tissue even if they were stained in separate batches. Postmortem intervals of up to 36 hours showed identical staining pattern in dog hearts. The critical step of the technique appeared to be the decolourisation process by picric acid-acetone. "*The relatively greater resistance to decolourisation by acetone of fuchsin-stained ischaemic myocardium, compared to normal myocardium, was not an all-or-none phenomenon but rather a time-dependent differential resistance. The optimal decolourization time was about 20 seconds for human tissue and about 15 seconds for animal tissue. Under-decolourization (less than 10 seconds) resulted in false-positive fuchsin staining of normal myocardium, whereas over-decolourization (more than 60 seconds) led to loss of positivity of ischaemic myocardium*" (Lie et al., 1971).

Aldehyde blocking and acetylation before staining by the HBFP technique did not produce changes in the results. However, de-amination before staining did not show positive fuchsin reaction of the ischaemic myocardium. Except for glutaraldehyde fixation, the results of HBFP staining were not altered by the use of different fixatives. Alum haematoxylin appeared to be an essential mordant in the HBFP staining method. Among the different haematoxylins tried, those alum haematoxylins which contain glycerol were the suitable substitutes. In case these solutions contain less than 0.5% haematoxylin, it was necessary to increase length of the staining time in order to obtain good results. All commercial brands of basic fuchsin and related compounds tested, except those containing para-rosaniline base, gave comparable and satisfactory results in the HBFP technique. It was shown by the authors that the commercially available dyes which are sold as basic fuchsin are not pure chemical compounds but are mixtures of three triaminotriphenylmethanes which differ from one another in the number of methyl groups attached to the benzene rings.

Although the HBFP staining method was found to be simple, rapid, and reproducible, it was advised by the authors that positive and negative controls had to be treated with each batch of sections under test until complete familiarization with the technique, especially the decolourization process, has been achieved.

The HBFP technique differs from acid fuchsin stain (Poley et al., 1964) in both method and results. In order to avoid confusion with term 'fuchsinophilia' which is commonly used for positive results with the acid fuchsin stain, the term 'fuchsinorrhagia' was suggested for the positive fuchsin staining of ischaemic myocardium by the HBFP stain. The authors suggested that "*the technique provided a vivid tinctorial demonstration of early myocardial ischaemia before the appearance of diastase-resistant PAS-positive material or definitive changes in H&E-stained sections. Normal myocardium and frankly necrotic myocardium did not stain. Positive results were reproducible and unaffected by postmortem intervals of up to 36 hours*" (Lie et al., 1971).

Bouchardy & Majno (1971/72) described a new histopathological feature in cardiac myocytes. Their study consisted of 65 cases of myocardial infarction with autopsy usually performed within 12 hours. The slices were stained with nitro-blue-tetrazolium (NBT) method and later were fixed in formalin. The sections were stained with H&E, and occasionally with Van Gieson, PTAH and reticulin. By light microscopical observation of the edge of the infarcted zones, as noted on the gross specimen by the NBT method, it was noticed that the myocardial fibres had become distinctly wavy. This wavy appearance developed into the more standard histopathological features of myocardial infarction. This waviness occurred in histologically obvious myocardial infarcts aged 48 hours or more. It was found in lesions which were recognized by the NBT method. It was also seen in cases clinically labelled as more recent than 6 hours, and did not show any of the known histological features of infarction.

This phenomenon could be explained by the fact that the heart wall at the site of an infarct tends to bulge; its histological counterpart must be a stretching of the fibres. Whether these wavy fibres are actually dead is not possible to conclude; however the stretching probably starts as soon as heart contraction stops and at that point, the fibre is not yet necessarily dead (Bouchardy & Majno, 1971/72).

Zugibe & Zugibe (1973) investigated the reliability of fuchsinophilia and fuchsinorrhagia by examining myocardial specimens of formalin-fixed, paraffin-embedded tissue taken from the centre of each myocardial sample and stained with the fuchsinophilia method of Nielsen et al. (1958) and by the fuchsinorrhagia method of Lie et al. (1971). The cases were subdivided into four groups: group 1 of 58 cases of histologically proven myocardial infarction aged less than 24 hours; group 2 of 70 cases of early myocardial infarction with no gross or histological evidence of myocardial infarction but in which the autopsy showed complete occlusion of a major branch of a coronary artery and with correlative clinical symptoms; group 3 of 25 cases with clinical myocardial infarction in persons dying in less than 12 hours as determined by combinations of clinical signs, electrocardiogram changes, or enzyme determinations or all three; group 4 of 147 cases without myocardial infarctions, the patients in this group dying of terminal cancer, traffic accidents (of infants, youths and young adults), multiple myeloma, hepatic cirrhosis, strangulation, stabbings, drug overdoses.

The results of staining with both the fuchsinophilia and fuchsinorrhagia methods using a double-blind technique were shown to be too erratic to be of any value in distinguishing an early MI. "*The decolourization process with picric acid-acetone in the fuchsinorrhagia method within the limitations set by the authors resulted in as many positive findings in the controls as in the cases with complete coronary occlusion and definitive myocardial infarctions less than 24 hours old*" (Zugibe & Zugibe, 1973).

Nayar & Olsen (1974) re-examined 60 formalin-fixed, paraffin-embedded blocks of myocardial tissue taken from autopsy cases of the Hammersmith Hospital, London, in which death was clinically attributed to MI. These were stained with H&E and the HBFP stain of Lie et al. (1971). The survival time of these patients after the clinical episode of myocardial infarction varied between 30 minutes to 5 hours. Based on the pathological changes seen in the coronary arteries and aided by the electro-cardiographs, the cases were divided into three groups: Group (1): 28 hearts with recent total coronary occlusion; Group (2): consisted of 17 hearts with atheroma which had resulted in narrowing the coronary artery lumen to half or less than that of the original size; Group (3): consisted of 15 hearts with no significant coronary atheroma. Old myocardial infarction was present in 29 hearts (18 in group 1, 9 in group 2, and 2 in group 3).

"*Haematoxylin-eosin preparations of the myocardium did not show the changes of early infarction in any of the cases. With the HBFP stain, fibres that stained crimson-red were present almost uniformly throughout the section in all cases belonging to group 1 with the intensity of staining was maximal in the central part of the fibre. Fourteen cases (82.3.%) in group 2 and 12 (60%) in group 3 stained positively with basic fuchsin. HBFP stain were observed in 42 of 45 cases with significant coronary atherosclerosis (groups 1 and 2). Fuchsinorrhagia was invariably present in the hearts with acute coronary occlusion (group 1). Its absence in 3 of 17 cases in group 2 could be attributed to the failure to obtain a representative area for histology*" (Nayer & Olsen, 1974).

Lichtig et al. (1975) induced myocardial ischaemia in eight pigs by ligating the anterior descending coronary artery for periods of 30 minutes to six hours. "*As emphasized by Lie et al. (1971) and confirmed by a personal visit to their laboratory by Lichtig, the critical stage in the procedure is the decolourisation with picric acid*". Ten serial sections were prepared from the same block of either normal or ischaemic myocardium and each section was stained separately with the only difference being the decolouriation time with picric acid. From a 15 second decolourisation time, the time was gradually increased by ten second intervals so that the decolourisation time by picric acid of the last slide was 105 seconds. Serial sections stained simultaneously and at different periods of the day in different days and sections from fresh frozen unfixed material from a few cases were also treated in the same manner and the results compared.

Generally, both normal control and ischaemic myocardium were totally decolourized by 55 seconds. "*Neither different periods of the day nor staining in fresh solutions on different days gave any reproducible pattern in two to five slides of normal control and ischaemic areas...Frozen sections of un-fixed material from both normal and ischaemic zones failed to reveal any relationship between depth in the block or fixation and the reproducibility of results; no consistent or dependable results could be recognised*". The authors concluded that the method is unreliable for demonstrating areas of ischaemia in swine hearts and cannot be used as a routine test of early myocardial ischaemia in such experimental work (Lichtig et al., 1975).

Rajs & Jakobsson (1976) in a study to evaluate the HBFP of Lie et al. (1971) investigated 148 medicolegal autopsies. The cases were divided into 3 groups: group 1 composed of 42 cases who had died with symptoms of coronary disease and

autopsy showed features of macroscopical and/or microscopical myocardial infarction with routine staining methods (H&E and PTAH stains). Group 2 composed of 50 cases who who died suddenly and showed severe coronary arteriosclerosis and a suspected myocardial infarction which was not proven by the routine histological methods applied. Group 3 composed of 56 cases who died of non cardiac conditions and the time between the accident or onset of symptoms and death ranged from several minutes up to 16 days. A comparative study was also performed on 47 rats with induced MI. All samples were formalin-fixed, and paraffin-embedded and stained with H&E, van Gieson, PTAH, acid fuchsin (Poley et al., 1964) and HBFP with each slide being treated individually.

In group 1 there was usually an intense crimson-red staining of the myofibres. In those with already well developed older infarcts, a positive HBFP staining could be seen only at the margins of the infarcted area. Different patterns of staining were seen in serial sections from the same block of tissue and thus it was impossible to achieve an objectively graded interpretation of the staining reactions. In group 2 the majority of the cases showed positive staining, often in areas which (due to lesions in the coronary arterial tree) were highly suspected of demonstrating myocardial ischaemia but positive staining was also seen in myofibres not likely to show any disturbances. The staining reaction was noted in the peri-nuclear regions and/or in the proximity of the intercalated discs. A comparison of H&E and HBFP stained sections often failed to demonstrate an identical distribution of positively stained areas. In group 3 many myocardial sections showed positively stained areas, especially in the sub-endocardial regions but also in this group serial sections did not show reproducibility of the stain. When the positively stained myofibres were examined at higher magnification, it was not possible to determine the validity of the reaction and whether the positive staining was artefactual e.g. due to poor fixation, haemolysis, insufficient decolourisation, mechanical impact, or due to ischaemic episodes. In all groups, when waviness of the myofibres (Bouchardy & Majno, 1971/1972) was noticed, there was no positive correlation with the positivity of the HBFP stain.

A comparison of the uptake of basic fuchsin stain of Lie et al. (1971) and acid fuchsin stain of Poley et al. (1964) showed inconsistent results. Four of the control rats and 10 of the 13 rats that died immediately upon coronary ligation showed positive staining often in close proximity to the area of the heart which had been handled or injured during the operation with the pattern of the HBFP-stain uptake

was very similar to that observed in human hearts. It was also difficult to obtain reproducible staining results with serial sections of rat hearts taken from the same block. The authors also noted that they failed in their efforts to standardise the differentiation time and they concluded that the HBFP stain is not adequate for routine use in forensic pathology to enable a decision to be taken as to the cause of death. It was also not easy to determine the cause of a positive HBFP staining of human myocardial tissue, whether it could have been due to an antemortem local or general hypoxia, an agonal lesion, or could it have been attributed to post-mortem changes (Rajs & Jakobsson, 1976).

Van Reempts et al. (1976) carried out another series of experiments to evaluate the HBFP method for the morphological diagnosis of early myocardial ischaemia. The experiments were performed on 14 dogs of varying age, weight, and sex. The tissue samples were fixed in one of the following: neutral buffered formalin 4%, neutral buffered formalin 10%, paraformaldehyde and Bouin's fluid with fixation times were 24 hours, 48 hours, 72 hours, 6 days, and 3 weeks. Staining was performed on four to six slides from each control case. Staining, rinsing and differentiation times were varied in a large number of combinations. "*The HBFP-stain gave no acceptable information about ischaemic and non-ischaemic cells or areas. A positive reaction was seen in controls as well as in ischaemic samples, while sometimes ischaemic tissue remained negative. Artificial staining on the edges of the sections was also observed*" (Van Reempts et al., 1976).

Armiger et al. (1977) assessed tinctorial demonstration of infarcts in a series of experimental infarcts of the posterior papillary muscle of the canine heart; six special stains were evaluated. The infarcts were of 5-720 minutes duration and were compared with normal control myocardium. The tissue was stained with H&E, PAS, PAS-diastase, a combined PAS-myofibril stain of Puchtler et al. (1969)-(Puchtler's PAS-tannic acid-phosphomolybdic acid-levanol, PTAH, Masson's trichrome, acid fuchsin, and Lie's haematoxylin-basic fuchsin-picric acid. In the H&E-stained sections the earliest changes (after 60 minutes of ischaemia) detectable within the ischaemic area following coronary occlusion were slight separation of the myocardial fibres and variable and patchy eosinophilia. These changes were more clearly evident after 120 minutes, when many nuclei showed obvious early pyknosis, more fibre separation; the paler staining fibres appeared granular and contrasted sharply with the adjacent more eosinophilic fibres. At this time, the infarct was defind by a zone of severely damaged myocardial fibres which showed contraction

bands. After 4-6 hours of ischaemia, margination of neutrophils was seen in the small vessels at the edges of infarcted areas in most cases , and neutrophilic infiltration of the necrotic tissue was evident after 8-12 hours of ischaemia. The PAS stain showed glycogen depletion, following 40-60 minutes of ischaemia or autolysis and total loss of stainable glycogen occurred following 2 hours of such conditions. Masson's trichrome stain did not help in distinguishing between the normal and the damaged areas in infarcts of less than 2-3 hours' duration. However early border zone damage and variations in the cytoplasmic eosiniophilia of myofibres within an infarct sometimes were easier to identify than in the H&E stained sections. PTAH demonstrated damage more clearly than H&E. In infarcts of 2 or more hours' duration the severe cellular disruption of the border zones was very distinctly seen as well as granularity of myofibres and apparent loss of cross striations. The combined PAS-myofibrillar stain, demonstrated both glycogen depletion and structural alterations in the myofibres, and in this aspect it performed relatively better than the other stains assessed. The acid fuchsin showed rather variable differentiation from batch to batch of serial sections. Lie's stain showed positive reaction in the border zones of infarcts which were 2 or more hours old, but ischaemic tissue within infarcts stained like normal myocardium and the diffuse fuchsinorrhagia described by Lie et al. (1971) was not noted.

To evaluate changes due to autolysis infarcts with different postmortem intervals were stained. They did not show any variation which could be attributed to autolysis with the exception that the differences in stainable glycogen between ischaemic and normal myocardium are difficult to distinguish from those occuring during autolysis. Therefore the PAS stain was of no value for early infarct detection in human post-mortem material but the combined PAS-myofibrillar stain appears to be of value for experimental investigations. Both the acid fuchsin and Lie's stains proved capable of demonstrating junctional damage very clearly, but failed to give the diffuse positive reaction of early infarcts described by their original authors (Armiger et al., 1977).

Sakurai (1977) studied lipid metabolism in infarcted myocardium of twenty three dogs both morphologically and biochemically. The study suggested that myocardial necrosis may be a contributing factor causing the elevation of serum free fatty acids (FFA) levels in acute myocardial infarction (Sakurai, 1977).

Fishbein et al. (1978) carried out a study to re-examine more quantitatively the evolution of myocardial infarction and to determine the prevalence of two

pathological features: wavy fibres and myoctyolysis. They reviewed the medical records of all autopsy-proven myocardial infarcts who died between January 1964 to December 1973 at Peter Bent Brigham, Boston. Only those cases in which the determination of the age of the infarct could be made on the basis of clinical data and had fulfilled the following criteria was included in this study: (1) adequate clinical history so that to determine the clinical age of the infarct (2) definite time of onset of symptoms (3) no clinical evidence of repeat or extension of infarction and (4) infarcts must have been less than 90 days old.

Histological sections of 192 cases who met these criteria were stained by H&E, Masson's trichrome, Mallory's stain for iron, von Kossa's for calcium salts. Sections included the junction of normal and infarcted tissue, as well as the central portions of the infarcts. The following features were specifically looked for: coagulation necrosis, wavy fibres and focal myocytolysis. Necrotic fibres were clearly evident in 90 % (45/50) of the patients died during the first day of infarction. These fibres showed increased eosinophilia, loss of cross-striations, granular cytoplasm and nuclear changes of necrosis but often, normal myocardial fibres were found within the areas of necrosis. In the other 10 % (5/50) of the patients who died during the first day of infarction, only thin wavy fibers separated by inter-cellular oedema were present. Such wavy fibres were present in 94 % (47/50) of the infarcts that were 24 hours or less in age and even in cases of one hour-old clinical infarcts. In 8 % (4/50) of the infarcts that were less than 24 hours old, there was focal myocytolysis characterized by the presence of vacuolated fibres. These degenerated myocardial fibres were accompanied by infiltration of macrophages and by deposition of collagen in older infarcts. Contraction bands were also seen in fibres at the edges of the infarct. Focal myocytolysis occurred in 16 % (19/121) of the infarcts that were 1 to 7 days old and in 19 % (36/192) of the infarcts of all ages. Seventy-six % (38/50) of the infarcts that were 24 hours old or less showed necrotic fibres surrounded by blood vessels revealing pavementation by polymorphonuclear leucocytes, in the peri-vascular connective tissue and between myocardial fibres. Congested blood vessels, interstitial oedema and focal haemorrhage were noted in infarcts by 24 hours.

The terms 'coagulative myocytolysis' & 'contraction band necrosis' (Baroldi, 1975) and 'myofibrillar degeneration' (Reichenbach & Benditt, 1968), have been used to describe degenerative changes of myocardial fibres that were found to result from hypercontraction or spasm of the fibres and from compression by adjacent

sarcomeres. These changes have been also observed in association with electrical shock, deficiency of potassium, administration of catecholamines, coronary arterial reperfusion and death after cardiac surgery (Morales et al., 1967). Although the primary event leading to the formation of the 'contraction bands' is unknown, most often they probably develop in areas of reflow after ischaemia (Kloner et al., 1974). The term 'focal myocytolysis' (Schlesinger & Reiner, 1955) has been used to describe focal abnormalities, mainly identifiable in the subendocardium and in peri-vascular regions, which were characterized by progressive vacuolization of fibres due to lysis of the contractile elements until empty sarcolemmal tubes are seen only. Since myocytolysis was present in only one-fifth of the cases studied, it was not easy to relate their occurance to ischaemia 'per se' as they could have been due to hypoxia, drug therapy, prolonged survival in patients with cardiogenic shock, or other factors. The high incidence of wavy fibres in recent myocardial infarcts provides supportive evidence that they are a result of ischaemia and this finding may thus serve as a useful criterion to aid in the morphological identification of early myocardial infarction; however, these wavy fibres may also be found at the edge of infarcts of different ages. The authors "*suggested that wavy fibres appear to be the earliest light-microscopic change present. While finding of myocytolysis is not useful in dating infarcts, this change probably indicates metabolic abnormalities of the myocardium that are not related to ischaemia per se*" (Fishbein et al., 1978).

Derias & Adams (1979) carried out a study to evaluate the phenomenon of wavy fibres in twenty-eight human hearts in whom there was no clinical history of angina, infarction or hypertension. Their ages were between 18-77 years and none of them showed evidence at autopsy of myocardial infarction and/or coronary occlusion. Another heart was also obtained from an infant aged 6 weeks, who did not die of cardiovascular disorder. Thirty-one hearts were obtained from subjects aged 25-84 years, in whom post-mortem and microscopical findings of myocardial infarction were evident. As controls, heart muscle was also obtained from 16 normal albino rats. All sections were stained by H&E. "*Wavy fibres were seen in about half the normal and half of the infarcted human hearts; they were even present in the heart of the 6-week-old infant and were prominent in older subjects. Such fibers were indistinguishable from those around areas of myocardial infarction... Wavy fibres were also seen in the myocardium in eleven out of sixteen normal rats examined*". The authors concluded that "*the wavy fibres are caused by uneven contraction of myocardial fibres brought about by patchy preservation or loss of ATP. Such*

changes may result from infarction or from post-mortem autolytic changes" (Derias & Adams, 1979).

Knight (1979) studied a series of 50 cases to evaluate the reliability of the HBFP stain by taking tissues at medicolegal autopsies. The post-mortem interval in almost all the cases selected for the study was of the order of one day. In those cases in which well-developed myocardial infarction of 12-24 hours' duration was obvious, demonstrable by both gross enzyme and histological stains such as H&E, PTAH, PAS, then the HBFP stain was uniformly strongly positive. In fact, as noted by Lie et al. (1971), the HBFP staining in the older infarcts, reverted to negative in many cases, though the changes were very patchy. The main interest in the study was in the detection of very early infarction. Its main disadvantage appears to be the very frequent occurrence of false positive results, where positive staining of the myocardial fibres, claimed to be evidence of abnormality was present without any other collateral evidence of cell damage using other techniques. Although the possibility that HBFP abnormalities could be genuine criteria of cell damage, until further proof is forthcoming of this possibly exquisite sensitivity of HBFP, the author suggested that "*its use must be critically evaluated and any interpretations based upon HBFP results used with extreme caution, especially in medicolegal situations*" (Knight, 1979).

2.I. Fluorescence studies.

Siegel & Fishbein (1982) examined whether the fluorescence microscopy would be of value in the evaluation of H&E-stained sections for the presence of myocardial necrosis in a series of 86 patients. Multiple histological sections from 16 cases demonstrating myocardial necrosis of different durations were selected for further examination by fluorescence microscopy. In addition, myocardial tissues from 20 dogs in which experimental myocardial infarction had been induced by closed-chest balloon occlusion of the left anterior descending coronary artery were also studied. Duration of MI was three hours in seven dogs, six hours in seven dogs, and seven days in six dogs. The following methods were performed: 1) triphenyltetrazolium chloride (TTC) staining for gross examination of myocardial necrosis 2) histological examination of whole-mount H & E-stained sections of TTC-stained slices, 3) histochemical evaluation of the extent of ischaemia using Carnoy's-fixed PAS-stained serial sections, 4) electron microscopical evaluation from normal areas and areas with TTC and histological evidence of necrosis.

In 13 of 16 human cases, all areas which showed features of coagulation necrosis by routine microscopy had corresponding yellow fluorescence of eosin stained areas. In three of the nine cases with infarcts of less than 24 hours' duration the abnormalities were patchy with only focal cytoplasmic hypereosinophilia. Examination of these areas by fluorescence microscopy also showed patchy yellow fluorescence that was was confined to those hypereosinophilic fibres. In the six dogs, non-necrotic areas also showed fluorescence, but not as brightly and the fluorescence had a green-to-brown tint rather than a bright yellow one seen in the necrotic foci. Examination of the seven hearts with 6 hour old infarct by fluorescence microscopy revealed yellow fluorescence of the necrotic regions in the five dogs in which hypereosinophilia of fibres was present. In the two dogs with infarcts in which hypereosinophilia was minimal, there was also only focal minimal yellow fluorescence, which corresponded to the hypereosinophic foci seen by routine light microscopy. Fluorescence microscopy of the seven dog hearts with occlusion of three hours' duration showed bright yellow fluorescence in only the three hearts in which hypereosinophilia of fibres had been observed by light microscopy. In those hearts with TTC positive staining and electron microscopical evidence of necrosis but no hypereosinophilia, there was no yellow fluorescence.

This study suggests that the fluorescence findings indicative of necrosis are related to hypereosinophilia of necrotic fibres and since only these hypereosinophilic fibres show the characteristic yellow fluorescence, the method appears to offer no distinct advantages over the routine light microscopical technique (Siegel & Fishbein, 1982).

Al-Rufaie et al. (1983) did a retrospective study on autopsy material from 30 patients who have died after a clinically diagnosed myocardial infarction to compare the reliability of the fluorescence of infarcted myocardium when stained by H&E and an adjacent section stained by the haematoxylin basic fuchsin picric acid (HBFP) method to detect early ischaemia. The cases were divided into 2 groups based upon the clinical history and on the histological abnormalities seen in the sections. In group 1 there were 14 cases with evidence of less than 6 hours of ischaemia and group II consisted of 16 cases with a history of more than 6 hours and up to 36 hours of ischaemia. In 28 of the cases the coronary arteries showed either complete occlusion or moderate to severe atherosclerotic changes of one or more of the major vessels. In 2 cases there was no vascular abnormalities. Careful analysis and by comparing group I with group II it was found that the colour changes were

independent of the duration of ischaemia, and were not related to the patient's age. Normally perfused myocardium was seen as dull olive green or red/brown with a clearly demarcated cell wall. "*The areas defined by fluorescence were then compared with the crimson red stained areas in the HBFP stained sections. It was found in all cases that they coincided exactly*" (Al-Rufaie et al., 1983).

2.J. The modified Luxol Fast Blue stain.

Arnold et al. (1985) introduced a modification of the Luxol Fast Blue (LFB) as a specific marker of myofibrillar degeneration. The myocardium of 137 patients was studied: 38 samples were taken as biopsies during open heart surgery and in 99 cases autopsy material was examined, the postmortem interval ranged from 18 to 133 hours with an average of 42 hours. The myocardial specimens were classified into 4 groups: 1) disseminated myofibrillar degeneration (59) cases: autopsy cases which were suggestive of showing a lot of these lesions were selected. According to the clinical findings death was caused by sustained cardiovascular shock, sudden raised intracranial pressure, and tumour-related cachexia 2) myocardial infarctions as proven by autopsy (17 cases) or biopsy specimens (18 cases) 3) randomly chosen autopsies (13 cases) 4) instantaneous death (10 cases) and control biopsies (20 cases) prior to or following short cardioplegic ischaemia: or due to massive pulmonary embolism, traumatic rupture of the aorta or a gunshot in the head.

The hearts of these 4 groups showed a range of a LFB-positive staining ranging from a marked staining intensity to a negative staining. "*According to the different forms of injury investigated the LFB-stain displays foci of intensely blue myocytes contrasting with the red normal fibres. Amount and distribution pattern of LFB-positive myocytes are clearly detectable even at low power and if myocytes are cut transversely... Even after an interval of 6 days between death and autopsy acceptable staining results can be obtained*".

Two basic staining features in myocytes were described: type (A) myocytes with irregular blue-staining transverse bands and type (B) myocytes of diffuse blue-staining colour. In all the cases studied, cross-band lesion is consistently stained by LFB giving a salient contrast with the red colour of unaltered myocytes and the interstitium. The PTAH-stain demonstrates a similar accuracy, but with this stain, at low power or in transversely cut myocytes, the lesions are not as clearly recognizable as with the LFB-positive reactions. When the authors commented on a

comparison between the HBFP-stain of Lie et al. (1971) they stated that the latter stain was superior and further stated that, "*The LFB-staining technique is highly reproducible and easy to be handled... The LFB-staining is not suitable for the detection of early stages of infarctions...Elective parenchymal necrosis due to coronary heart disease or transient ischaemia, however, can be exhibited by LFB as it is made up of myofibrillar degeneration*" (Arnold et al., 1985).

2.K. Electrolytic changes.

Varga & Zsonda (1988) analysed the K/Na ion ratio of myocardium for the post-mortal determination of the early myocardial infarction. It is well established that the transport mechanism for these 2 ions, which is usually called the 'membrane pump' is energy-dependent. It is postulated that "*If hypoxia caused by an arterial occlusion deteriorates the energy producing oxidation in the Krebs cycle the K/Na ion balance will change*". According to the authors' method infarction had not occurred if ratio was above 1, but if the ratio was 1 or below it was likely that infarction had taken place.

Myocardial specimens were taken from autopsy cases and histological evaluation was performed in all the cases chosen for the study. Cases for controls were chosen from those with non-cardiac causes of death. Some cases were chosen after well-documented ischaemic cardiac disease followed by death regardless of the autopsy findings in respect to ischaemia and often cases were taken from areas of old MI. In total, 58 samples were selected in which the clinical and autopsy data were known. "*In 23 cases of acute MI proved by clinical signs, autopsy and histological alternations, the K/Na ion ratio always suggested acute ischaemic tissue deterioration. The K/Na ion ratio ranged between 0.2 and 0.92. In 4 cases there were clinical symptoms and electrocardiographical signs of ischaemia but the autopsy and the histological examinations did not suggest MI; the K/Na ion ratio indicated acute ischaemic tissue deterioration. The ion ratio ranged between 0.5 and 0.69. In 4 cases neither the clinical diagnosis nor the autopsy or histological findings suggested MI and there were no obvious causes of death. The K/Na ion ratio definitely indicated acute ischaemic tissue deterioration. The ion ratio ranged between 0.62 and 0.96. In 13 cases there were no suggested cause of death at all and the autopsy revealed not a cardiac cause of death. The K/Na ion ratio was not altered in any of these cases. The ion ratio ranged between 1.34 and 2.3. In 9 cases which were chosen on the basis of negative clinical anamnesis and autopsy*

concerning with cardiac diseases the K/Na ion ratio was always high above 1. These were so-called controls in which the ion ratio ranged between 1.15 and 2.1. In 5 cases the samples were taken from the myocardial scar of old infarcts: the K/Na ion ratio did not indicate acute ischaemic tissue deterioration and the ion ratio ranged between 1.11 and 1.39". Based on this the authors suggested that they "*consider death with an unknown cause as an acute hypoxic cardiac death if the K/Na ion ratio was below 1*". Though the authors did not have an opportunity to compare this method to any other, they stated "*that beside and parallel to other classical methods, this K/Na ion ratio determination widens the diagnostic arsenal*" (Varga & Zsonda, 1988).

Saukko & Knight (1989) evaluated eosin-fluorescence in the diagnosis of sudden cardiac death, eosin in itself being a fluorescent dye. Most of the previous reports concerning eosin fluorescence mainly dealt with the well established and more advanced human or experimental myocardial infarction in H&E stained sections (Siegel & Fishbein, 1982; Al-Rufaie et al., 1983) and have connected the shift from the olive green fluorescence of the normal myocardium to the yellow fluorescence of the damaged fibres with the presence or with the absence of hypereosinophilia (Siegel & Fishbein, 1982) or with the positive reaction by the haematoxylin basic fuschin picric acid (HBFP) method of Lie et al. (1971) (Al-Rufaie et al., 1983). The authors compared the findings on the enzyme-histochemical β-hydroxybutyrate-dehydrogenase method (β-HBDH) with the green eosin induced fluorescence, and increased eosin staining in areas of ischaemia. "*Although both phenomena are quite obviously related to myocardial injury, the difference in their topographical distribution suggests that they may reflect different aspects of the cell injury and need not to be directly related to each other. The slightly greenish fluorescent observed in some of the control hearts... shows that even in non-cardiac deaths the agonal ischaemia may last long enough to produce changes in fluorescence. This is further supported by the presence of the 78% wrong positive subjects in the control group which demonstrates that eosin fluorescence as well as hyperchromasia are two sensitive indicators of injury and not reliable enough to be used for medicolegal purposes*" (Saukko & Knight, 1989).

Stahl (1990) carried out a study on 60 autopsies to compare two histological stains: PTAH and modified LFB for screening of myocardial contraction bands (CBs). The study consisted of 34 males and 26 females with a median age of 78.5 years (range

50-94 years). The procedure for the modified LFB was identical to that of Arnold et al. (1985) except that the time of differentiation was shortened from 5 to 3 minutes.

LFB seemed to be useful in controlled experimental situations, but when introduced into routine human use did fail to meet the expected results and was not as sensitive as PTAH. "*LFB is somewhat less time consuming than PTAH but on the other hand it includes a differentiation step which can be a significant source of error in routine use when handled by different laboratory technicians with variable skill and judgement. Moreover, artefactual blue discolourations at the edges of the sections and sometimes in other locations made assessment difficult*" (Stahl, 1990).

Oehmichen et al. (1990) investigated the incidence of myofibrillar degeneration (MFD) in different forensic-pathological diagnostic groups: the selection of their case material was based on the results of investigations, past history, morphological and toxicological analyses. The purpose behind these extensive determinations was to exclude all influences which might have caused changes in the agonal period. All cases with features of early decomposition and those in which resuscitation had been attempted were not included in their study. The following diagnostic groups were examined: (1) acute morphine intoxication (2) carbon monoxide intoxication (3) drug intoxication (4) hanging (5) strangulation by hand/ligature (6) drowning (7) acute haemorrhagic shock (8) acute brain injury (9) sudden infant death syndrome (10) infant and children with explainable acute cause of death. Twenty five cases was studied in each group, except in the third group (drug intoxication where 18 cases were examined).

All cases were stained with Luxol-Fast-Blue as described by Arnold et al. (1985). The expression of LFB-positive staining was absent in a total of 22% of cases and in about half cases of morphine and CO intoxication, acute brain injury and sudden infant death syndrome. By contrast, there was LFB-positive staining in almost all cases of drug intoxication, hanging, strangulation and acute haemorrhagic shock. LFB-positive myocytes appeared in disseminated foci, but both types i.e. cross-band striations and diffuse staining of MFD were easily identifiable in the subendocardial region.

MFD has been shown to occur in a variety of conditions and thus these observations are in agreement with previous findings in other studies; no other diagnostic conclusions can be reached. The authors noted that "*No completely negative group*

could be found, although the morphine and CO intoxication groups showed comparatively fewer cases of LFB staining" (Oehmichen et al., 1990).

Al-Alousi (1990) re-evaluated tinctorial stains in a study on 33 selected cases of IHD with MI at different stages of development to investigate the medico-legal problems of these conditions. IHD and previous attacks of MI were diagnosed clinically in 13 cases; in all other cases, IHD and MI were found incidentally at the time of performing the autopsy, and were either the sole cause of death or had significantly contributed to death. In one case, myocardial infarction had occurred in a context of heat stroke. The condition of the coronary arteries and the myocardium was studied on gross and microscopical levels. The interval between the MI (as shown by the date of admission to the hospital and the time of death) was more than 24 hours in 24 cases. But the lapse between the attack and death was less than 24 hours in 3 cases, in 2 of which, acute MI could not be seen on the gross examination. All cases were formalin-fixed, paraffin-embedded and were stained with H&E and, in selected cases, with other special stains such as periodic-acid Schiff (PAS) and Mallory's Phosphotungstic-acid haematoxylin (PTAH). "*There was no good correlation between the observed pathological changes and the clinical age of the infarction. In fact in the 9 correlated cases, the clinical age was almost always younger than the pathological age*" (Al-Alousi, 1990).

Teraoka et a. (1991) investigated the relation between the contraction band lesion (CBL) and acute myocardial infarction, and also unexplained sudden death. The frequency of CBL occurrence and nature of CBL localisation patterns were analysed. Five hundred hearts were obtained from autopsy cases over a three year period at the First Department of Pathology, Tokyo Women's Medical College. Acute MI patients had been diagnosed clinically on the basis of severe chest pain that continued for 30 minutes and changes on electrocardiography. A few deaths had occurred within 1 hour of the onset of symptoms, and it was not possible to determine the direct clinical or pathological cause of death; these cases were grouped into one category and termed 'unexplained sudden death (USD)'. In addition, deaths of a patient that occurred 1 month following cardiac surgery were also grouped as a separate category termed 'cardiac surgery death' (CSD). The distribution of the major causes of death was as follows: cancer (247 cases); infection (53); acute MI (26); CSD (30); USD (28); General Heart Disease, i.e. various heart conditions outside acute MI, CSD, and USD, (33); cerebrovascular disease (26); ruptured aortic aneurysm (10); and others (47). CBL was observed in 11.4% of the 500 i.e. in 26.7% of 30

cases of CSD, 67.9% of 28 cases of USD, and in 100% cases of AMI. CBL was observed in only one 0.4% of the 247 cases of cancer and one 3.0% of 33 cases of general heart disease. CBL was not seen in the 50 cases of infectious disease and in 10 cases of aortic aneurysmal rupture. CBL was seen either diffusely or locally distributed; diffusely in 50.0% and localised in 50.0% of the 26 cases of acute MI. In the group USD, 68.4% exhibited diffuse-type CBL. CBL occurred in 90.9% of USD cases with history of previous MI. The diffuse type of CBL was more predominant in USD than AMI. It was concluded "*that CBL is not an artificially induced artefact but rather, a natural condition of the heart myofibrils that develop prior to death and it was also proposed that considering the high rate of CBL incidence in both AMI and manifestation patterns of CBL, AMI and USD marked by CBL belonged to the same disease group*" (Teraoka et al., 1991).

Burns et al. (1992) measured cardiac enzymes at autopsy to predict early MI. A total of 72 cases performed for the Coroner were collected in which full data were included in their study. From the results of the autopsy combined with the histological evidence 29 out of 72 cases were classified as non-cardiac deaths, 15 as definite MI, and 28 as possible MI (occlusive coronary artery atheroma only). Multiple blocks of paraffin-embedded myocardium stained with H&E were examined to identify or exclude infarction. Blood from the right atrium (into which the coronary sinus drains), blood from a peripheral vein, and pericardial fluid were collected, and cardiac enzyme activities measured in each. The following enzymes were measured: aspartate aminotransferase (AST); hydroxybutarate dehydrogenase (HBD); total creatine phosphokinase (CPK); creatine phosphokinase isoenzymes CPK-MM, CPK-MB, and CPK-BB. The post-mortem examination interval varied from 2-99 hours (mean 31.8 hours, median 21 hours).

"*The enzyme activities varied widely and there was a steady rise of enzyme activities the longer the interval between death and post-mortem examination... It was shown that death caused by early MI can be predicted from cardiac enzyme activities at necropsy. Most non-cardiac deaths were correctly predicted (24 out of 26). Five of the 13 cases in which there was histological evidence of infarction were predicted as cardiac deaths using this model... In the cases of possible MI 10 cases were classified as infarction, based on the enzyme activities... In the other 17 cases there was no histological or biochemical evidence of infarction. In many of these cases there was probably a cardiac arrhythmia and very rapid death without any infarction. Very early infarction, with no rise in enzymes, or an insignificant rise,*

however, cannot be excluded... Early MI can be identified at autopsy using cardiac enzyme measurements of body fluids. The accuracy of this method is increased if blood from the right atrium and peripheral venous blood is used in addition to pericardial fluid. If the post-mortem examination interval is included in the analysis, the accuracy is increased still further... Although not all cases of early myocardial infarction can be predicted using this model, any additional information when only occlusive coronary artery atheroma is present may be useful in controversial cases" (Burns et al., 1992).

Hougen et al. (1992) compared the morphological, histochemical and biochemical methods as applied to the detection of MI in 150 consecutive cases autopsied at the Copenhagen University Institute of Forensic Pathology. The mean age of the deceased was 54.6 years (ranging from 17-88) and comprised 48 females and 102 males. The morphological examination was based on H&E and van Gieson-stained slides of the heart and pericardial fluid was used for biochemical analysis. Slices from the heart were studied with the nitro-blue tetrazolium (NBT) method. In the cases of grossly obvious infarction, samples for microscopical and biochemical analyses and the NBT-test were taken from these zones. In cases where no grossly detectable infarction, 2 slices from that heart were stained by the NBT-test and the gross section in between them was used for the microscopy and biochemistry. Both macroscopical and microscopical analysis were classified into different groups for easier statistical analysis: (I) no pathological changes; (II) pathological changes other than early MI and (III) early MI. Slides were also stained with acridine orange for fluorescence microscopy. Sections of known early infarction served as positive controls and normal myocardium as negative ones. Determination of K/Na ratio was also performed and biochemically a case was regarded as positive for infarction if the K/Na ratio was equal to or less than 0.7 in at least two different areas on the myocardium.

Morphological and histochemical examinations resulted in 52 positive cases (early MI recognized) and 98 negative cases (no infarction was recognized). The results of the biochemical analysis showed that 72 cases were positive for MI and 77 cases were negative; one case was inconclusive because of lack of pericardial fluid. These results were compared with the results of the morphological and histochemical examinations and there was full concordance in 96 (64%) of the cases. The concordance comprised 35 of the 52 morphologically positive cases and 61 of the 98 cases regarded as negative by the pathologist. Final concordance after comparison of

results was reached in 86% of cases, while discrepancy remained on in 13.3% of cases. Of the 20 cases where discrepancy remained after comparison of results, 8 were regarded as MI by the pathologist and no infarction by the biochemist, and in 12, vice versa.

"The mean values of all the variables (morphological, histochemical and biochemical) show that both macroscopical and microscopical examination and K/Na determination were suitable for discriminating between infarction/no infarction... The NBT-test and acridine orange-staining resulted in many false negatives... In 22 cases the pathologist could not diagnose any MI but the biochemical analyses were able to detect ischaemic changes. On the other hand, biochemical methods do not always reveal the whole truth; in 11 cases the morphological examinations left no doubt and the interpretation of doubtful biochemical results had to be modified. Nevertheless, in 20 cases agreement could not be reached. These comprised 8 cases where the pathologist had no doubt about the positive diagnosis of MI. In 5 of these, MI was detected microscopically. In another 3 cases, the MI was diagnosed by an NBT-test or by macroscopy ".

The authors concluded "*that although no existing methods are ideal for diagnosing early MI in forensic medicine, their results clearly showed that a combination of various morphological and biochemical analyses are of great value in their cases; by combining efforts they were able to diagnose 22% more cases than the pathologist or the biochemist could diagnose alone* " (Hougen et al., 1992).

Reid et al. (1992) devised an 'elution method' in which the ionic ratio in the myocardium could be more readily determined by elution of ions from tissue samples and assay of the eluates by an auto-analyser; this was in an attempt at validating the method by comparison of the results with those obtained by 'homogenisation method' and then to apply the technique to the investigation of autopsy cases of ischaemic heart disease, to determine whether the K/Na ratio was reduced in myocardium which otherwise showed no morphological abnormality.

In the first part of the study, grossly normal-looking samples from the left ventricular myocardium from persons who died from non-cardiac causes were obtained at autopsy, stored at -20^{o}C for up to several weeks, and these thawed for study. In the second part of the study, cases were selected if following autopsy the main cause of death was considered to be ischaemic heart disease if the clinical evidence was also

supportive. A transverse slice, 1cm thick, was taken through both the ventricles midway between the apex and atrioventricular valves and was examined histologically. The K/Na ratios were also measured and diagramatically represented in each section of heart, areas of four types of ischaemic change, namely myocyte vacuolation, acute infarction, healing infarction, or fibrosis, were similarly mapped.

Eluting K & Na ions from the myocardium, followed by a biochemical assay of the elutes produced results which were essentially similar if myocardial samples had been homogenised and the K/Na ratio measured. The mapping of the changed ionic ratio showed also K/Na change in areas corresponding to ischaemia identified by other methods, but also some extension beyond the limits of the ischaemic zones defined by other methods (Reid et al., 1992).

2.L. Enzyme immuno-histochemistry in tissue sections

Elevation of serum enzymes, particularly of aspartate amino-transferase (AspAT) has been put forward as a further useful and accurate clinical biochemical method of diagnosing acute myocardial infarction for several decades (Galen, 1975; Lott & Stang, 1980) and indeed the levels of this enzyme have been used as a prognostic index of the severity of an acute infarction (Thompson et al., 1979). To refine further the use of this enzymatic assay, attempts were therefore made to look at the changes in different iso-enzymes of AspAT in relation to one another in the early post-infarction period, particularly a comparison of cytosolic or soluble AspAT as compared with mitochondrial AspAT. A further scope in such studies was to devise a diagnostic tool for ischaemia that would prove useful on a blood sample. The hypothesis which was put forward was that ischaemic damage would result in relative differences of elevation of the two isoenzymes. However immunohistochemical studies on formalin-fixed, paraffin-embedded blocks from both animal and human tissues were unable to demonstrate any differences in the selective losses of these enzymes from myocytes in that both were lost simultaneously when infarction occured; no well-defined and reproducible changes could be identified with ischaemia in the absence of obvious necrosis (Siegel et al., 1984)

Immunoperoxidase staining methods utilising other enzymes were also carried out. In both the canine cardiac ischaemia/infarction model and in human cardiac tissues. The sequential loss of LDH-1 (H4) i.e. lactic dehydrogenase (Herscher et al., 1984)

and of CK, creatinine kinase B and M (Siegel et al., 1984) were studied by immuno-peroxidase techniques, and in both instances no specific changes associated with pre-infarction ischaemia could be demonstrated.

These methods were later adapted to studies of pericardial fluid at postmortem: attempts were made to correlate CK, LDH and other iso-enzymes levels with the presence of recent ischaemic myocardial damage and have to be found to have a limited application in such a context (Luna et al., 1982 & 1983; Stewart et al., 1984, Burns et al., 1992).

CHAPTER 3.

DETAILED MORPHOLOGY OF THE HEART MUSCLE CELL (MYOCYTE).

3. A. The vascular component and extracellular space.

A little over half of the extracellular space is occupied by the coronary arteries, capillaries, and veins. The smallest blood vessels, the capillaries, lie close to the surface of the cells and come into contact with about one third of the total cell surface. The purpose of this large area of surface contact is to allow diffusion of oxygen and nutrients into cells and to facilitate removal of metabolic waste products. The other half of the extracellular space in the heart not occupied by the coronary vessels is filled with interstitial fluid, some of it entering into the interior of the cell by means of the T-tubular system (Opie, 1991).

A population of stem cells, analogous to satellite cells of skeletal muscle is not present in cardiac muscle, hence regeneration following damage cannot occur.

3.B. The cardiac myocyte.

Most of the heart is made up of muscle cells (also known as cardiocytes or myocytes). The individual muscle cells that account for more than half of the heart's weight are roughly cylindrical in shape. Those in the atrium are quite small, being less than 10 μm in diameter and about 20 μm in length, while the ventricular myocytes are larger, measuring about 10-25 μm in diameter and 50-100 μm in length (Opie, 1991). Although cardiac muscle is striated it is involuntary (Stevens & Lowe, 1992).

The cardiac muscle cells are long and narrow, they branch and anastomose, and are therefore known as muscle fibres. When the term fibre is used in connection with heart muscle, however, it means a series of cells, of no fixed lengths, joined end to end by cell junctions. This arrangement of cell-cell contact allows myocardial muscle to contain straight fibres and fibres that branch to effectively construct a hollow organ capable of pumping blood. Although cardiac muscle fibres have many lateral and end-to-side connections, the contractile cells are not in a reality a true anatomic syncytium. Most cardiac muscle cells possess a single nucleus, but a few

of them contain two. Occupying a central position in the cell, the nucleus is relatively large and pale staining, with a bare or cleared zone of cytoplasm surrounding it- the perinuclear space - which arises as a consequence of the myofilaments arranging themselves in such a manner that they detour around the nuclear compartment (Cormack, 1993).

3.B.1. Sarcolemma.

The whole of the complex surface structure that surrounds each muscle cell can be subdivided into three layers, from the periphery inward. First, there is the glycocalyx, which can in turn be split into an outer coating, or external lamina, and an inner coat. Each coat is only about 20-30 nm thick (1nm = 1 x 10^{-9}m = 10 Ångstrom units). Second, immediately within the surface coat there is the true sarcolemma, which consists of a lipid bilayer with its associated proteins, similar in composition to all other external cell membranes for which the general term 'plasmalemma' is used. Third, just beneath the sarcolemma, there is a thin, mat-like layer of fine microfilaments that provides structural support. The term 'sarcolemma' includes all these three layers (Opie, 1991).

The external lamina and the surface coat together constitute the glycocalyx. This term literally means 'sweet husk'; the sweet refers to the complex carbohydrates of polysaccharides that are fundamental to its compositions. Synonyms for the glycocalyx include basement membrane, basal lamina, surface coat, and boundary layer. The major function of the glycocalyx is to provide an ionic trap to ensure that the external surface of the sarcolemma has a reasonably constant ionic environment.

The sarcolemma of the myocyte invaginates to form an extensive tubular network (T-tubules) that extends into the extracellular space into the interior of the cell. This system involves the invagination of the sarcolemma to provide a series of hollow tubules that penetrate inward, usually at the level of a Z-line. The important features of the T-tubules are as follows:

(1) Their lumen is an extension of the extracellular space. Nonetheless, it does not contain the same extracellular fluid but a modified interstitial fluid because communication with the external extracellular space is not entirely free.

(2) The lumen of the T-tubules at the surface of the cell is about 250 nm wide. This makes them 10 times wider than their skeletal muscle counterparts. The existence of these very wide T-tubules in ventricular myocytes may ensure that an adequate supply of oxygen and nutrients is available to satisfy the needs of the mitochondria. Other possible advantages include the rapid removal by diffusion of metabolic waste products and a rapid and adequate supply of extracellular calcium for the contractile cycle.

(3) The T-tubules are extensions of and have the same ultrastructure as the cell surface, they thus increase the surface area of the cell, at least by 30% and possibly much more, thereby facilitating the spread of the excitatory stimulus to within the cell.

(4) They may contain a certain type of calcium channel, the L-type, that responds to the wave of electrical excitation by releasing a messenger, which in turn activates the sarcoplasmic reticulum to release much more calcium. The close juxtaposition of the T-tubules and the sarcoplasmic reticulum is crucial to the links between the wave of depolarization and the contractile process (excitation-contraction coupling). The main function of the sarcoplasmic reticulum is to regulate the intracellular movements of calcium ions (Opie, 1991).

3.B.2. Sarcoplasm-myoglobin.

The cytoplasmic component of the myocytes is referred to as the sarcoplasm. It contains a reddish-brown protein called myoglobin, which in several respects is similar to the red protein haemoglobin in erythrocytes. Myoglobin is a respiratory protein with the property of reversibly binding molecular oxygen. It is a small molecule (17,000 daltons), which takes up oxygen from the blood and stores it in muscle fibres. It is the single-polypeptide, oxygen-binding haeme protein found (in man) only in skeletal and cardiac muscle. Isoenzymes have not been described and it is presumed that cardiac and skeletal muscle myoglobin are identical, but that has not been proven definitively. It may enter the circulation after muscle injury or stress and following renal clearance, may appear in the urine. Application of immunoassay techniques has proven useful not only in identifying patients with disorders which involve skeletal muscle, but with cardiac muscle involvement as well (Kagen, 1978).

Myoglobin's role both in skeletal and cardiac-muscle is centrally related to its ability to reversibly bind oxygen. Millikan (1939) outlined three possible areas, or mechanisms, of action: oxygen transport, oxygen storage, and other 'catalytic' functions: transport of oxygen from the muscle cell membrane to the mitochondrion appears to be its central, or dominant, function.

The myoglobin may also function as an oxygen store for the muscle cell. This represents approximately 10% of the oxygen stores of the body and would supply the oxygen need for only a very short time, in the range of a few minutes, if atmospheric oxygen were not available. In this sense, the function of oxygen storage in man seems a minor one. However, in another sense, myoglobin may have a dynamic storage function, serving as an oxygen buffer (Wittenberg, 1970), which can supply oxygen to mitochondria during contraction and take on oxygen delivered from the capillary blood during relaxation. It has also been suggested, that myoglobin may be involved catalytically in the transport of fatty acids though the muscle cell cytoplasm.

There are two ways of conceptualising possible relationships between myoglobin and human muscle disease. Firstly, changes in the function of myoglobin, perhaps based upon qualitative changes in the molecule or quantitative changes in the amount of myoglobin available for oxygen transport, might be responsible for the production of muscle cell dysfunction. Alterations of these sorts may play roles analogous to those of abnormal haemoglobins of man where physiological changes results from the presence of either abnormal globin types with altered stability, solubility, or oxygen binding properties or from decreased amounts of haemoglobin available for oxygen transport. To date, however, there is no known clinical situation in which myopathy has resulted from defects or changes in the function or amount of the myoglobin molecule within the muscle cell.

Secondly, as a result of muscle disorders due to other factors, myoglobin may leak from muscle tissue and appear in the blood circulation or in the urine. The detection of myoglobinemia and myoglobinuria, and the relation of these phenomena to the diagnosis and outcome of myopathies and cardiopathies has been the chief clinical use to which knowledge of the myoglobin molecule has to date been applied. Myoglobin appeared in the serum after myocardial infarction in rapid short 'staccato' bursts or episodes, often lasting just a few hours. These bursts, frequently multiple, occur within the first day in all patients, and are repeated on the second day in

patients who initially released greater amounts of myoglobin into the circulation. It was hypothesized that release of myoglobin from reperfused areas may result in some of the cardiac damage in the hours following an ischaemic episode. Myoglobin colours the urine in concentrations near 1mg/ml or greater. Below these concentrations, pigmenturia may not be noticed, and if the patient is less acutely ill, myoglobinuria may not be suspected (Kagen, 1978).

3.B.3. Lipofuchsin.

Lipofuchsin is an insoluble pigment, also known as lipochrome, 'wear-and-tear' or ageing pigment. Its importance lies in its being the telltale sign of free radical injury and lipid peroxidation. The term is derived from Latin *fuscus* means brown and thus brown lipid. In tissue sections it appears as a yellow-brown, finely granular intracytoplasmic pigment. It is seen in cells undergoing slow, regressive changes and is particularly prominent in the heart of ageing patients it is often accompanied by organ shrinkage (brown atrophy). On electron microscopy, the granules are highly electron dense, often have membranous structures in their midst, and are usually in a perinuclear location (Cotran et al., 1989).

No functional importance has been attributed to lipofuchsin, the yellowish-brown pigment that accumulates linearly with age around the nuclei of myocardial fibres (Strehler et al., 1959). Chio et al. (1969) have described lipofuchsin formation in isolated mitochondria as a function of lipid peroxidation; it could be prevented by the addition of an antioxidant into the incubation medium (Chio et al., 1969). Analyses of lipofuchsin have demonstrated the presence within it of polyunsaturated lipids and protein (Hendley et al., 1963).

Much of the evidence which bears on the nature of age pigment (lipofuchsin) in heart and other tissues has been derived from histochemical studies (Pearse, 1961). These studies include negative tests for iron, cholesterol, DNA, ceroid, and bile. Acid fast staining, as well as staining with Sudan III, Sudan IV, Nile Blue sulfate, and basic fuchsin, suggested the presence of anionic lipids, or fatty acids. The pigment is also auto-fluorescent (Hendley et al., 1963).

Examination by light and electron microscopy of human myocardium from necropsies and biopsy specimens has revealed that mitochondria can be transformed into granules of lipofuchsin (Koobs et al., 1978).

3.B.4. Myofibrils.

The sarcoplasm (Greek, *sarkos*: flesh) also contains the striated contractile elements, the myofibrils (Cormack, 1993). Any one muscle fibre has hundreds of myofibrils running parallel along its length, the alternating zones of thick and thin filaments giving rise to the descriptive term striated muscle. Each myofibril is assembled from a large number of small filaments, the myofilaments. The first recorded description of myofibrils was probably made by Felix Fontana in 1787; he noted the presence of "*fleshy threads . . . solids cylinders equal to each other and very perceptibly marked at equal distances*". In 1840, Bowman actually measured the distance between adjacent striations and reported it as being 0.001 inch, a measurement that agrees fairly closely with that obtained using electron micrographs 2.2 μm for resting muscle (Opie, 1991).

Fibres cut in longitudinal sections show a distinctive pattern of alternation dark- and light-staining transverse bands. Under polarized light, the dark-staining bands are birefringent (anisotropic) whereas the light-staining bands are isotropic. The dark bands are therefore called A (for anisotropic) bands, and the light ones, I (for isotropic) bands (Cormack, 1993).

The ultimate contractile unit is the sarcomere (Greek *meros*, part), which is that part of a myofibril that lies between consecutive Z lines. Under much higher magnification fine dark lines called Z bands (Zwischenscheiben) can be seen bisecting the light I bands. The Z bands divide each myofibril into numerous contractile units, called sarcomeres, arranged end to end. The dark A band is bisected by the lighter H (from German *helle*: clear) band, which is further bisected by a more dense M (Mittelscheibe) band. Irrespective of the degree of contraction of the muscle fibre, the A band remains constant in width. In contrast, the I and H bands narrow during contraction and the Z bands are drawn closer together (Opie, 1991; Burkitt et al., 1993).

Each sarcomere consist of thick myosin filaments and thin filaments containing several different proteins, including actin (which accounts for 60% of the thin filaments), troponin, and tropomyosin. Myosin and actin usually are classed together as contractile proteins, whereas troponin and tropomyosin are the regulatory proteins

because they regulate the actual physical process of contraction, which involves the displacement of the thin actin filaments along the myosin-containing thick filaments.

3.B.5. Mitochondria.

The cardiac mitochondria are located beneath the sarcolemma, they are wedged in between the myofibrils, presumably so that the chief source of energy supply is close to the chief site of energy use. Beside generation of ATP, they have another important role, the accumulation of calcium. Thereafter, the mitochondria must again release such calcium to prevent the damage to mitochondria that comes from excess sustained accumulation of calcium. Mitochondria that are isolated from heart muscle that has been made ischaemic and then reperfused are overloaded with calcium and hence produce ATP at a very slow rate (Opie, 1991).

3.B.6. Cytoskeleton.

Cytoskeleton is the system of fibrillar structures in the cytoplasm of eukaryotic cells. It forms the structural backbone of the cell, to which other, more easily extractable proteins and organelles are associated. These structures are unique because they can be highly dynamic and are directly in contact with all the other cellular organelles from the nucleus to the external membrane (Bershadsky & Vasiliev, 1988). The cytoskeleton is not simply a passive feature of the cell that provides structural integrity; it is a dynamic structure that is responsible for whole-cell movement, changes in cell shape, contraction of muscle cells, and provides the machinery to move organelles from one place to another in the cytoplasm. In addition, recent studies have provided evidence that the cytoskeleton is the master organizer of the cell's cytoplasm, furnishing binding sites for the specific localization of RNAs and proteins (Goodman, 1994). Historically, the concept of cytoskeleton is very old one; the first fibrillar cytoskeletal structure, the mitotic spindle, was discovered by Walter Flemming in 1879 when he first described the stages of mitosis and introduced this term (Greek *mitos* , thread) (Bershadsky & Vasiliev, 1988).

The cardiomyocyte's cytoskeleton is composed of a highly organised complex array of specific proteins, arranged to transmit mechanical forces within the cell, to adjacent cells and the extra-cellular matrix, as well as to maintain internal organisation of cellular organelles. Although most of the published reports on cytoskeletal proteins refer to non-myocyte and smooth muscle cells, there seem

significant homologies with cardiac structures. But the roles of many proteins are uncertain, and the list of proteins that compose the cytoskeleton is likely to be incomplete. Currently, the term myocyte cytoskeleton includes the inter-connections of the contractile system, the submembrane cortical lattice, microtubules, and trans-membrane attachments to extra-cellular components (Ganote & Armstrong, 1993).

The many activities of the cytoskeleton in the heart muscle depend on three principal types of protein filaments: the micro filaments, microtubules, and intermediate filaments. Each type of filament is formed from specific association of protein monomers. The dynamic aspects of the cytoskeleton arise from accessory proteins that control the length of the filaments, their position within the cell and the specific binding sites along the filaments for association with protein complexes, organelles, and the cell membrane (Goodman, 1994). By far the greater percentage of myofibrillar protein is that concerned with contraction, with about 10% concerned with its regulation and another 10% concerned with maintenance of the structure of the myofibril (Opie, 1991).

3.B.7. Microfilaments.

The thin filaments of cardiac muscle are constructed from actin, tropomyosin, and troponin. Although these proteins form the same complex as that found in skeletal muscle, they are different biochemically from the polypeptides found in their skeletal muscle counterparts i.e. they are cardiac-specific isoproteins (Goodman, 1994).

Actin

These are thin filaments (about 1μm and only 5-7 nm wide) with a molecular weight of 47,000 daltons (Pollard & Cooper, 1986), each contains two helical chains of actin units (actin accounts for 60% of the thin filaments), each carried on a backbone of tropomyosin, with troponin complexes placed at intervals of about 38 nm (Opie, 1991).

Each thin filament (F-actin) is formed by the polymerization of many single molecules of globular actin (G-actin). To form a complete thin filament, two actin filaments became attached by their tails ends to α-actinin (which is another protein associated with actin) in the Z line so that they face in opposite directions (i.e. away

from the Z line) (Stevens & Lowe, 1992). This immobilizes the thin filaments with their plus (+) ends at the Z disc, and their minus (-) ends extending to the central region of the sarcomere. Therefore, a sarcomere unit contains actin filaments that extend from the Z disc and exhibit polarity opposite that of the centre regions of the saracomere. Actin filament networks also provide mechanical support to the cell membrane by attachment to it via membrane anchoring proteins, the best characterised of which are spectrin and ankyrin (Stevens & Lowe, 1992).

Tropomyosin

Tropomyosin and troponin are regulatory proteins that constitute about 10 per cent of total myofibrillar protein and are associated with the thin filaments. Tropomyosin is a rodlike protein, so-called because of its similarities with myosin, specifically the rodlike tail domain of the myosin molecule. It is 400 nm in length and 20 to 30 nm in width, with a molecular weight of 70,000 daltons. Tropomyosin forms a continuing strand through the centre of the thin filament, while the troponin complex is located at intervals of 365 nm. Tropomyosin binds along the length of the actin filament, thereby stabilising and stiffening the filament (Opie, 1991).

Troponin

The troponin complex that comprises three components: (1) troponin C, (C for calcium), a calcium-sensitizing factor that binds Ca^{++}. (2) troponin I, (I for inhibitor), an inhibitory factor that inhibits the Mg^{++} -stimulated ATPase of actomyosin; and (3) troponin T, which links the whole troponin complex to tropomyosin (Opie, 1991).

The elongated troponin T molecule (Mw 37,000) binds to the COOH-terminal region of tropomyosin and links both TnI and TnC to the tropomyosin. Troponin I (Mw 22,000) binds the TnT as well as actin and, in concert with tropomyosin, causes a change in the conformation of F-actin such that it interacts only weakly with myosin head groups, this weak interaction cannot activate the myosin ATPase activity.

The distribution of troponin isoforms varies between cardiac muscle and slow–and fast-twitch skeletal muscle. Their importance regarding the diagnosis of myocardial disease comes from the fact that some isoforms show a high degree of cardiac specificity. Cardiac-specific troponin-T and troponin-I isoforms have been

identified. On the basis of the current literature, troponin-T and troponin-I offer the most exciting potential as very specific serum markers for the diagnosis of acute MI. An enzyme immunoassay for the cardiac troponin-T, which showed a cross-reactivity with troponin-T extracted from mixed skeletal muscle fibres of less than 2%, has been reported (Katus et al., 1989; Gerhardt et al., 1991). Using this immunoassay, troponin-T was elevated in the serum of patients with acute MI from 3.5 hours to more than 10 days after the onset of chest pain (Apple, 1992).

3.B.8. Thick filaments.

The thick filaments are composed mainly of the protein myosin. Myosin, like actin, was first described in muscle cells, but is now known to be a ubiquitous component of nonmuscle cells. It is the second most abundant protein of striated muscle but constitutes a much smaller fraction of the protein of non-muscle cells (Stossel, 1978). The thick filament of myosin (about 1.5 μm long and 10-15 nm wide), is composed of an orderly aggregation of 300 longitudinally stacked molecules of myosin proteins with a molecular weight of approximately 500,000 daltons, held parallel and in register by centrally located connections at the M line (Opie, 1991).

Myosin itself can be separated into three isoenzyme components (Mahdavi et al., 1984). The human atrium and ventricle each contain at least two molecular variants of myosin heavy chains; heterogeneity of cardiac muscle fibres with respects to myosin composition consequently appears to be a general property of vertebrate hearts regardless of the size of the animal (Léger et al., 1984). There are also two myosin light chains (MLCs) located at each amino-terminal end of the myosin heavy chain molecule and each can be differentiated by specific monoclonal antibodies. However, there is evidence that the cardiac MLC 1 is also expressed in slow-twitch skeletal muscle. Thus, the use of specific monoclonal anti-cardiac MLC antibodies may not differentiate heart damage from skeletal muscle damage. Only a small amount (less than 1%) of MLC exists as the free, unbound cytosolic portion.

Myosin filaments are polar, to form a complete heavy filament, two myosin filaments become attached by their tail ends so that they face in opposite directions (i.e. away from the M line) (Apple, 1992).

3.B.9. Microtubules.

The microtubules (MTs) are non-contractile structures of variable undefined length with an exterior diameter in cardiac cells in the range of 24 to 30 nm, the largest diameter of all cytoskeletal fibrils. Microtubules are universal components of all eukaryotic cells except red blood cells. The wall of the microtubules is made of the single protein tubulin which is one of the most highly conserved proteins known (Bershadsky & Vasiliev, 1988).

In the ventricles, myocardial cells yield images of MTs dispersed longitudinally or transversely in the sarcoplasm adjacent to the I band and at the periphery of nuclear profiles. Longitudinal MTs surround the nuclei without displaying any connections with myofibrils in this area. The fact that few studies have been carried out on MTs in cardiac tissue seems to be secondary to technical difficulties in the preservation of tissues and microtubular structures by fixation procedures (Goldstein & Entman, 1979).

MTs are longitudinally oriented in the inter-myofibrillar spaces and sometimes pass through the desmin filaments located between the Z discs when extending along several sarcomeres, and occasionally appear to surround the Z discs and the sarcomere, but MTs never display a striated pattern. Some MTs run closely apposed to the outer membranes of the mitochondria and along the sarcolemmal borders (Rappaport & Samuel 1988).

MTs are constantly polymerizing and depolymerizing in the cell and are labile structures that have the capacity to undergo rapid assembly and disassembly. The picture that forms when considering dynamic instability is one of a cytoplasm that is constantly changing because of the rapid turnover of microtubules. At any one instance, many microtubules would be rapidly growing, whereas others would be quickly and catastrophically disassembling. The average life span of cytoplasmic microtubules is about 10 minutes. Microtubules can exist for longer periods because the cell has several mechanisms for stabilizing cytoplasmic microtubules i.e. microtubule-associated proteins, which convert the unstable microtubular network into a relatively permanent framework. Indeed microtubule inhibitors are commonly used for cancer chemotherapy (Goodman, 1994).

Tubulin

The structural subunit protein of microtubules, is a 100-kD dimer of two 50-kD polypeptides designated α and β, which are 36-42% homologous to each other. A third type of tubulin has been discovered, γ-tubulin, which interacts with β-tubulin and microtubules in an, as yet, unknown way (Oakley & Oakley, 1989). Different tissues seem to express different tubulins when examined by isoelectric focusing, and double labelling experiments showed that all detectable microtubules contained β-tubulin (Gozes & Barnstable, 1982).

There is both structural and functional evidence for the interaction of microtubules with membranes. Studies of the endogenous tubulin content of purified plasma membranes and of the interaction of exogenous tubulin with plasma membranes reveal a rather complex system; not only evidence for a specific interaction of tubulin with isolated membranes has been found, but in addition purified plasma membranes contain very tightly bound tubulin-like molecules, which can only be removed by detergents. An attractive hypothesis views membrane tubulin as a receptor for binding cytoplasmic tubulin, initiating its polymerization at the membrane. This binding represents assembly of tubulin at the membrane, in agreement with this hypothesis, erythrocyte plasma membrane vesicles that lack membrane tubulin do bind much less exogenous tubulin (Niggli & Burger, 1987).

3.B.10. Intermediate filaments.

The intermediate filaments (IFs) as indicated by their name, are thicker than actin filaments, but thinner than microtubules. They are tubular structures with a diameter of 8-12 nm. In striated muscle and cardiac myocytes, IFs are attached to the periphery of the Z discs. Intermediate filaments were proposed to be mechanical integrators of cellular space (Lazarides, 1980), that is, they play a major structural role as scaffolding within the cell. This view is confirmed by Schaper et al. (1991) who showed that desmin filaments do indeed connect all Z discs, thereby preventing the sarcomeres from slipping during contraction (Schaper et al., 1991).

Many properties of IFs set them apart from the two other main groups of cytoskeletal fibrils. IFs are much more stable than actin filaments and microtubules, and their polymerization is usually irreversible. In contrast to the actin-myosin and dynein-

tubulin systems, the systems of IF seem to be unable to move actively and independently of other structures (Bershadsky & Vasiliev, 1988).

The contractile elements are not only fixed by desmin filaments at the Z band level, but they also are connected to the intercalated discs and to the sarcolemma by intermediate filaments as well as by vinculin (a cytoskeletal supporting protein). The nucleus, on the other hand, is held in its central position in the cell mainly by the presence of microtubules and also by intermediate filaments (Schaper et al., 1991).

Desmin:

Desmin is a protein that belongs to the IF class of cytoskeletal proteins, referred to as the round filament, it was first extracted and visualized by electron microscopy, and also differentiated from other components of the cytoskeleton by Cooke & Chase in 1971 (Cooke & Chase, 1971). The term desmin was introduced subsequently by Lazarides and Hubbard (Lazarides & Hubbard, 1976).

The protein monomers that constitute intermediate filaments (IFs) differ from the components of microfilaments and microtubules in several important ways: 1. They are fibrous proteins 2. Almost all of the IFs subunits are incorporated into stable IFs within various cells: no energy in the form of ATP or GTP hydrolysis is required for intermediate filament polymerization 3. Intermediate filaments have no polarity, whereas microfilaments and microtubules have plus and minus ends. They have cell type-specificity of which has been proven useful to histo-pathologists, who use specific monoclonal antibodies to identify the tissue of origin and metastatic cancer cells.

Until recently, the cytoskeleton had been regarded as a purely cytoplasmic structure. However, new data suggest that the nucleus may also contain structures that are similar to or even identical to the cytoskeletal fibres in the cytoplasm. One such important group of data was obtained in the studies of the nuclear envelope separating the nucleoplasm from the cytoplasm. The major structural component of this envelope is the nuclear lamina, a fibrous layer on the internal surface of the nuclear membrane. Electron microscopical examination had confirmed that the nuclear lamina is a meshwork of intermediate-type filaments (Aebi et al., 1986).

3.B.11. Accessory proteins.

Accessory proteins link the different components of the myofibrils and hold them in register with each other. These proteins can be visualized through immunohistochemistry.

These include α-actinin which holds actin filaments in a lattice arrangement in the Z disc, Fimbrin which bundles actin filaments, and Profilin binds actin monomers. Other disc proteins include: Filamin, Cap Z protein (which caps plus-ends of filaments), Amorphin, and Z protein. Other proteins are Myomesin which holds myosin filaments in a lattice arrangement in the region of the M line, Titin (Connectin) which is an extremely long elastic protein, which runs parallel to the filament array and links the ends of the thick filaments to the Z disc, maintaining their ends in register with the lattice of thin filaments, C protein (a myosin binding protein running parallel to the M band in the first half of the A band) and Vinculin.

α-Actinin

It is a fibrous protein composed of two identical 190-kDa, about 4 nm in diameter and 40 nm in length. It is the major component of the Z disc, α-actinin provides the ability to bind tightly to the sides of the actin filaments, allowing the bundling together of adjacent thin filaments at the Z disc (Goodman, 1994). It is also seen on the cytoplasmic side of the vinculin (Pollard & Cooper, 1986).

Vinculin

Vinculin is defined as a 130-kd soluble protein that was first purified by Geiger (1979). It has been demonstrated, by immunocytochemical methods, to be present in a variety of cells, including fibroblasts (Geiger, 1979; Burridge & Connell, 1983). Pardo et al. (1983) showed that in cardiac muscle vinculin connects actin filaments to either the sarcolemma or the intercalated disc. They described the localization of vinculin at the sarcolemma as part of an attachment system of myofibrils to the plasma membrane, and they described the presence of vinculin in longitudinal section along the Z line and in transverse sections within tubular invaginations, most probably the T tubular system. Schaper et al. (1991) showed that vinculin immunofluorescence was regularly present at the level of the intercalated disc and at the lateral sarcolemma.

Vinculin was not found on internal myofibrils. Thus, additionally to the reported localization of vinculin in intercalated discs (Tokuyasu et al., 1981), a new type of vinculin localization in cardiac muscle was found; the protein overlies the I bands around the Z-line region of external myofibrils near sarcolemma. It is important that vinculin, not being an integral myofibrillar protein, is periodically distributed along the cardiomyocyte myofibrils with the same periodicity as I bands of sacromeres containing actin filaments. It is generally assumed that in all objects tested, vinculin can be involved in the attachment of actin bundles to the membrane (Geiger, 1979; Geiger et al., 1980; Tokuyasu et al., 1981). However, *in vitro* vinculin can interact with F-actin. Thus, it was suggested that in cardiac muscle vinculin plays an important role in linkages between contractile structures (external myofibrils) and membranes of sarcolemma. Vinculin is the first non-membrane component which can link sarcolemma and myofibrils. Sarcolemmal membranes do not simply cover the myofibrils bundles but physically associate with external myofibrils through the periodically distributed structures containing vinculin (Koteliansky & Gneushev, 1983). These myofibril-sarcolemma attachment sites containing vinculin were named costameres (Pardo et al., 1983).

Costameres (Latin *costa*, rib; Greek *meros*, part) are transverse, rib-like bands at the sarcolemma which are precisely in register with the underlying I bands. These costameres encircle the cardiac muscle perpendicular to the long axis of the fibre and overlie the I bands of the immediately subjacent sarcomeres (Pardo et al., 1983). In the cardiac myocyte, the lateral costamere junctions are sites of cell matrix adhesion. In contrast to the cell to cell junctions, the lateral cell matrix junctions specifically contain integrin as the trans-membrane protein and talin as the linker molecule between integrin and vinculin (Burridge et al., 1988; Burridge et al., 1990). Vinculin in turn binds α-actinin, finally linking to actin. The lateral costamere junctions are also sites of inter-action between the intermediate filament system and the extra-cellular matrix (Bershadsky et al., 1987).

In sustained ischaemia, vinculin is destroyed, a process that probably allows the sarcolemma to rupture more easily, thereby liberating intra-cellular enzymes and hastening cell death (Opie, 1991).

Disturbances of the cytoskeleton during ischaemia may produce alterations in cell structural integrity that could account for cell injury and death. Although

mechanisms both of cytoskeletal assembly in normal cells and of cytoskeletal injury in ischaemic cells are currently poorly understood, research into the interactions of cytoskeletal proteins during ischaemia includes new approaches that may increase our understanding of the pathophysiology of the cardiac myocyte (Ganote & Armstrong, 1993).

3.B.12. Intercellular junctions.

Cardiac muscle cells are richly endowed with intercellular junctions. The three distinct types of junction are present – the gap junction *nexus*, the 'spot' desmosome, and the 'sheet' desmosome (fascia adherens), and they are widely known as: gap junction, desmosome and fascia adherens respectively. These junctions are not scattered at random over the plasma membrane surface, but are confined to a specialized portion of this surface, the intercalated disc (Severs, 1985).

Gap Junctions

Gap junctions (also called *nexus* junctions, Latin: *nexus* means bind), link neighbouring cytoplasmic compartments and form low-resistance pathways along which the action potential can spread from one cell to the next such that there is a synchronization of contraction among these cells. To fulfil these functions, the plasma membranes at the gap junction are closely apposed, leaving an inter-cellular gap of only 2 nm. These contacts also allow the cardiac cells to exchange small cytoplasmic solutes (Goodman, 1994).

Desmosomes

Desmosomes are rivet-like structures that fasten adjacent plasma membranes firmly together. They are most abundant in tissues–like the myocardium–that are subject to severe mechanical stress. Intermediate filaments, which in the cardiac muscle cell consists of desmin and vimentin (Lazarides, 1980), are anchored to the desmosomal plaques. In this way, forces acting on the supportive structural framework provided by the intermediate filament system in the cytoplasm can be distributed throughout the tissue as a whole. In this region of the plasma membrane is a second type of junctional complex, termed the fasciae adherens, which functions to connect the thin filaments of adjacent cells and hold them in register with the myosin thick filaments (Goodman,1994).

Fascia Adherens

The true fascia adherens (Latin: *fascia* means band; the adherent band). The fascia adherens, like the Z-disc, contains actin, α-actinin, filamin, and a range of polypeptides that do not appear to have counterparts in the Z-disc (Koteliansky et al., 1983). Notable among these is the vinculin that has been localized using immunochemical labelling in the filamentous mat and is thought to be directly responsible for linking the myofilament bundles to the plasma membrane (Geiger et al., 1980; Koteliansky et al., 1983).

The fascia adherens of cardiomyocytes is the site of cell to cell contact which links the sarcomere chains of adjacent cells across the intercalated disc. It consists of a complex of proteins, that link the trans-membrane cell adhesion protein N-cadherin, to sarcomeric actin (Ganote & Armstrong, 1993).

3.B.13. Intercalated discs.

The chains of cardiac muscle cells are joined end to end by anchoring cell junctions and are traversed at intervals by intercalated discs, which are unique to cardiac muscle fibres. In longitudinal sections, intercalated discs appear as darkly stained irregular lines, some of which traverse the fibre in a step-wise manner (Cormack, 1993).

Intercalated discs are found where the plasma membranes of adjacent cardiac myocytes come into close contact, usually at or near the ends of neighbouring cells. Each disc is thus composed of two apposed plasma membranes and their associated intra-cellular and extra-cellular structures. Intercalated discs are highly specialized macrodomains of the plasma membrane the function of which centres on the transmission of both the signal for and the force of contraction from one myocyte to the next (Severs, 1985).

The major part of the intercalated disc is where the actin filaments are actually inserted into the fascia adherens. Not all thin actin filaments bind to this disc. Some actually penetrate the disc and run into the neighbouring cell through the desmosomes, which occupy only about 5% of the disc surface. Thus, the fascia adherens and the desmosomes have the important function of linking the thin

filaments of one cell to that of another. The depolarizing current spreads directly from cell to cell, explaining the heart's capacity to function as a group of cells with similar behaviour (this is called a syncytium; Latin, *syn* : together; *cytium* : cell), although anatomically the heart consists of discrete cells (Opie, 1991).

Different regions of the intercalated disc contain specific junctional complexes. In the transverse sections of the intercalated disc, there are two junctional complexes: the desmosomes and the fascia adherens. In the longitudinal regions of the intercalated disc are the gap junctions (Goodman, 1994).

3.B.14. Contraction.

The heart muscle's contraction is myogenic: it is an intrinsic and spontaneous rhythmic activity of the cardiac muscle cells themselves. Because contractile activity is an inherent property of cardiac muscle cells, only the rate of contraction (the heart rate) is regulated by the autonomic nervous system (Cormack, 1993). The contractile apparatus consists of partially over-lapping rodlike myofilaments that are fixed in length, both at rest and during contraction (Braunwald, 1992).

Microanatomy of Contraction

Control of muscle contraction is achieved by proteins that bind to actin and prevent muscle contraction by blocking the myosin-actin interaction. This is reversed by high concentrations of Ca^{++} ions in the cell cytoplasm (Stevens & Lowe, 1992).

In muscle contraction, the actin filaments slide along the myosin filaments. These are driven by the heads of myosin molecules, which bind to actin and, in a sequence of binding and release movements, 'walk' along the actin filament. This repetitive binding and release is powered by the hydrolysis of ATP; myosin can be regarded as an ATPase that is activated by the binding of actin.

The tropomyosin-troponin complex is responsible for the calcium sensitivity of the contractile apparatus. It regulates the interaction between the heads of the myosin molecule and actin. Although much is known about this interaction, the precise molecular mechanisms responsible for the biochemical-mechanical transduction have not yet been fully elucidated (Opie, 1991).

These ultrastructural findings explain the 'sliding filament theory' which proposes that under the influence of energy released from ATP, the thick and thin filaments slide over one another, thus causing shortening of the sarcomere (Burkitt et al., 1993).

CHAPTER 4.

IMMUNO-HISTOCHEMICAL STAINING OF THE MYOCARDIAL ISCHAEMIA.

4.A. Immunohistochemical staining of the myocytic components.

Khaw et al. showed for the first time that antibody (ab) (Fab)2 specific for cardiac myosin could localise selectively and specifically in infarcted myocardium; this was demonstrated in an *in vivo* canine experimental model. They suggested that labelled specific abs or fragments of abs may represent a conceptually new imaging approach to the localisation and sizing of MI. Their data support the hypothesis that ischaemia induces alterations in myocardial cell membrane permeability and thus allows the efflux of intra-cellular macro-molecules into the environment and circulation and the influx of extra-cellular macromolecules to the intra-cellular space of the cardiac myocytes (Khaw et al., 1976). This research group described the use of polyclonal abs or sub-fragments coupled with ^{125}Iodine, ^{131}Iodine or ^{111}Indium, as well as the use of monoclonal abs specific for the human myosin heavy chains and labelled with ^{111}Indium (Khaw et al., 1976; Khaw et al., 1978; Khaw et al., 1979; Khaw et al., 1980; Khaw et al., 1983; Khaw et al., 1984).

Khaw et al. (1986) successfully injected anti-myosin ab labelled with ^{99}Technetium-into patients with acute MI. They have also shown that Fab labelling can be used to identify necrotic myocardial cells which do not appear dead by previous histological criteria and that non-ischaemic cells do not take antibody up. As early as forty minutes following coronary artery ligation and forty-five minutes of reperfusion it is possible to identify by anti-myosin staining, cells which are necrotic. These antibodies label cardiac myocytes by penetrating defects in the sarcolemma sufficiently large to admit a molecule of 30,000 Mw. This group of researchers also provided unequivocal evidence from tissue culture of cardiac myocytes which were similarly labelled with anti-cardiac myosin Fab fragments (Khaw et al., 1986).

Katagiri (1977) studied changes in dogs after producing myocardial infarctions following ligation of the left anterior descending artery, at intervals post-ligation varying from 12 hours to 28 days. Infarcted tissue and normal portions of hearts obtained from 5 cadavers who died of myocardial infarction after periods ranging from 24 hours after the attacks of infarction up to 4 years were also studied. Changes

of structural proteins were determined by looking at myosin, α-actinin and actomyosin-ATPase activities. It was shown that in dogs, the relative amounts of myocardial cell myosin and α-actinin decreased at 24 to 48 hours after coronary ligation, and that these changes of structural proteins became more prominent at around 48 to 72 hours after coronary occlusion (Katagiri, 1977).

Kent (1982) carried out an experimental study of myocardial ischaemia on 14 dogs, and formalin-fixed, paraffin-embedded myocardial tissues were stained with H & E, PAS, diastase digestion followed by the PAS (D-PAS) and by the basic fuchsin stain of Lie et al. (1971); they demonstrated intra-cellular IgG and myoglobin reaction by indirect immuno-fluorescence. It was shown that the pattern of myoglobin loss in the ischaemic areas is different from that seen with autolysis. Myoglobin diffused into the interstitial spaces as a result of ischaemia and subsequently into other circulating body fluids. After 30 minutes of ischaemia, myocardial fibres appear to have lost their myoglobin content, while adjacent interstitial spaces contained demonstrable myogobin with relative distribution between the two sites increasing as the length of ischaemic period increased. These alterations in cell permeability shown by myoglobin loss and intra-cellular diffusion of IgG were suggested as markers of irreversible injury (Kent, 1982).

Haber et al. (1982) isolated human cardiac and skeletal myosin from ventricular myocardium and Psoas muscle which were obtained at necropsy, and examined the leakage of cardiac-myosin light chains from damaged myocardium as well as the entry and fixation of radioactively labelled cardiac myosin-specific antibody into cells. They used these antibodies singly in a conventional radio-immunoassay, or in pairs, in a sandwich immuno-radiometric assay. Cardiac-light chain antibodies were shown to provide a powerful tool for the localisation and quantification of myocardial ischaemic necrosis *in vivo*. (Haber et al., 1982).

Ishiyama et al. (1982) applied the immuno-peroxidase technique to investigate the myoglobin staining reaction on formalin-fixed, paraffin-embedded heart tissues of typical cases of acute cardiac failure in five autopsy cases of apparently healthy and well-developed males who were found dead in bed in a Tokyo district and in whom there was no coronary disorder; slight hypoplasia of the heart and aorta and poorly developed coronary arteries being noted in such cases. In these 5 cases myoglobin was lost from the myocardial fibres in multiple and widespread foci together with a deposition of the released myoglobin into the interstitial connective tissue. The

ordinary tinctorial stains including the H &E and the haematoxylin basic fuchsin picric acid stain of Lie et al. (1971) showed no structural abnormalities of myocardial fibres and no oedema or inflammatory changes in any myocardial layer (Ishiyama et al., 1982) .

Block et al. (1983) studied 13 human cases of coronary artery disease, ten obtained from patients who died from MI and 3 from excised cardiac muscle in patients undergoing surgery for complications of MI (i.e. acquired ventricular septal defect, papillary muscle dysfunction). These infarcts ranged in age from less than 24 hours to several weeks and showed some significant scar formation. Cardiac tissue from 28 dogs were also used for comparison. In addition to examination of gross specimens by triphenyltetrazolium chloride (TTC) H&E, Periodic Acid-Schiff (PAS) staining for glycogen and myoglobin immuno-peroxidase studies were utilized. In 3 dogs with only 3 hours of occlusion, glutaraldehyde-fixed tissue was studied by electron microscopy to confirm evidence of necrosis shown by (TTC). The slice incubation technique of TTC originated in Germany in 1957 with Sandritter and Jestat , who first used a solution of TTC and exogenous substrate to macroscopically demonstrate infarcts in guinea pig and human hearts as early as 4 hours after experimental MI or the onset of clinical symptoms in patients who died. The theoretical basis for the tetrazolium staining of myocardium derives from the observation that when viable tissue is exposed to a tetrazolium salt, a coloured formazan is deposited at the site where the following reduction reaction is taking place. This reduction reaction depends on the availability of an appropriate substrate and an intact oxidative enzyme system, e.g., succinate dehydrogenase, within the myocyte. Oxidation of the substrate, e.g., succinate, produces a reduced coenzyme that then passes electrons to the tetrazolium, causing its reduction to formazan. It is important to note that by definition, 'ischaemic' tissue contains active enzymes and therefore will stain. However, 'infarcted' or necrotic tissue does not contain active oxidation enzymes and therefore remains unstained (Fallon, 1981).

In all human tissue the areas of necrosis could be defined by the use of the H&E and triphenyltetrazolium chloride (TTC) stains. Normal myocardial cells adjacent to necrotic areas stained darkly. Sub-endocardial sparing was demonstrable as a darkly stained narrow band of cells adjacent to the endocardium. In 4 dogs with 50% flow reduction for 3 hours and in 2 others with a total occlusion for 45 minutes no detectable reduction of immuno-staining for myoglobin was shown. TTC and H&E stains showed no evidence of necrosis; PAS staining showed marked glycogen

depletion, indicating that some ischaemic damage had occurred. In 3 of the 8 dogs with infarcts which were 3 hours old, decreased immuno-staining for myoglobin was detected. This was shown initially as a patchy decrease in staining in sub-endocardial areas with longer periods of coronary occlusion resulting in increasingly myoglobin losses. In the other 5 dogs with infarcts 3 hours old, no definite myoglobin loss was observed. In ischaemia with a five to six hour coronary occlusion a clear and definite loss of myoglobin could be demonstrated. Ischaemic myocardium unless clearly necrotic does not show any diminution in immuno-staining for myoglobin; actual necrosis is required before myoglobin loss from myocardium can be detected (Block et al., 1983).

Nolan et al. (1983) examined the development of cellular injury in the rat left ventricle by immuno-fluorescence after intravenous injection of monoclonal antimyosin. It was shown that cardiac muscle cells which have bound antimyosin during ischaemia were localised by staining sections with fluorescein-conjugated anti-mouse IgG. Three hours after occlusion and the injection of antimyosin fluorescent staining was detectable within the ischaemic region of the left ventricle. After 6 hours of ischaemia, the margin of the ischaemic zone was delineated clearly by fluorescent cells. At 3-6 hours after occlusion, the cellular staining in the epicardial half of the ischaemic area varied intensely, and fluorescent cells were mixed in with cells of much less fluorescence. By 24 hours, the ischaemic areas showed a homogeneous pattern of staining. The fluorescence was of a striated pattern indicating that anti-myosin penetrates cell membranes to bind specifically to myosin in A bands and the presence of eccentric staining in some cells suggests that the membrane may only be leaky at specific sites. The heterogeneous staining seen only in the epicardial half in early ischaemia, suggests that collateral arterial circulation may be sufficient to support a few cells but not others in the sub-epicardium for a period. The boundary between presumably irreversibly injured ischaemic cells (fluorescent) and the non-fluorescent surrounding cells (with intact membranes) is extremely sharp, both at 6 and 24 hours after occlusion. Although the exact mechanisms accounting for this heterogeneity are not established, it appeared that certain cardiac myocytes are more resistant to ischaemia than others, by virtue of specific characteristics such as their enzymatic and contractile properties (Nolan et al., 1983).

Fulghum et al. (1986) in an experimental study on dogs injected large amounts of non-radiolabelled monoclonal anti-cardiac myosin Fab fragments systemically

following varying periods of myocardial ischaemia by ligating the circumflex coronary artery. Immuno-fluorescent and immuno-peroxidase staining techniques were applied to hearts of these animals. Necrotic and viable myocardium could be differentiated at a cellular level by staining with anti-myosin antibody. The intra-cellular cyto-skeleton, in terms of myosin bands appeared to be preserved early in the necrotic process, even though the cell is dead. It was also noted that early in the necrotic process labelling with Fab did not necessarily occur throughout the entire cell: some cells demonstrated labelling at specific points beneath the membrane indicating that in such an early phase, the loss of membrane integrity or the number of membrane defects are inadequate to permit diffusion of larger amounts of Fab into cells in the time allowed. Following the shortest period of occlusion (i.e. forty minutes) and a brief period of reflow, single isolated necrotic cells were demonstratable within sheets of cells that were not yet necrotic (as determined by the Fab labelling) suggesting that the necrosis due to ischaemia starts by affecting scattered individual cells. Contraction bands were noted within some labelled cells but did not label with Fab (possibly because their density failed to allow the Fab to enter). All cells with contraction bands were labelled with Fab; but also of areas of antibody stained myocardial cells showed no contraction bands, indicating that contraction bands are not essential requisite of necrosis (Fulghum et al., 1986).

Hashimoto et al. (1986) studied the immuno-reactivity of myocardial actin filaments with actin ab on 16 autopsied cases diagnosed as myocardial infarction. The patients had died between 2 hours and 30 days after clinical onset of symptoms; with all autopsies which were performed with a postmortem interval of less than 2 hours. All specimens were formalin-fixed, paraffin-embedded and were stained with H&E, Azan-Mallory (Azan), acid fuchsin (Poley et al., 1964), hematoxylin-basic-fuchsin-picric acid (HBFP) (Lie et al., 1971) and the avidin-biotin-peroxidase complex (ABC) method with actin ab. Heart slices from three cases were stained by the triphenyltetrazolium chloride (TTC) reaction. Based on the comparison of gross findings of TTC reactions with actin ab, it was shown that the extent of loss of the actin reaction was larger than the area of loss of the TTC reaction with this disparity being more pronounced in fresh cases of infarction. All preparations examined showed constant, diffuse and reproducible patterns of reaction to the actin antibody.

The reaction of actin ab observed on these sections, was that the smooth muscle cells of the blood vessel walls in the myocardium showed maximal staining intensity. Myocardial fibres were stained pale brown to dark brown and retained their

striations in the normal areas. Intercalated discs were also stained, but to a lesser degree than that in the smooth muscle cells. The intensity of the staining reaction within myocardial fibres was not related to the duration of the postmortem interval upto 28 hours. The reactivity of the actin ab on muscle fibres which showed necrosis was completely lost. The most important finding to be emphasised in the study was the complete disappearance of reaction to actin ab, not only in completely necrotic muscle fibres, but also in fibres showing eosinophilic cytoplasm but retaining cross-striations and with very slightly pyknotic nuclei. Muscle fibres showing compensatory hypertrophy adjacent to granulation and scar tissue showed a more intense reaction to this ab staining (Hashimoto et al., 1986).

Hoffmann et al. (1987) investigated the distribution of human atrial and ventricular myosins after myocardial infarction. They prepared myosins from autopsy specimens from subjects with coronary heart disease. Cardiac myosin light chain isotypes were resolved using two-dimensional gel electrophoresis, whereas myosin isozymes were detected by pyrophosphate gel electrophoresis. Ventricular myosin light chains were found in pressure overloaded atria and atrial light chains have been identified in the infarcted ventricle of the human heart. It could be shown that in myocardial infarction, the ventricular light chain isomyosin-1 (VLC-1) becomes partially replaced by the atrial light chain isomyosins (ALC-1 & ALC-2). For this reason, it is likely that the ALC-1 and ALC-2 exhibit a specific occurrence in cardiac disease thus serving as markers at the beginning of the infarct (Hoffmann et al., 1987).

Nishida et al. (1987), investigated the effectiveness of diagnosing myocardial infarction at the earliest (possible) stage and the changes of actin filaments on 106 rats using immunohistochemical methods involving anti-actin antibodies after their left coronary arteries were ligated. At specified intervals after occlusion, the heart was removed and divided into three parts with the central ischaemic region being used exclusively for electron microscopy . The upper and lower parts were fixed in formalin and embedded in paraffin after triphenyltetrazolium chloride (TTC) reaction. The sections were stained with H&E, and were also reacted with anti-actin antibodies.

In sections with a 5-minute ischaemic period the dehydrogenases were still present. However, when reacted with anti-actin antibody they showed a sporadic presence of fibres which were negative.

Very early in the acute stage i.e. 5 and 15 minutes after coronary occlusion, some fibres already showed a negative reaction to anti-actin antibodies, and that these fibres because were prominent and frequent with the increasing duration of the occlusion: extensive disappearance of the reaction was seen 30 minutes after occlusion. However, in animals with early infarction i.e. five minutes to 3 hours after occlusion, in which enzyme-histochemical detection of ischaemia was not possible, despite the disappearance of the anti-actin antibody reaction, electron microscopy still revealed the existence of actin fibres (Nishida et al., 1987).

Nomoto et al. (1987) studied the pattern of myoglobin loss in 105 rats following occlusion of the coronary artery. The hearts were fixed in formalin and stained with H&E or by the peroxidase-antiperoxidase (PAP) for myoglobin. With myoglobin loss in each slice being calculated by an image analyzer. Staining with a rabbit anti-human myoglobin ab resulted in diffuse, dark brown immunostaining of the myocardium of the control rats. Tissues without myoglobin, such as vessel walls, failed to show staining. Myoglobin depletion, as well as interstitial oedema, was evident in the subendocardium of the left ventricle as early as 0.5 hour after coronary ligation and when only interstitial oedema could be identified with H&E stain. No irreversible changes of ischaemia e.g. eosinophilic cytoplasm, loss of nucleus could be detected in the H&E stain of sections until 6 hours after the commencement of ischaemia. The ultrastructure of the myocardial sections obtained from the subendocardial myoglobin-depleted area at 0.5 hour of ischaemia showed characteristic ischaemic changes, i.e., widened I bands, loss of glycogen, swelling of sarcoplasmic reticulums, intra-myofibrillar oedema and breaking, clumping and margination of nuclear chromatin, loss of matrix density of mitochondria and with separation of cristae. It was suggested "*that the loss of myoglobin from the myocardial cells could serve as an early and sensitive marker of irreversible cell damage after myocardial ischaemia*" (Nomoto et al., 1987).

Fujiwara et al. (1988) in an experimental study on 23 farm pigs examined their hearts 20 minutes after occlusion by the H&E stain, Masson trichrome stain, nitrotetrazolium blue stain and an immunohistochemical method using myoglobin antibody. Two hours after coronary occlusion, all the five hearts examined showed slight losses of myoglobin with the myoglobin depletion being moderate at 4 hours and 6 hours after occlusion (Fujiwara et al., 1988). This study was extended by Fujiwara et al. (1989) and similar features were shown.

Leadbeatter et al. (1989) studied 12 human hearts with macroscopic myocardial infarction and 17 other cases without myocardial infarction (10 with and 7 without significant coronary artery atherosclerosis) to characterise the patterns of immunocytochemical staining in deaths with myocardial infarction visible to the naked eye, in deaths without macroscopic infarction but with significant coronary artery atherosclerosis (denoted by greater than 50% obstruction of the diameter of the lumen in at least one vessel) and in deaths with neither coronary artery atherosclerosis nor evidence of prolonged terminal hypoxia and comparing them with other tinctorial methods (with those seen with H&E, PTAH, and HBFP staining), on formalin-fixed, paraffin-embedded tissue. The tissue blocks were selected from the region of the left ventricle which was considered to be the most vulnerable to ischaemic/hypoxic damage. The primary antibodies used in their study were commercially obtained polyclonal antibodies labelled with DNP imidoester: anti-myosin, anti-myoglobin, anti-caeruloplasmin, anti-CRP, anti-pre-albumin, anti-α-1 anti-trypsin (anti-α-1 AT) and anti-$C3_b$.

In the 12 deaths with MI visible to the naked eye: Histological examination showed that in all the cases the apparently healthy muscle was without abnormality visible on either H&E and PTAH staining. The subendocardial zone which generally escaped ischaemic damage showed a convenient and reliable in-built control and these zones consistently showed strong staining with antibodies to myoglobin, myosin, caeruloplasmin and CRP, slightly weaker staining with anti-pre-albumin but no significant staining with anti-α-1 AT. Results with anti-$C3_b$ were variable with the staining in these areas being generally strong. In the six cases which showed areas of coagulative necrosis, no staining with antibodies to myoglobin, myosin, caeruloplasmin, CRP, pre-albumin or $C3_b$ was seen, but there was strong staining with anti-α-1 AT. All cases which contained areas of infarction in the reparative phase (six without concomitant coagulative necrosis) showed no staining with any antibody except that to $C3_b$, which gave strongly positive immuno-staining.

In 10 cases with no macroscopic infarction but with significant artery atherosclerosis; there was no abnormality on H&E and PTAH staining but these areas showed staining with abs to myoglobin, myosin, caeruloplasmin, CRP and pre-albumin. Antibody to α-1AT was not found to be useful, there being no significant staining with antibody to this protein in all cases without coagulative necrosis. Anti-$C3_b$ staining was found to be too variable for useful interpretation.

In the 7 hearts in which there was neither coronary artery atherosclerosis nor any history of prolonged terminal hypoxia, it was shown that in five of these deaths the pattern of immunohistochemical staining seen in the sub-endocardial zone was strong with antibodies to myoglobin, myosin, caeruloplasmin and CRP, slightly weaker staining with antibody to pre-albumin but no significant staining with antibody to α-1AT and variable staining with antibody to $C3_b$. In two deaths in which there had been periods of active resuscitation, focal fuchsinorrhagia with the HBFP technique was shown but in these foci there was no staining with antibodies to any of the markers. Anti-α-1 AT was a strong 'positive marker' of infarction, consistent with α-1 AT being an acute phase reactant, while, pre-albumin and CRP were consistently 'negative markers'; $C3_b$ was found to be an unreliable marker. This study also demonstrated that HBFP results correlated well with immunohistochemical results for cases with suspected early myocardial damage (Leadbeatter et al., 1989).

In an another study Leadbeatter et al. (1990) subdivided deaths in to one of three populations: Population-1: 15 cases with macroscopic evidence of myocardial infarction. Population-11: 26 cases with no macroscopic evidence of myocardial infarction but significant coronary arterial atherosclerosis i.e. obstruction of more than 50% of the diameter of the lumen of at least one major artery. Population-111: 33 cases with neither macroscopic evidence of myocardial infarction nor significant coronary artery atherosclerosis. Serial sections of myocardium were stained respectively with H&E, PTAH, HBFP and immunoperoxidase technique with antibodies to caeruloplasmin, myosin, myoglobin and CRP. In all 15 cases of population-1, the immediately subendocardial myocytes provided a reliable 'in-built control' consistently showing no abnormality and strongly positive staining with all four antibodies. No histological abnormality was seen in the myocardium (stained with H&E and PTAH) taken from the hearts in populations 11 and 111. In 19 of 26 cases in population-11 the myocardium which was fuchsinorrhagic did not stain. In 6 of the remaining 7 cases there was strong positive staining with the antibodies but no fuchsinorrhagia was demonstrable. Of the 33 cases in population-111, 15 cases showed fuchsinorrhagia which did not stain with the antibodies, and 15 cases contained myocardium which showed strong positive antibody staining and did not show fuchsinorrhagia with only 3 cases showed discordant results. In 9 of 15 cases with fuchsinorrhagic myocardium, a clinical history of ischaemia or hypoxia was present, which did not stain with the antibodies. In several of the cases in population

11 and 111 where the myocardium showed fuchsinorrhagia and no staining with the antibodies, histological examination at high power showed contraction band necrosis.

From this study it was concluded that loss of antibody staining to caeruloplasmin, myosin, myoglobin and C-reactive protein is a valid indicator of myocardial damage and that this correlating well with HBFP staining. Both techniques are more sensitive than H&E and PTAH staining, but their sensitivity is such that they may also detect immediately-terminal or agonal changes, regardless of cause of death and thus cannot be used alone for the specific diagnosis of myocardial ischaemia as a cause of sudden death (Leadbeatter et al., 1990).

Iwai et al. (1990) performed an in situ experimental study on twenty-nine canine hearts to delineate the cytoskeletal changes during myocardial ischaemia and reperfusion. Myocardial tissue perfused by the LAD was fixed by perfusion with a periodate-lysine-paraformaldehyde (PLPD). The sections were incubated with monoclonal antibodies against actin, ß-tubulin and vinculin and examined by both immunofluorescence and electron microscopy. There was no immuno-reactivities of microtubules with ischaemia of less than 20 minutes. Initial detectable changes with a loss of characteristic reticular network in microtubules were observed 20 minutes after coronary occlusion; the immuno-reactivities were decreased or lost in patchy lesions, whereas actin filaments appeared unaltered. The disruption of vinculin was less marked than that of microtubules, suggesting that the disruption of microtubules which occurred before the irreversible change, may be an early sign of irreversible injury. Thus, disruption of the microtubules may largely contribute to the cellular fragility and dislocation of the organelles, which could accelerate the membranous damage of the cell. Vinculin, which is tightly associated with the plasma membrane, may directly support the sarcolemmal integrity, and its disruption may result in sarcolemmal damage (Iwai et al., 1990).

Armstrong & Ganote (1992) isolated cardiomyocytes from adult rats and examined them under ischaemic incubation by using monoclonal antibodies against tubulin and vinculin applying immunofluorescence and quantitative flow cytometry. Fluorescence microscopy of control vinculin-stained myocytes showed that the specific fluorescence was found at the intercalated discs and in a ribbed pattern along Z-bands. Ischaemic myocytes showed small changes in tubulin fluorescence, but with inhibition of contracture (by using butanedione monoxime) there was a rapid

and comparatively large loss of fluorescence by 15 min of ischaemia. Vinculin fluorescence also began to drop by 15 min, and progressively decreased. Osmotic fragility was also tested by trypan blue staining. A temporal relationship was shown between loss of vinculin fluorescence and increasing osmotic fragility suggesting a relationship between the two events, but the structural and metabolic events which cause decreased fluorescence were unexplained. The loss of vinculin fluorescence may be due to a variety of causes: loss or degradation of this specific protein, redistribution of the protein within the cell, structural modification of the protein with masking of binding sites and any other combination of these causes. The close temporal correlation between decreased fluorescence of vinculin and increased myocytic fragility suggests that diminished fluorescence may reflect structural and functional disorganization of the cytoskeleton. Alterations in the cytoskeleton could be demonstrated to occur prior to or coincident with increasing cell fragility which suggests that the findings of early fluorescence changes of vinculin represent membrane and cytoskeletal abnormalities. These results are apparently in conflict with the findings of Iwai et al. (1990), who reported that early disappearance of tubulin fluorescence correlated more closely with irreversibility of ischaemia than did a later decrease of vinculin (Armstrong & Ganote, 1992).

Brinkmann et al. (1993) in a recent study investigated the validity of a large 'battery' of histochemical and immunohistochemical methods in the diagnosis of early myocardial damage. Forty eight hearts were systematically studied of which 35 were cardiac deaths and the others were controls which were sub-divided into 3 groups: 1. infarction visible in gross examination (N=15), 2. coronary thrombosis without infarction (N=11), 3. stenosing coronary atherosclerosis without infarction (N=9). {The control group (group 4) consisted of un-natural deaths with presumed short agonal periods (N=13)}. Postmortem intervals varied between 6 and 144 hours and did not differ significantly between sub-groups 1-4. Formalin-fixed, paraffin-embedded 5 mm sections were stained with H&E, a modified luxol fast blue, chromotrope aniline blue (CAB) and immunohistochemical staining for desmin, myoglobin, fibrinogen, and $C5_b$-9 complement complex. In the infarction sub-group (1) the histochemical and immunohistochemical features were fairly evenly distributed. Serial sections from infarction areas showed a significant loss of desmin and myoglobin, and an accumulation of fibrinogen and $C5_b$-9 within the necrotic fibres and/or in their intimate surroundings. In cases of thrombosis without infarction (2), 4 cases (out of 11) lacked the histological/histochemical signs of myocardial necrosis and 3 showed positive reactions for the antigens tested. In group (3) staining

alterations were patchy and in single fibres or in small groups of fibres with the staining intensity being quite low (Brinkmann et al., 1993).

4.B. Coronary microcirculation.

Knowledge about the metabolic and synthetic properties of endothelial cells has substantially increased in recent years and certain key functions of endothelial cells in thrombosis, haemostasis, regulation of vascular tone and wound healing have been described (Gräfe et al., 1993). Substantial progress has been also made in clarifying questions about capillary transport. The vascular endothelium consists of a single layer of flattened, fairly uniform, polygonal, and elongated cells that are approximately 25 μm to 50 μm long and 10 μm to 15 μm wide. Their long axis is oriented in the direction of the blood flow. Because of its location, the endothelial layer assumes (in most areas) the role of a barrier which selectively regulates transfer of substances of varied molecular size between the circulating blood and the surrounding tissues (Thorgeirsson & Robertson, 1978).

4.B.1. The reaction of microcirculation to ischaemia.

The coronary capillary bed consist of a network of relatively long (up to 1-2 mm) capillaries. These capillary networks inter-connect through extensive anastomoses, but capillary loops from different large epicardial coronary arteries do not interconnect (Okun et al., 1979). An increase in the functional intercapillary distance of 30% can lead to a partial pressure of oxygen of 0 mm Hg at some tissue locations (Henquell et al., 1976). It was demonstrated that if 60-68% of capillaries are blocked there is the possibility of local ischaemia despite adequate coronary arteriolar pressure upon reperfusion. In the normal myocardium multiple intercapillary anastomoses minimize the effect of blockage of a single feeding arteriole or capillary, they result in a functional capillary length equivalent to the unbranched length, and help maintain a uniform short diffusion path (Engler et al., 1983).

Early studies of in-vitro myocardial ischaemia showed that vascular injury was only found after prolonged ischaemia and appears to develop later than injury to the myocardium itself. Kloner et al. (1980) in ultrastructural studies confirmed that vascular injury was not present till after 60 minutes of ischaemia, at which time some myocyte injury was already evident. These morphological changes were confirmed in studies of myocardial albumin distribution and coronary vascular

resistance measurements. Dauber et al. (1990) indicated that these studies reflected the relative insensitivity of the experimental methods which were being used and suggested an assessment of vascular injury by analysing endothelial permeability as a functional index of vascular damage. Using a double indicator method i.e. (^{113}indium transferrin and ^{99}technetium erythrocyte), Dauber et al. (1990) were able to quantitatively assess an increased microvascular permeability with protein leakage in anaesthesised dogs in which the left anterior descending coronary artery has been occluded for varying periods. Permeability increased after 15 minutes of ischaemia and there was also an impairment of endothelial dependent vasodilation. No changes were demonstrable by electron microscopy in vessels which had been subjected to less than 60 minutes of ischaemia (Dauber et al., 1990).

An added phenomenon in myocardial capillary damage is associated with the flocking of polymophonuclear neutrophils to the area of ischaemia as a direct effect of chemical mediators. Engler et al. (1983) described 'plugging of microvessels' by neutrophils in the ischaemic dog heart supposedly leading to additional injury of myocytes. Very early in irreversible injury the neutrophils are seen to adhere and stick to the capillary endothelium; thereafter they leave the capillaries and migrate into the extravascular space (Engler et al., 1983). The microcirculation becomes plugged by leukocytes which have a natural intrinsic tendency to adhere to vascular endothelium, especially in post-capillary venules. When arterial pressure is reduced and when capillaries are narrowed by perivascular oedema the leukocytes will lodge within blood vessels; this capillary plugging is not sorted out even when there is re-perfusion of the area. This suggests a further potential for damage resulting from vascular-related phenomenon, or from the endothelial dysfunction resulting from ischaemia and re-perfusion which also contribute further to the dysfunction of the myocardium. Furthermore interstitial oedema resulting from circulatory and inflammatory reactions further impair myocardial function by impairing the exchange of metabolites and myocardial contractile function. The endothelial dysfunction may eventually lead to spasm, and even to local thrombosis with further compromise of nutrient delivery to the myocardium and through the release of the inflammatory mediators from this side to further endothelial injury with a viscous circle of further damage (Ryan, 1986; Dauber et al., 1990).

Migration of activated polymorphs from the plugged blood vessels then ensues. These mobile cells attach themselves to cardiac muscle fibres and can be directly cytotoxic, partly due to local activation of the complement cascade with the

production of chemotactic complement factors such as $C3_a$ and $C5_a$ and of other factors which damage membranes such as the membrane attack complex, $C5_b$-9 (Mullane & Young, 1992).

4.C. Complement.

The term complement was originally applied by Ehrlich in the year 1899 to describe the activity in serum which, combined with specific antibody, would cause lysis of bacteria. Ehrlich & Morgenroth paper was published and reprinted as a part of a monograph in 1910 (Ehrlich & Morgenroth, 1910). The developments leading to the recognition of the individual complement proteins span the period from 1907, when Ferrata (1907) described C1 and C2, to the mid-1960s when the resolution of the terminal complement proteins C5, C6, C7, C8 and C9 was completed (Nelson et al., 1966).

The pivotal step in complement activation is the cleavage of C3 by two C3 convertases which are produced by both the classical and alternative pathways. The larger product of C3 cleavage, $C3_b$, combines with the C3 convertases, forming a $C5_a$. The larger product of C5 cleavage, $C5_b$, spontaneously binds C6, C7, C8 and C9 (Williams & Rampart, 1987). The consequences of complement activation are: i) opsonization, ii) activation of leucocytes and iii) lysis of target cells. The latter results from the insertion of a hydrophobic 'plug' into lipid membrane bilayers, allowing osmotic disruption of the target (Roitt et al., 1993).

When the macromolecular product, $C5_b$-9, forms on the surface of a susceptible cell, it can cause cell lysis. This final phase of activation of the complement cascade is called the: Membrane Attack Complex (MAC). The first step towards MAC formation is the enzymatic cleavage of C5 but the remainder of the formation of the MAC is non-enzymatic. $C5_b$ binds C6, forming $C5_b6$, and this then binds C7 to form a $C5_b67$ complex. Binding to C7 marks the transition of the complex from a hydrophilic to a hydrophobic state that preferentially inserts into lipid bilayers. C8 and C9 then sequentially bind to this complex resulting in the formation of the lytic 'plug', or pore-forming molecule. $C5_b6789$ is usually abbreviated to $C5_b$-9. This MAC is not detectable in normal muscle fibers (Engel & Biesecker, 1982; Biesecker, 1983; Williams & Rampart, 1987; Roitt et al., 1993).

4.C.1. Regulation of activation of membrane attack complex (MAC).

Once this complex has formed, it can insert itself into other membranes close to the primary surface on which complement activation is focused. This is the process of 'reactive lysis' which, if un-regulated, could have damaging consequences to self/host tissues. There are a number of proteins that inhibit this process by binding to fluid phase $C5_b67$ before it can attach to self membranes. Host cells also bear membrane proteins that protect them against lysis by the MAC. One such protein which has been characterised is protectin (CD 59). CD59 is a protein anchored by a glycophospholipid foot and it is widely distributed in cell membranes. It inhibits the insertion and polymerization of C9 into cell membranes bearing $C5_b$-8 (Simpson et al., 1993).

The MAC formed following complement activation in tissue may directly cause injury to cellular or membrane structures and may also serve as a marker for tissue damage by complement-dependent cellular mediators. The MAC has been localised at sites of tissue injury in humans and in animals with experimentally induced diseases, and has also been demonstrated in renal tissue of individuals with systemic lupus erythematosus (SLE) nephritis (Biesecker et al., 1981), in skin lesions of SLE (Biesecker et al., 1982), and in an experimental model of immune-complex glomerulonephritis (Koffler et al., 1983; Biesecker, 1983).

4.C.2. Protectin and MAC in myocardial infarction.

Deposition of the MAC has been shown in infarcted myocardium of both human patients and experimental animals (Schäfer et al., 1986; Rus et al., 1987; Vlaicu et al., 1988; Väkevä et al., 1992 &1993; Brinkmann et al., 1993). The most intensive staining with anti-$C5_b$-9 antibodies was observed in the peripheral areas of infarctions indicating that the terminal complement components were deposited mainly in the form of membrane-damaging $C5_b$-9 complexes. Immunohistochemical staining shows that the whole cytoplasm is stained in most necrotic cells in infarcted areas. Single necrotic cells that may be easily overlooked by conventional histology are clearly visible even at low-power magnification. $C5_b$-9 staining was also positive in the subendothelial layer of myocardial arteries, however, this vascular staining was not restricted to myocardial infarctions. In rare instances, $C5_b$-9 staining can be restricted to discrete granular or linear deposits at the cell membrane. Staining was especially intense in a peripheral rim of larger infarcted areas.

$C5_b$-9 investigations on paraffin sections of formalin-fixed material showed that the polyclonal antibody to $C5_b$-9 is also able to stain myocardial infarctions under these conditions. This, is only possible after prior pronase digestion of the sections. Although it is not possible to exclude that $C5_b$-9 deposition had occurred as a secondary reaction on irreversibly necrotic myocardial cells, immunostaining for $C5_b$-9 should represent a sensitive tool for detection of ischaemic myocardial lesions, permitting easy detection even of single cell necrosis (Schäfer et al., 1986).

In the normal myocardium the sarcolemma stained positively for protectin throughout the cardiac muscle. Protectin was also present on vascular endothelia of arteries, veins, and capillaries throughout the body. Analysis of cross-sections of arteries showed positive staining in the luminal aspect of endothelia, whereas the surrounding smooth muscle layers were negative (Meri et al., 1991). A clear association was shown between the absence of protectin histopathologically documented infarction (Väkevä et al., 1992).

Examination of serial sections revealed that depositions of MAC were co-localised with the protectin-negative lesions. Deposition of MAC was also demonstrated with a monoclonal antibody against the $C5_b$-9 complex. The protectin-negative area was often somewhat larger than the area of MAC deposition. MAC was also deposited within clearly demarcated borders where protectin showed a gradual disappearance towards the histopathologically demonstrable area of infarction. It was suggested therefore that the lack of full overlap between the protectin-negative and MAC-positive areas indicates that the loss of antibody reactivity against protectin is a specific feature of the infarcted cells. These results are in accordance with those of Schäfer et al. (1986), who noted that demonstration of $C5_b$-9 deposition is the most sensitive tool for detecting ischaemic myocardial lesions. Despite the demonstrated association between loss of protectin and MAC deposition in myocardial infarcts, this did not of course establish a 'cause and effect' relationship between the loss of protectin and MAC-mediated cell damage (Väkevä et al., 1992).

To explain the loss of protectin (CD59) Väkevä et al. (1992) suggested originally that this was the result of exocytotic shedding from the myocardial cells but in their paper of (1993) it was suggested that this loss was due to endogenous phospholipases or proteolytic enzymes with, the release of CD59 being physiologically useful in assisting MAC-mediated clearance of non-viable tissue

with the complement system, including the $C5_b$-9 complex and its regulators being a clearance mechanism to remove non-viable cells after tissue injury. Cells damaged by ischaemia become the targets for attack by complement components because of changes in the local micro-environment and alterations in the properties of the cell surface; the formation of the MAC, possibly enhanced by loss of CD59, further contributes to the dissolution of cell membranes of the ischaemic cells (Väkevä et al., 1993).

Brinkmann et al. (1993) showed a highly specific accumulation of fibrinogen and $C5_b$-9 using immunohistochemistry within the necrotic fibres and/or their intimate surroundings. In the group with virtually no or only weak signs of myocardial necrosis, $C5_b$-9 seemed to react even more slowly than other histochemical markers (Brinkmann et al., 1993).

4.D. CD 34.

The CD34 monoclonal antibody recognizes a cell surface antigen that appears to be expressed selectively on human haematopoietic progenitor cells (Watt et al., 1987); foetal tissue capillaries from most tissues were also CD34 positive. In vivo, CD34 molecules have a striking ultrastructural localisation on endothelial cells being concentrated primarily on the luminal side and, in particular, on membrane processes, many of which interdigitate between adjacent endothelial cells. This distribution accords with a possible role in cell interaction (Fina et al., 1990).

A limited number of reagents for the identification of endothelium are available. Factor VIII-related antigen (Mukai et al., 1980), now referred to as von Willebrand factor (VW factor) and Ulex europaeus agglutinin, type 1 (UEA-1) (Holthofer et al., 1982; Miettinen et al., 1983)) are the two markers widely used for identification of endothelium in routinely processed tissue. Both these markers have limitations. UEA-1 may be a more sensitive means of recognizing endothelial differentiation (Ordonez & Batsakis, 1984), it is also less specific, since binding may also occur in many epithelial tumours (Leader et al., 1986). Recently, a new monoclonal antibody QBEND/10, directed against endothelium, has been described (Watt et al., 1987). It is an IgG_1 antibody and was raised by injecting mice with endothelial cell membranes. QBEND/10 detects the formalin-resistant epitope on the endothelial cells lining all types of vessels and is therefore applicable to routinely processed tissues (Ramani et al., 1990).

CHAPTER 5.

LECTIN BINDING IN THE MYOCARDIUM.

5.A. LECTINS: A definition and historical background.

The name 'lectin' was suggested originally by William Boyd & Elizabeth Shapleigh in 1954, to denote antibody-like substances that are not formed as a result of an antigenic stimulus, *"...it would appear to be a matter of semantics as to whether a substance not produced in presence to an antigen should be called an antibody even though it is a protein and combines specifically with certain antigens only. It might be better to have a different word for these substances and the term: 'LECTIN', from the Latin lectus, the past participle of 'legere' meaning to pick, choose or select"* was proposed. 'Lectins' are therefore a class of proteins, glycoproteins different from immunoglobulins which have binding sites for carbohydrates (CHOs) without inducing any chemical changes on them (Boyd & Shapleigh, 1954).

In 1949, Boyd working at Boston University School of Medicine, and Renkonen working at the University of Helsinki, discovered independently that certain plant agglutinins (or phytagglutinins, as they were referred to at the time) were blood type-specific (Boyd & Reguera, 1949; Renkonen, 1948). The research in this area owes its origins to a thesis published in 1888 by Hermann Stillmark, then a doctoral student of Rudolf Kobert, at the University of Dorpat in Estonia (Sharon & Lis, 1987) who while studying the toxicity of the castor bean (Ricinus communis) observed that extracts of the beans agglutinated erythrocytes and the material causing the agglutination was a protein, and gave it the name 'ricin'. In 1891, Hellin another student of Kobert, discovered that the toxic extract of the jequirity bean (Abrus precatorius) which also caused red cells to clump together and this new agglutinin was named 'abrin' (Sharon & Lis, 1987).

These reports immediately attracted the attention of the father of modern immunology, Paul Ehrlich at the Royal Institute of Experimental Therapy in Frankfurt; he recognised the value of abrin and ricin as antigenic model substances and their advantages over the then-used diphtheria toxin. Ehrlich carried out a number of experiments with abrin and ricin that established some of the fundamental and elementary concepts of immunology. Rabbits fed with small amounts of jequirity seeds developed a certain degree of immunity against abrin. Immunity

could be increased by the additional parenteral administration of the toxic protein. The potential of plant agglutinins as model antigens lay in the ease by which they could be prepared in large quantities, their stablity, and the fact that when injected into animals they caused the production of antibodies that inhibited both their toxic and agglutinating activities (Kocourek, 1986; Sharon & Lis, 1987).

Early studies had indicated some selectivity in the lectin-mediated agglutination of red cells from different animals. In 1908 Karl Landsteiner, (the discoverer of blood groups), together with Raubitschek added further weight to this observation (Sharon & Lis, 1987).

The sugar specificity of lectins was discovered by James Sumner (from Cornell University, Ithaca, New York), also well known for being the first to crystallise an enzyme (urease) from jack beans in 1926. As early as 1919, he isolated concanavalin A from extracts of the jack bean and obtained for the first time a pure lectin (Sumner, 1919).

5. B. Characteristics of lectins.

The main characteristics of lectins are their ability to bind sugars, to agglutinate cells, and also to stimulate lymphocytes: the latter phenomenon was discovered by Peter Nowell (Nowell, 1960). In all their activities, lectins exhibit varying degrees of specificity. Most lectins interact preferentially with a single sugar structure, e.g. galactopyranose or L-fucopyranose. For some lectins, the specificity is broader and includes a number of closely related sugars e.g. mannopyranose, glucopyranose, and arabinose. Other lectins will interact only with complex CHO structures such as those that occur in glycoproteins or on cell surfaces. It is noteworthy that the sugars with which lectins interact best are those that are typical constituents of glycoproteins or glycolipids (Lis & Sharon, 1977).

Despite their wide range, lectins can be classified in five groups on the basis of their preferential binding to terminal sugars in the CHO side chains: 1) α-D-mannosyl and α-D-glucosyl residues; 2) β-D-galactose and β-D-galactose/N-acetyl galactosomine group; 3) N-acetylglucosamine group; 4) L-fucose group and 5) sialic acid group. Lectins commonly used as histochemical probes (Danguy et al., 1988).

The ready availability of lectins, their ease of preparation in purified form, their amenability to chemical manipulation and the observation that the activity of many of them is inhibited by simple sugars, make them a most attractive tool in biological research (Lis & Sharon, 1977).

Since their discovery, numerous lectin-like substances have been identified, purified, and characterised from diverse sources including plants, bacteria, fungi, invertebrates, and vertebrates. They have been utilized extensively to isolate glycoproteins and polysaccharides, and as tools to investigate the sugar residues of the glycocalyx. The glycocalyx is the morphological term which describes the membrane-associated complex of polysaccharides, proteoglycans, glycolipids, and glycoproteins; these are anchored in the plasmalemma, but have their bound oligosaccharides exposed outside the lipid barrier. These CHO moieties are critical to surface membrane phenomena e.g. cell adhesion, intercellular linkage, contact inhibition, growth control, antigenicity of cells, uptake of nutrients, and receptor activity for numerous biologically active agents. Lectins are powerful and specific histochemical probes, and assist with the study at both light and electron microscopical level of specific constituent sugars of the oligosaccharides of the glycocalyx (Alroy et al., 1984).

Whatever it can be, lectins have proved to be very valuable in a wide variety of both *in vitro* and *in vivo* applications including: mitogenic stimulation of lymphocytes and their subpopulations studies; blood grouping; isolation; purification; and studies of CHO containing molecules; histochemical studies of many tissue and organs (Lis & Sharon, 1986a & 1986b; Danguy et al., 1988).

5.C. Nomenclature and properties.

Goldstein et al. (1980) suggested that all these diverse organic compounds should be grouped together under the term lectin. Other sugar-binding proteins such as sugar-specific enzymes, transport proteins, hormones and toxins are well known. It has been suggested that only when the latter can act as lectins biochemically and immunologically they are to be included in the same definition which is thus 'an operational one'. In contrast, Kocourek & Horejst (1981) suggested that it was inappropriate to define lectins by their *in vitro* biological activities but that these should be defined under more important physicochemical properties in their interactions with CHO. Dixon (1981) then, the Secretary of the Nomenclature

Committee of International Union of Biochemistry decided in favour of Goldstein et al.'s (1980) definition.

Lectins attracted only limited attention until the early 1960s but two discoveries dramatically altered this. The first was made in 1960 by Peter C. Nowell at the University of Pennsylvania, Philadelphia, who found that the lectin originating from the red kidney bean *(Phaseolus vulgaris)*, known as phytohaemagglutinin or PHA, is mitogenic to lymphocytes (Nowell, 1960). This discovery had a revolutionary impact on immunology in that it shattered the view held until then that lymphocytes were 'dead-end' cells that could nor divide or differentiate further (Sharon & Lis, 1987). The second discovery was that *in vitro* lectins had an ability to preferentially agglutinate malignant cells, as first demonstrated in 1963 by Joseph Aub at the Massachusetts General Hospital in Boston (Aub et al., 1963). Indeed the purification of wheat-germ agglutinin by Max Burger at Princeton University, New Jersey, led to extensive studies of the surface changes that accompany the malignant transformation of cells (Burger & Goldberg, 1967; Rapin & Burger, 1974).

These discoveries rekindled interest in the properties of known lectins and led to an intense search for, and purification of, new ones (Sharon & Lis, 1987). Work carried out by Nathan Sharon in collaboration with Itzhak Ofek from Tel Aviv University has shown that the mannose-specific bacterial surface lectins may also mediate attachment of bacteria to phagocytic cells in the absence of antibodies and complement leading to their engulfment and killing (Sharon, 1987): this process was designated as 'lectinophagocytosis'. Lectins were thus postulated also as recognition molecules (Sharon & Lis, 1987). Their occurrence in various organisms from virus to human, suggests that they do have important roles in cell recognition, cell adhesion, intra-cellular traffic, internalisation of external molecules, and transmembrane signalling (Zanetta et al., 1992).

The ability of lectins to induce erythrocytic agglutination results from the lectin's polyvalent nature, and many lectins having either two or four CHO binding sites. They have been used in agglutination techniques since the results of such tests can be determined visually and the specificity of each lectin can be assessed by inhibition of agglutination with specific haptenic sugars.

The agglutinating ability of lectins has also been utilised in developing a variety of gel precipitation in assays starting with a simple double diffusion method similar to Ouchterlony immunodiffusion technique.

5.D. Specialised applications.

5.D.1. Fluorescence.

The differential binding capacity of lectins coupled with the fact that they can be conjugated with either fluorescein isothiocyanate (FITC) or tetramethylrhodamine (TMRITC) and examined with a UV-microscope equipped with appropriate filters (Holthofer et al., 1981; Watanabe et al., 1981), either horseradish peroxidase (HRP) (Stoward et al., 1980; Schulte and Spicer, 1983) and further more the availability to obtain biotinylated lectins have made it possible to localise sugar sequences at the morphological level (Danguy et al., 1988). Early work that used this methodology employed fluorochrome-conjugated lectins as histochemical reagents on tissue sections or immobilised cell preparations (Pratt & Gibson, 1973). These fluorescent assays with subjective manual evaluation of binding have found widespread use as assays utilising lectins as CHO probes.

Fluorochrome-conjugated lectins have been adapted for use in flow cytometric techniques. A common problem in adapting fluorochrome-conjugated lectins for flow cytometric analyses is the inherent tendency of lectins to induce agglutination. Agglutination of lectin-stained cells precludes analysis by flow cytometry as this technique requires single cell suspensions.

5.D.2. Radioisotopes.

Radioisotopes conjugated to lectins have been used extensively for localisation and quantitation of lectin binding sites on cell surfaces. Autoradiographic techniques are particularly useful in studies in which low numbers of lectin binding sites need to be localised and the enhancement gained by radiography is required. A fascinating, albeit non-laboratory, use of radiolabelled lectins is in the scintigraphic localisation of tumours *in vivo*. In this application, a radiolabelled lectin is injected directly into a tumour-bearing animal, and with appropriate sodium iodide blockage of the thyroid and time for clearance of the unbound radiolabelled lectin, the tumour can

theoretically be localised using a gamma camera. This method has been attempted, using PNA to locate tumours in man and mice (Holt et al., 1984).

5.D.3. Affinity chromatography.

Another area in which lectins have been used successfully is affinity chromatography. Similar to antibody-based affinity chromatography, this methodology relies upon immobilisation of a lectin to a matrix such as Sepharose. A significant advantage of this procedure for cell separation over flow cytometric separation based on lectin binding is the relatively low cost of affinity chromatography for the separation of large numbers of cells.

5.D.4. Enzyme-linkage.

The conjugation of enzymes as labels to lectins has enabled the development of several detection laboratory assays. The most common applications of enzyme-lectin conjugates are as histochemical probes, as a direct one step method, (Enzyme-conjugated anti-lectin antibodies), reagents in ELLA (Enzyme Linked Lectin Assay)-type reactions, and as probes for glycoconjugates in gels and blots.

The use of enzyme-conjugated lectins in histochemistry is technically similar to fluorescence methods used to detect glycoconjugates in tissue sections. Peroxidase-histochemical methods allow the use of these reagents in electron microscopy as well as light microscopy, with the advantage that appropriate counterstaining enables concomitant morphological examinations. Early reports concerning peroxidase-lectin conjugates (Stobo & Rosenthal, 1972; Gonatas & Avrameas, 1973) emphasized the use of these reagents as probes for cell membrane CHO structures in electron microscopy.

Lectins were also biotinylated and binding can be visualised using an avidin biotin-peroxidase complex technique (Hsu and Raine, 1982; LIoyd et al, 1984). The latter method increased the sensitivity of the assays while simultaneously allowing a wider variety of applications for the conjugated lectin. In one experiment, the biotinylated lectin could be used in conjunction with avidin-peroxidase for histochemical staining of tissue sections, while in a second experiment the same biotinylated lectin could be used with avidin-fluorescein for flow cytometric analysis of blood cells, and in a

third experiment the same lectin could be used with avidin-alkaline phosphatase in an ELLA-type reaction.

Colloidal Gold, a label useful in several assay systems, has been successfully adapted for use with lectins, largely through the efforts of Roth and colleagues (Roth, 1983a & 1983b; Roth et al., 1984).

5.D.5. Electron microscopy.

Lectins have long been used as histochemical reagents in electron microscopy for the localisation of specific CHO-bearing structures. Any of several electron-dense labels, including ferritin (Nicolson & Singer, 1971), peroxidase (Bernardes & Avrameas, 1971; Stobo & Rosenthal, 1972; Gonatas & Avrameas, 1973), and colloidal gold (Roth et al., 1984), may be conjugated to lectins for use in this technique. Although this technique involves simple staining of tissue with label-conjugated lectins, many procedural variations exist such as staining either prior to or post-embedding, and labelling of intact cells, isolated cells organelles, or tissue sections are stained.

Lectins have been utilised as markers for CHO residues in a number of tissues particularly the endothelium. Thin sections can be exposed to different lectins with known CHO affinities with or without pre-incubation with enzymes that act on the same biochemical components. These studies have provided evidence for the superficial localisation of sialic acid relative to other sugar residues in the endothelial glycocalyx with changes demonstrable with ageing and vascular degenerative disease (Sarphie, 1985).

Rat atrial myocytes were cultured and after fixation with gluteraldehyde were exposed to WGA conjugated to HRP. This enabled the Golgi region, and the associated tubules and cisternae, to be identified showing that glycoproteins and/or glycolipids recognized by WGA can be separated from atrial natriuretic peptides also packed in intracytoplasmic granules thus enabling a better identification of the mechanisms of release of this hormone and its intracellular transfer (Iida & Page, 1989).

Milici & Porter (1991) also used endothelial cells and developed localisation techniques for the structural components using lectins as the markers. GS I, LEA and

PHA were the lectins used in these studies. The rationale for this was to identify the role of endothelial cells and their receptors involved in interactions with leukocytes, the coagulation cascade and the inflammatory mediators (Milici & Porter, 1991).

5.D.6. Histochemistry.

The interaction between a lectin and its specific sugar residues has been compared to that of an antibody and its respective antigen. This affinity provides the basis for the cytochemical localization of these CHO residues at both the light and electron-microscopic levels (Bernardes & Avrameas, 1971). The CHO residues of the cell surface have been classified into two broad categories: free polysaccharides, which are loosely attached to the membrane; and membrane CHOs, which are strongly linked by covalent bonds to membrane proteins (glycoproteins) or membrane lipids (glycolipids) (Roseman, 1975). It has become increasingly evident that complex CHOs are involved in fundamental cellular processes, in particular, glycoproteins and glycolipids have been shown to be constituents of receptor sites as well as serving as surface antigens which play an essential role in such basic phenomena as recognition, adhesion, immunological defence, and permeability regulation (Sharon & Lis, 1972; Bonilla et al., 1980).

Hapten inhibition techniques are employed to determine the nature of the specific sugar-binding sites of lectins; different sugars are tested for their ability to inhibit either haemagglutination or glycoconjugate precipitation by the lectin. A major drawback in using paraffin sections is the loss of glycolipids receptors during tissue processing prior to staining which suggested the use of frozen tissue sections for visualising these receptors (Alroy et al., 1984).

Various attempts at the histochemical demonstration of lectins appeared in the literature. Hsu et al. (1981) developed an immunohistochemical technique utilising a biotin-labelled antibody and an avidin-biotin-peroxidase complex (ABC) for demonstrating antigenic epitopes in tissues and this was shown to be more specific than the unlabelled antibody method. Hsu and Raine (1982) then extended this study and produced a biotin-labelled lectin by which to demonstrate specific CHO moieties in formalin-fixed tissue, and also compared the ABC method with two other techniques for demonstrating the same compounds, the peroxidase-conjugate and peroxidase-antiperoxidase (PAP) methods. Their results showed the ABC

method to be far superior in terms of its sensitivity and specificity (Hsu & Raine, 1982).

Stoward et al. (1980) carried out a study using a peanut lectin-horseradish peroxidase (PL-HRP) conjugate as a histochemical staining on composite paraffin blocks of various adult mouse organs. Sections were fixed in Carnoy's fluid, and postfixed in absolute ethanol or in a calcium acetate-paraformaldehyde solution. A number of histological sites showed striking and selective affinity for the PL-HRP conjugate with staining of stored secretory bodies, Golgi zones, or the apical surface of epithelial cells. Reactivity with the PL-HRP conjugate coincided with PAS staining, although some PAS-positive sites failed to bind PL-HRP conjugate irrespective of whether they were pre-digested with sialidase or otherwise. All sites containing glycoprotein with neuraminic acid as a component, failed to stain with the PL-HRP method. Pre-treatment with sialidase, also had the effect of imparting affinity for PL-HRP conjugate to several non binding sites (Stoward et al., 1980).

Watanabe et al. (1981) investigated the distinctive distribution of binding sites for *Dolichos biflorus* agglutinin (DBA) and PNA in adult mouse tissues. DBA was labelled with fluorescein isothiocyanate (FITC-DBA) or with horseradish peroxidase (HRP-DBA), PNA labelled with FITC (FITC-PNA). Samples were fixed with 95% ethanol and 4 μm paraffin sections were cut. For frozen sections, unfixed fresh tissue was frozen in dry ice-acetone and stored at -80°C until used. Both paraffin and frozen sections were incubated with one or more drops of FITC-lectin solution. Binding sites for DBA and PNA were discretely distributed among various tissues of the mouse. But, the regions stained with these labelled lectins did not always exhibit positive PAS reactions.

From the appearances of staining patterns, it can be inferred that materials of different categories are included in DBA binding sites. Such as membrane-bound materials (as observed in collecting tubules and Bowman's capsule of the kidney, pancreatic ducts, bile ducts, sperm, oocytes, and capillary vessels of the brain) mucin, (although it should be noted that mucin in some organs does not express DBA binding sites) and intracellular materials observed in some cells, which might be biosythetic intermediates. In all cases the staining patterns obtained for membrane-bound materials were discrete with positive cells being intensely stained and cells around the positive cells completely unstained. PNA binding sites were more widely distributed as compared to DBA binding sites. In the kidney and genital

organs both PNA binding sites and DBA binding sites were expressed in significant amounts and these most probably represent mixtures of glycoconjugates with entirely different structures (Watanbe et al., 1981).

5.E. Voluntary muscle staining.

Bonilla et al. (1978) reported on a study on the muscle cell surface in 6 patients with Duchenne dystrophy using the Con A peroxidase labelling technique and electron-microscopy: the cell surface was found to be coated by a regular and continuous layer of electron-dense material, which covered the plasma membrane and basal lamina. The caveolae were also heavily stained.

Focal alterations in Con A binding were identified at the cell surface of abnormal muscle in contrast with the regular and continuous pattern of Con A binding observed in normal individuals. These indicate the presence of focal abnormalities at the muscle cell surface of Con A receptors in Duchenne dystrophy (Bonilla et al., 1978).

Bonilla et al. (1980) applied lectin cytochemistry to investigate the muscle cell surface abnormality in a further 8 Duchenne muscular dystrophy (DMD) patients. The specimens were fixed immediately in glutaraldehyde and Con A labelling of CHOs was performed. A sequential study of the focal abnormalities of Con A binding at the cell surface of these fibres suggested that the earlier damage was characterized by loss of regularity of the reaction pattern at the cell coat in association with focal increase in extracellular collagen. The more advanced focal lesion comprised interruptions of the regular pattern of the reaction and a disrupted plasma membrane. Beneath these focal defects dilatation of the sarcotubular systems and structural changes in the mitochondria were also observed. The late focal lesion consisted of interruptions in the regular pattern of the reaction at the cell surface with focal penetration of the Con A-peroxidase with the intracellular structures beneath these focal defects showing profound ultrastructural changes (Bonilla et al., 1980).

Pena et al. (1981) applied biotinyl derivatives of seven plant lectins to muscle: *Ricinus communis* agglutinin 1 (RCA 1), Wheat germ agglutinin (WGA), *Concanavalin* A (Con A), Peanut agglutinin (PNA), *Dolichos biflorus* agglutinin (DBA), *Ulex europeus* agglutinin 1 (UEA 1), and Soybean agglutinin (SBA); avidin-horse-radish peroxidase conjugates were obtained commercially. For the lectin

binding, 4 μm thick cryostat sections of biopsies of the quadriceps muscle of four different patients were used. These biopsies were considered to be normal by light microscopy after a routine battery of histochemical tests (Karpati, 1979). It was shown that the lectins bound to the muscle cryostat sections and provided a variety of staining patterns that were specific for each lectin. The authors noted that "*In several cases, neuraminidase pre-treatment modified the observed staining pattern, often dramatically, while with other lectins no modification was observed*".

The results were as follows:

Con A produced heavy staining of the sarcolemma, connective tissue, and blood vessels. The sarcoplasm showed extensive fine punctate staining reduced after α-amylase pre-treatment as expected from the known avidity of Con A for glycogen. Con A staining was not altered by neuraminidase pre-treatment but was completely blocked by incubation with α-methylmannoside.

PNA stained sarcolemma, blood vessels, and connective tissue with no intra-fibre staining; the capillary staining seemed to be mostly at the external surface. After neuraminidase treatment, the pattern of staining became stronger and more generalized, resembling that of RCA 1. Incubation with PNA in the presence of D-galactose ablished the staining completely.

RCA 1 provided strong staining of the sarcolemma, connective tissue, and blood vessels. Several annular profiles inside the muscle fibres were outlined by the staining and these were referred to as 'intra-fibre profiles'. Pre-treatment with neuraminidase only intensified very slightly the RCA 1 staining pattern. Incubation with β-lactose inhibited partially the staining while, incubation with α-methylmannoside did not alter it.

UEA 1 stained blood vessels preferentially; there was also good staining of intra-fibre profiles, some staining of the sarcolemma, and faint staining of occasional nuclei. Neuraminidase treatment did not modify the staining, but incubation with L-fucose blocked it completely.

SBA gave preferential staining of blood vessels, similarly to UEA 1. Neuraminidase pre-treatment changed the staining pattern of SBA, to make it become similar to that of RCA 1, presumably due to the exposure of galactose and N-acetyl-galactosamine

(galNAc) residues after removal of sialic acid. Staining with SBA could be completely blocked by incubation with D-galactose or N-acetyl-galactosamine.

DBA was unique in that it appeared to stain preferentially the myonuclei and some of the muscle satellite cells. After neuraminidase treatment, there was a dramatic intensification of staining of sarcolemma, blood vessels, and connective tissue. Incubation with N-acetyl-galactosamine blocked completely DBA staining.

WGA produced strong staining of sarcolemma, intra-fibre profiles, blood vessels, and connective tissue. Neuraminidase pre-treatment resulted in a significantly decreased the sarcolemmal and intra-fibre profiles staining with a relative accentuation of capillary visualisation. WGA was only partially blocked by incubation with N-acetyl-neuraminic acid or N acetyl-glucosamine.

The seven different lectins employed in the study provided highly individual patterns of staining with PNA giving the clearest sarcolemmal staining, UEA 1 and SBA staining blood vessels, and DBA staining structures resembling myonuclei or satellite cells. By the ability of these lectins to stain selectively specific structures, especially membranes, and by their capacity to probe specifically the glycoconjugate composition and distribution in cells of the human muscle, it was suggested that the application of lectin histochemistry proved to be a useful new tool in the investigation of muscle disorders (Pena et al., 1981).

Paljärvi et al. (1982) carried out a study on muscle from eight patients with X-linked muscular dystrophy (Duchenne and Becker types), eight patients with other myopathies or denervation and six normal controls. Cryostat sections of each were incubated with fluorescein isothiocyanate conjugated lectins. The following lectins were used: Con A, SBA, DBA, PNA, and WGA. Con A gave a bright fluorescence sharply, located on the cell surface while SBA and DBA staining was interpreted as universally negative. Differences emerged with the use of PNA and WGA, which gave a weaker staining than Con A but a diffuse distribution on the cell surface and endomysium. With PNA, muscle from X-linked dystrophy gave more positive staining than muscle from other diseases or normal controls. All lectins produced a non-specific net-like or tubular pattern in the cell's interior, probably corresponding to the endoplasmic reticulum or T-tubules with no essential differences between the diagnostic categories were detected in this respect.

It was shown that in X-linked muscular dystrophy, glycoproteins and/or glycolipids with glucose and galactose moieties are increased in the muscle but comparison with a study of normal muscle reveals that these differences are likely to be quantitative rather than qualitative (Paljärvi et al., 1982).

Dunn et al. (1982) investigated the binding characteristics of lectins with varying sugar specificities in specimens of muscle tissue obtained by needle biopsy of the Vastus lateralis from patients with a variety of neuromuscular disorders. Young controls were patients from the muscle clinic whose biopsies showed no morphological abnormalities, while adult controls were healthy volunteers. Horseradish peroxidase (HRPA) and fluorescein isothiocyanate (FITC)-conjugated lectins were used for light microscopy and ferritin-conjugated Con A for electron microscopy.

On light microscopy no staining of the interior of individual fibres was observed when frozen sections of normal muscle were stained with FITC-Con A. Both perimysial and endomysial areas between the fibres were heavily stained as well the blood vessels and capillaries associated with individual muscle fibres. Incubation with the competing sugar, α–methyl-mannoside abolished FITC-Con A staining in both normal and Duchenne muscular dystrophy (DMD) biopsies. HRPA-conjugated lectins specific for β-D-galactose residues (PNA, Osage orange agglutinin (OOA), Abrus agglutinin (ABA), RCA 120) gave similar staining patterns to those observed with FITC-Con A. In normal muscle unstained muscle fibres were outlined by the positively stained perimysial and endomysial areas and the blood vessels including the capillaries were also stained. In biopsies from patients with DMD the excess connective tissues were strongly stained by the lectins with the majority of fibres remaining unstained and with occasional fibres positively stained. A similar staining patterns were observed in biopsies from patients with limb-girdle muscular dystrophy (LGD) and Becker muscular dystrophy (BMD). Areas of excess connective tissue in DMD carrier biopsies were also strongly stained by the galactose-specific lectins. In some biopsies from a variety of disorders there was a discontinuity of staining at the periphery of occasional fibres. Staining by this group of lectins was abolished by the presence of the inhibitory sugar, D-galactose.

Wheat germ agglutinin (WGA) which is known to react with both N-acetyl-glucosamine and N-acetyl-neuraminic acid residues did not stain the interior of individual normal muscle fibres, but stained intensely the perimysial and endomysial

areas of connective tissue. Capillaries associated with the muscle fibres appeared to be particularly strongly stained by this lectin, but in biopsies from dystrophic patients the capillaries were not as distinct with this difference being apparent with all lectins which stained blood vessels. Staining was inhibited by N-acetyl-Neuraminic acid and N-acetyl-glucoasamine.

Lectins specific for α-L-fucose residues (Lotus agglutinin, (LOA), and (UEA) and α-acetyl-galactosamine residues (DBA, Lima agglutinin, (LIA), and SBA), gave a markedly different staining pattern with no staining of the fibres and little stain associated with the perimysial and endomysial areas of connective tissue, blood vessels and capillaries. A few individual isolated cells within the perimysial areas showed only a positive reaction; these were more common in biopsies of diseased muscle. This staining was not inhibited by incubation with the competing sugar L-fucose or N-acetyl-galactosamine. Treatment of these sections with DAB alone also demonstrated the presence of these cells indicating that the staining was not due to a specific reaction with the lectins and that these cells were exhibiting endogenous peroxidase activity suggesting that these cells were macrophages.

Although the results are in general agreement with Pena et al. (1981), Dunn et al. (1982) found that Con A, RCA 120 and WGA stained annular profiles (termed infra-fibre profiles) within muscle fibres but felt that this type of intra-fibre staining is a non-specific phenomenon. Con A, WGA and the lectins specific for β-D-galactose (PNA, OOA, ABA, RCA 120) clearly demonstrate excess of connective tissue.

Ferritin labelled-Con A binding was found on ultrastructural studies external to the plasma membrane of the muscle fibres with no ferritin granules on the plasma membrane itself. Ferritin Con A did bind to the basement membrane and connective tissues immediately adjacent to the sarcolemma (Dunn et al., 1982).

Gulati and Zalewski (1985) applied ten different fluorescein-conjugated lectins of various sugar specificities to study cell surface glycoconjugates of normal and regenerating rat skeletal muscle. In normal muscle, *Canavalia ensiformis* agglutinin, *Triticum vulgaris* agglutinin (wheat germ agglutinin, WGA), *Ricinus communis* agglutinin-I, and *Maclura pomifera* agglutinin bound strongly to the endomysial region of the myofibres. No binding was observed in the cytoplasm of the myofibres. Other lectins (Dolichos biflourus agglutinin, Griffonia simplifolia agglutinin I and II,

Ulex europaeus agglutinin, *Arachis hypogaea* agglutinin, *Glycine max* agglutinin) bound very poorly or not at all in the normal muscle.

In each animal the Extensor Digitorum Longus (EDL) muscle was autotranplanted according to the procedure described by Gulati et al. (1982; 1983). Frozen cross sections of 6 μm thickness were cut from different regions of each muscle. A few of these slides were stained with periodic acid-Schiff (PAS)-haematoxylin for histological analysis, whereas the remaining were stored at -20°C until used for staining with specific lectins. The results in the rat concurred with the results obtained in normal human muscle. Of the additional lectins positive endomysial binding was seen for MPA (sugar specificity: galactose) and negative binding for GS I and GS II. No intra-fibre binding was seen in this study and it was suggested that this dissimilarity may be due to the differences in the species studied and the staining procedure (Gulati & Zalewski, 1985).

Capaldi et al. (1985) carried out a comprehensive histochemical study on human skeletal muscle using 15 lectin-horseradish peroxidase conjugates to investigate the lectin binding. Samples were obtained by needle biopsy of the *Vastus lateralis* from healthy volunteers or from patients attending the muscle clinic whose biopsies showed no morphological abnormalities. Muscle samples were rapidly frozen, unfixed transverse cryostat sections were mounted and incubated with biotinylated lectin or peroxidase-conjugated lectin. Their results were as follows:

Con A, Maclura pomifera (MPA), Phaseolus vulgaris (PHA-L), PNA, Pisum sativum (PSA), RCA I, SBA, Visea villosa (VVA) and WGA consistently produced similar patterns of staining although relative intensities of staining varied. All these lectins delineated muscle fibre peripheries clearly (albeit very weakly in the case of SBA), and endo- and peri-mysial collagen was always positively stained as were blood vessels and capillaries. Staining of the interior of muscle fibres was not observed in normal muscle. The staining of myonuclei by DBA observed by Pena et al. (1981) could not be confirmed by this study even in sections which were not counterstained with haematoxylin (Capaldi et al., 1985).

Bonilla & Moggio (1987) in an experimental study on the rat, used the lectins wheat germ agglutinin (WGA) and Limulus polyphemus (LP) as cytochemical probes to study the ultrastructural localisation of sialic acid at the cell surface. In addition cytochemical studies employing strontium as an electron-dense marker were also

carried out to investigate cation binding sites at the muscle cell surface. All these autoradiography experiments were carried out using the Extensor Digitorium Longus (EDL) muscle.

Studies with WGA (coupled to peroxidase) showed that the muscle plasma membrane, basal lamina, caveolae and collagen fibrils had enhanced electron density. At higher magnification, lectin binding sites were identified at the basal lamina, caveolae and at the glycocalyx or cell coat of the muscle fibre. Using WGA and LP, sialic acid was shown to be preferentially localised at the glycocalyx or cell caveolae and at the basal lamina suggesting that the glycocalyx, caveolae and basal lamina typically provide the negative charge of the muscle fibre and may be involved in the binding of calcium ions (Bonilla & Moggio, 1987).

Helliwell (1988) carried out a study on 21 rats whom they received subcutaneous injections of bupivicaine hydrochloride over the right anterior tibial muscle. Cryostat sections 6 μm thick were stained with H&E to assess the overall morphological changes in the muscle and a panel of 11 biotinylated lectins was used. The non-injected left Tibialis anterior muscles of all animals and the saline-injected Tibialis anterior muscles of the control animals had a normal morphology.

Normal muscle showed strong uniform staining of the periphery (plasmalemma and basal lamina) of muscle fibres, of perineurial, endoneurial, endomysial, and perimysial connective tissue, and of capillary endothelium by WGA, Con A, RCA, PNA, BPA, MPA, and GSI, with BPA and GSI showed stronger staining of the periphery of fibres which had oxidative enzyme activity. SBA gave a weak peripheral staining combined with stronger staining of connective tissue and endothelium, DBA only stained the endothelium. UEA was completely negative. Weak diffuse cytoplasmic staining was only seen with Con A, probably due to its affinity for glucose. The superficial zone of damaged muscle showed strong, slightly granular cytoplasmic staining by WGA, Con A, RCA, BPA, SBA, GS I, and UEA whether or not the fibres showed other features of necrosis. The pattern of peripheral staining was preserved in necrotic fibres even when the cytoplasm was replaced by macrophages; only a few fibres showed localised areas of disruption. As the macrophages removed the necrotic cytoplasm, the residual basal lamina tubes developed a wrinkled contour. Weak lectin staining of the interstitial tissues was observed for all lectins except UEA and DBA. This study demonstrated that the binding of lectins to normal rat muscle is similar to their binding to human striated

muscle (Dunn et al., 1982; Capaldi et al., 1985) with the exception of UEA which binds to human endothelium but not to rat tissue.

The cytoplasm of necrotic fibres bound lectins with a range of affinities likely to be due to the breakdown of glycoconjugates which revealed previously hidden saccharides e.g. the binding of UEA to fucose. These changes occurred in fibres which did not show morphological features of necrosis and these may serve as a useful indicator of early muscle fibre damage. While lectin-binding components of the basal lamina persist during necrosis, most of the binding is probably due to newly synthesized glycoproteins and the binding of lectins to the cytoplasm of regenerating fibres probably reflects the synthesis of new proteins containing galactose (RCA, MPA), fucose (UEA), N-acetyl glucosamine or sialic acid (WGA), and glucose or mannose (Con A) (Helliwell, 1988).

Helliwell et al. (1989) published another study on muscle biopsy specimens which were obtained using local anaesthesia from patients attending the muscle clinic; diagnostic groups were established by assessing the clinical, electrophysiological, and histological findings in each cases. Specimens were frozen in liquid nitrogen and 6 μm thick frozen sections were used for all staining methods. Each specimen was examined by standard histochemical methods for diagnostic purposes and by a panel of eleven biotinylated lectins. Appropriate lectin concentrations were determined so as to provide clear peripheral staining and a little or no cytoplasmic staining of normal fibres.

The intensity of cytoplasmic lectin staining was assessed as negative, weak (just evident), moderate (clearly evident at x100), or strong (clearly evident at x 40). In normal muscle, UEA bound only to endothelium. DBA bound consistently to arteriolar endothelium and weakly to connective tissue; capillary staining was observed in some cases but not in others. GS1 showed a similar variation in capillary endothelial binding to DBA although some cases were GS I-positive and DBA-negative. All other lectins showed uniform binding to the periphery of fibres, to endo-mysial and peri-mysial connective tissues and to endothelium, although PNA and SBA bound less strongly than other lectins. SBA bound to endothelium more strongly than to interstitial connective tissue. Weak cytoplasmic staining was seen in all fibres with Con A and in scattered fibres with both GS I and GS II and this was not clearly related to fibre type and there was no staining differences were seen in different muscles.

Necrotic fibres were identified in 26 cases with their cytoplasm usually binding Con A, WGA and the D-galactose binding lectins RCA, PNA, BPA, MPA, and SBA. No major differences in lectin binding to necrotic fibres were seen in the different disease groups, except that DBA identified necrotic fibres more often in Duchenne dystrophy than in polymyositis. Most lectins bound with moderate intensity, although SBA was rather weaker,, and BPA slightly stronger, than the other lectins. Lectin binding to the periphery of the fibres was patchy in necrotic fibres and, while focal defects of peripheral lectin binding were observed in other non-necrotic fibres, these changes were not related to necrosis, regeneration, or cytoplasmic lectin binding. Con A was the lectin which bound most consistently to necrotic fibres and identified these fibres as compared with other lectins e.g. RCA. Macrophages were readily identified within necrotic fibres by most lectins but were not easily seen in the interstitium due to the strong staining of connective tissue. Regeneration fibres showed a granular or reticular staining of the cytoplasm with all lectins except UEA; DBA was only positive in regenerating fibres in one case. Con A was the lectin which bound most consistently to regenerating fibres but staining was not strong for any lectin and was generally weaker than seen in necrotic fibres. The binding of lectins to necrotic human muscle fibres is similar to that seen in necrotic rat muscle; there are qualitative differences in that UEA bound to necrotic rat muscle but not to human muscle fibres. Lectin binding to regenerating fibres is weak and was only seen in those fibres that could be identified easily on H&E stained sections. This increased staining is probably related to the synthesis of new CHO-containing glycolipids and glycoproteins as well as to the affinity of Con A for rough endoplasmic reticulum.

These studies have suggested that lectin binding to the cytoplasm of damaged skeletal muscle fibres may help to identify early degenerative changes in them and lectins may supplement existing histochemical methods. Lectin binding also provided evidence of more extensive damage than was suspected by other methods, but did not reveal damage in biopsies which were otherwise thought to be normal (Helliwell et al., 1989).

Kirkeby et al. (1991) carried out a study on rats which were killed by cervical dislocation and specimens were taken from the Gracilis muscle. The muscle samples were rapidly frozen in isopentane cooled to -150°C with liquid nitrogen and cut in 6μm transverse sections. The following biotinylated lectins were used in their study:

Con A, WGA, SBA, GS I and GS II, LCA, PNA, PSA, and the PAS reaction was also performed. Muscle sections were treated with 0.3% α-amylase. Furthermore demonstration of Con-A, GS II and WGA binding was carried out in serial sections using the indoxylphosphate nitro-BT method.

Binding of lectins could be observed in sarcoplasma, fibre periphery, inter-fibre connective tissue, capillaries and blood vessels but the nuclei in the muscle fibres were unstained. The staining of the interior of muscle fibres was present both as discrete granules and as a diffuse cytoplasmic reaction.

A number of experimental conditions altered lectin binding results. The localisation of the reaction product was the same, but its intensity was always stronger after 24 hours incubation, than after only 1 hour. The reaction products were well localised in the sections, although some artefacts due to over-incubation were seen sometimes in sections as needle-like deposits after prolonged incubation. The lectin binding patterns observed varied much with the different substrate media used for visualising alkaline phosphatase activity with the strongest and most distinct reaction in sections incubated in the indoxyl phosphate-tetrazolium medium. The localisation and intensity of the reaction product were the same whether NBT or tetra-NBT were used as tetrazolium salts.

WGA gave the strongest sarcoplasmic staining. With Con A and GS II all of the fibres were equally well stained. Incubation with PNA, GS II and SBA did not show any sarcoplasmic reactions. The fibre periphery was outlined with GS I and PNA while the sarcolemmal reaction with SBA was inconspicuous. The connective tissue, blood vessels and capillaries were prominent with all the lectins used in this study, except for GS II (Kirkeby et al., 1991).

5.F. Heart muscle-lectin staining.

Grahl et al. (1984) carried out a study to characterise the cell surface CHOs of Ca^{++} stable cardiac myocytes of adult rats by lectin binding. Incubations of the myocytes with fluorescein isothiocyanate-conjugated lectins showed a specific binding of WGA, Con A, RCA, Maclura pomifera agglutinin (MPA) and Griffonia simplicifolia agglutiin I (GSA I) to the plasma membrane. WGA indicates the presence of N-acetylneuraminic acid and N-acetyl-D-glucosamine the former of which homogeneously covers the cell surface. This assumption was confirmed by

neuraminidase pre-treatment of the myocytes, these split off the sialic acid residues with a remarkable reduction of the fluorescence.

The occurrence of α- and β-D-galactose and α-D-galactose was detected by binding of RCA, Con A, MPA and GSA I, the fluorescence intensity by MPA and GSA I was weaker than by the other ones. The lectins DBA, PNA, and UEA showed no binding and SBA showed a very weak fluorescence (Grahl et al., 1984).

Stegemann et al. (1990) carried out a study on isolated guinea-pig and also rat ventricular myocytes to investigate the occurrence and distribution of CHO components of this surface coat. Cells were stored as a thin layer for several hours at 37°C under 100% O_2 atmosphere. FITC (Fluorescein Isothiocyanate) or TRITC (Tetramethylrhodamine Isothiocyanate) labelled lectins were used.

The results of lectin binding to guinea pig cardiac myocytes were as follows:

Con A: cardiac myocytes incubated with Con A revealed strong fluorescence along the whole cell's surface and spots and lines within the sarcoplasm forming a regular striated pattern which coincided with the sarcomeric Z-line pattern of myofibrils. Fluorescence was also present at the cell poles and the regions of intercellular connections (intercalated discs). Pre-incubation of Con A with D-glucose inhibited lectin binding by no more than about 17%. Higher degrees of inhibition were achieved by D-mannose or α-methyl-D-glucoside and α-methyl-D-mannoside.

WGA and succinylated WGA (sWGA): WGA and sWGA (succinylated wheat germ agglutinin) gave similar patterns to those of Con A, but of a weaker intensity. The succinylated form of this lectin lacks the specificity for sialic acids and the stronger fluorescence of WGA relative to sWGA might be due to it picking out a considerable amount of sialic acids on the cell surface of cardiac myocytes.

RCA 1: stains moderately the whole surface. Intracytoplasmic staining is slightly pronounced at the level of Z-lines, with previous incubation of the lectin with D-galactose yielding 83% inhibition.

DBA and SBA: the binding patterns of both DBA and SBA were distinctly different from those of Con A, WGA, sWGA, LFA and RCA-I in that they were preferentially

bound at the cell poles while the lateral cell surface was only slightly stained. These positive reactions were reduced by pre-incubation with N-acetylgalactosamine.

PNA: this lectin showed no positive reaction with cardiac myocytes. PNA, however, was shown to bind to cells pre-treated with neuraminidase to produce a fluorescence pattern in close correlation to that of LFA. Simultaneous pre-incubation with D-galactose and N-acetyl-D-galactosamine strongly inhibited PNA-binding to cells pre-treated with neuraminidase.

Lectin binding to rat cardiac myocytes was as follows:

Rat cardiac myocytes were stained with the lectins Con A, WGA, sWGA, RCA-I, LFA, and UEA-I with the binding patterns being almost identical to those observed on guinea-pig cardiac myocytes. The lectins Con A, WGA, sWGA, LFA, RCA-I, and UEA-I bound to the whole cell surface and also produced a striated fluorescence pattern with a periodicity similar to the cross striation of rat cardiac myocytes. Fluorescence intensities of cardiac cells covered with Con A, WGA, sWGA, LFA, RCA-I, and UEA-I were very different: WGA showed strong fluorescence intensity, sWGA was much less effective. DBA, SBA, and PNA were not bound by rat cardiac myocytes.

From these results the overall distribution of the different sugars that is suggested by the binding patterns of Con A, WGA, sWGA, and LFA, is that the CHOs α-mannose, α-glucose, N-acetylglucosamine and N-acetylneuraminic acid are evenly distributed throughout the cell surface of both guinea-pig and rat cardiac myocytes. The same is true for α-L-fucose on rat cardiac myocytes. β-Galactosyl residues are also distributed all over the cell surface but seem to be slightly concentrated at the sarcomeric Z-lines. The Sarcolemma above the Z-lines contains attaching sites for intercellular connecting filamentous material and the RCA 1 binding sites may colocalise with these regions. In contrast to the lectins mentioned above, DBA and SBA binding sites were concentrated in regions corresponding to intercellular junctions. Transverse striations were observed with fluorescent Con A, WGA, sWGA, LFA, and RCA 1 which are similar to those produced by the lipophilic membrane probe (TMA-DPH), which indicates that the lectins may bind to the tubular sarcolemmal surface. These findings are consistent with those of Gros et al. (1982) where WGA and Con A binding sites were demonstrated on the tubular sarcolemma of mouse myocardium (Stegemann et al., 1990).

Dolber et al. (1992) carried out a study on 6 dog hearts, and hearts from 17 rats, to assess the distribution of gap junctions in relation to cardiac myocytes. From dogs, large preparations from the anterior wall of the left and right ventricles were taken roughly midway between the apex and base of the heart. All preparations were immersion fixed in paraformaldehyde, and subsequently processed routinely for paraffin embedding. In the double-label procedure the sarcolemma was first labelled with WGA. The gap junctions were next labelled using the indirect immunofluorescence method.

WGA labelled the sarcolemma, capillaries, neurons, cells of the extracellular matrix and some extracellular matrix constituents. Myocyte cross sections showed labelling of the ordinary sarcolemma also of the sarcolemma lining the intercalated discs and within the transverse tubules. Labelling of the ordinary sarcolemma was strongest between adjacent myocytes and sometimes weak in proximity to vascular spaces with interruptions in sarcolemma labelling being sometimes present between adjacent myocyte profiles. In transversely striated myocytes the intercalated discs also stained. In longitudinal sections, sarcolemmal labelling revealed individual myocytes with step-like ends with transverse tubules also being easily seen in longitudinal sections; some fibrillar extracellular matrix constituents were also labelled with WGA and it was therefore, not possible to determine exactly which individual profiles were transverse tubules and which were not.

The WGA label completely outlined the sarcolemma lobes within the intercalated discs and interruptions could be seen in the WGA-labelled saracolemma corresponding to the sites of gap junctions (Dolber et al., 1992).

5.G. Blood vessels-lectin staining.

The vascular endothelium is a highly specialized secondary epithelium forming a continuous lining which separates circulating blood from surrounding tissues. It serves as a semi-permeable barrier to water and solutes. Its surface coat (the glycocalyx) is negatively charged and contains specific domains with characteristic carbohydrate residues (Simionescu et al., 1982). It is a regulatory organ that mediates haemostasis, contractility, cellular proliferation, and inflammatory mechanisms. Injury to the endothelium from hypertension, smoking, hyperlipdemia, and diabetes mellitus disrupts normal regulatory properties and results in abnormal

endothelial cell function. The endothelial cell regulates smooth muscle cell contractility by the production of relaxing and constricting factors in response to physiological stimuli and signals the release of mitogens from platelets, macrophages, and endothelial cells, which stimulate smooth muscle cell proliferation. Endothelial cells also undergo morphological and functional alterations in response to cytokine signals (Nabel, 1991). Its vasodilator mechanisms are counter-balanced by vasoconstrictor substances for example, endothelin is a potent vasconstrictor produced by the endothelial cells in response to hypoxia and other stimuli. Endothelial cells become activated by the binding of adhesion molecules. For example, interleukin-1 can induce the synthesis of endothelial leukocyte adhesion molecule-1 (ELAM-1), which stimulates neutrophil adhesion to endothelial cells (Bevilacqua et al., 1989; Nabel, 1991).

One important means of distinguishing endothelial cells is to identify cell surface-associated lectins binding sites, because these reflect glycoconjugates likely to be important in cell-to-cell and cell-to-matrix interactions (Holthöfer et al., 1982; Auerbach et al., 1985; Belloni & Nicolson, 1988; Fujimoto & Singer, 1988; Laitinen et al., 1990).

Stein et al. (1976) carried a study on rats to investigate the staining of the surface coat of heart capillaries and aorta by the binding of Con A and phytohaemagglutinin (PHA) and also applied perfusion techniques to introduce both the lectin and the peroxidase into the lumen of heart capillaries. The presence of lectin was quite uniform and could be seen at the light microscopic level over all the capillaries encountered. The reaction was confined to the outer surface of the luminal plasma membrane and to the lumen of plasmalemmal vesicles on the luminal surface of the endothelium with no reaction being seen in other regions of the cell (Stein et al., 1976) .

Holthöfer et al. (1982) carried out a study on different human tissue samples: kidney, lung, liver, pancreas, and skin. These were obtained either from patients during surgical procedures in the University Central Hospital (Helsinki, Finland), or, in the case of the brain tissue from autopsy, a few hours after death. The tissues were snap frozen in isopentane cooled with liquid nitrogen, and stored at -70º C. Commercially available fluorescein isothiocyanate (FITC)- or tetramethylrhodamine isothiocyanate (TRITC)-labelled UEA 1 and Lotus tetragonolobus (LTA) lectins were used. Fluorochrome-coupled UEA 1 lectin stained endothelium homogeneously in all

human tissues which were studied but, LTA (with the same nominal specificity) failed to react with any endothelial structures.

The binding sites of UEA 1 lectin appear to be structural α-L-fucosyl-containing glycocompounds; these seem to be specific for endothelial cells with the fucose residues are readily accessible for UEA-I lectin (Holthöfer et al., 1982).

Ponder and Wilkinson (1983) carried out a study on 10 mice, on paraffin embedded tissues which were fixed in methacarn overnight then transferred to 70% ethanol. In many of the organs examined, arteries, capillaries, and veins were uniformly stained by DBA-peroxidase; no negative blood vessels or patchy staining were seen but the endocardium was negative. Myocardial vessels were strongly positive.

These observations indicate with the presence of biochemical differences between the endothelial cell membranes of the blood vessels in different tissues *in vivo*: this organ-related patterns of the heterogeneity raises the possibility that the endothelial cell surface carries a 'tissue address' (Ponder & Wilkinson, 1983).

Immunofluorescence methods have indicated that UEA 1 has specific affinity for α-L-fucose residues of sugar moieties (Debray et al., 1981), and UEA I appears to react preferentially with vascular endothelium independently of blood group antigen type of the tissue donor (Holthöfer et al., 1982). Ordóñez & Batsakis (1984) showed that UEA 1 is a more sensitive marker for endothelial cells than factor V111-related antigen which in both normal and neoplastic tissue can be used in formaldehyde-fixed, paraffin-embedded tissue as the lectin-binding sites are not altered during tissue processing (Ordóñez & Batsakis, 1984).

Kadota et al. (1986) performed a study on 14 human autopsied brains from patients who had died of ischaemic cerebrovascular diseases. Electron microscopy was utilised to confirm the reactive site of UEA 1 on the vascular endothelium. UEA 1 was shown to bind to the surface of the capillary endothelial cell membranes of the normal cerebrum. Endothelial cytoplasm also showed a positive reaction. Many morphological findings have been reported in ischaemic changes of the endothelium, such as the disappearance of the endothelium, crater formation, flattening of convolutions and fibrin or platelets covering the endothelial surfaces (Fonkalsrud et al., 1976). In addition, increased numbers of endothelial vacuoles have been reported under ischaemic conditions (Nag et al., 1981). Partial absence of UEA 1 reactivity

was observed after 6-hour ischaemia with a reperfusion period of 1 hour (Nishida et al., 1986). These ultrastructural alterations of the reactivity could not be recognised by light microscopy. The UEA 1 reactivity was variable and heterogeneous; individual capillaries even in the same visual field of the damaged cerebral cortex showed different staining. This may may be related to the so-called 'microdomains' generated by the preferential distribution of chemically different anionic sites through the vessel wall (Simionescu et al., 1981). The authors therefore speculated that a reduction of L-α-fucose may result in changes of 'microdomains', resulting in functional changes in vascular permeability. They also remarked that the lectin UEA 1 could be used to evaluate endothelial functional states and permeability in the central nervous system (Kadota et al., 1986).

Alroy et al. (1987) applied 10 biotinylated lectins as probes on formalin-fixed, paraffin-embedded tissue specimens of placenta, skeletal, and cardiac muscle of animal and human tissue to demonstrate the pattern of CHO residues in the vascular endothelium. The lectin staining pattern of vascular endothelium from different sources (capillaries, arterioles, arteries, veins and venules) in tissues obtained from one individual was identical. In all specimens studied, only two lectins stained the vascular endothelium identically, regardless of species.

RCA-I, which binds specifically to β-galactosyl residues, stained vascular endothelium in all the tissues studied other than human but not that of any other species studied. Con A, DBA, LCA, PNA, and SBA did not stain vascular endothelium in any of the species. Only following an incubation of the tissue with neuraminidase, did PNA stain vascular endothelium in all specimens studied. DBA (which binds only to α-GalNAc), did not stain vascular endothelium in any of the specimens studied.

These results demonstrate that, vascular endothelium in paraffin sections stains specifically with RCA-I, indicating the presence of non-reducing β-galactosyl residues. Staining with WGA, as opposed to sWGA indicates the presence of sialyl residues in the glycocalyx of mammalian endothelium: as WGA is positively charged it binds to sialyl residues which are negatively charged; sWGA is neutral does not bind to sialyl residues. The UEA 1 staining of human vascular endothelium only, regardless of the blood group, indicates the presence of non-reducing α-L-fucosyl residues which are not blood group specific but unique to humans (Alroy et al., 1987).

Darr et al. (1990) did an experimental study to investigate angiogenesis on the hairless Yucatan strain of mini-pig for studying cutaneous wound healing because its skin is histologically and functionally very similar to that of man and unfortunately, excellent markers for vascular endothelium in human tissues exhibit little or no staining of endothelia in tissue of other animal species, including the pig. To find a specific marker to identify vascular endothelium in the mini-pig, a battery of 11 different lectin-horseradish peroxidase conjugates have been applied. Based on specificity and staining intensity, Dolichos biflorus agglutinin (DBA) was chosen and when compared with routine histological sections stained with H&E, blood vessels were much easier to identify in sections stained with it.

Angiogenesis plays a key role in numerous pathological events. Certain lectins have been found to bind specifically to glycoconjugates on large and small blood vessels in a number of species (Holthofer et al., 1982; Holthofer, 1983; Ponder & Wilkinson, 1983; Ordonez & Batsakis, 1984; Schulte et al., 1984; Stephenson et al., 1986; and Spicer et al., 1987). In human tissues histochemical staining with lectin from Ulex europaeus (UEA 1) specific for fucose, has been shown to be a more reliable procedure for recognizing endothelial cells than immunocytochemical staining of factor VIII related antigen (FVIII-RAg) (Holthofer et al., 1982; and Stephenson et al., 1986). Although UEA 1 is an excellent marker for vascular endothelium in human tissues, it stains only weakly or not at all the endothelium in tissues of other animal species, including the pig (Ponder & Wilkinson, 1983; and Holthofer, 1983) but DBA proved to be a more sensitive and reliable marker for endothelial cells in the of mini-pig with an uniform and reproducible binding pattern with endothelial cells of vessels of all calibers (Darr et al., 1990).

Plendl et al., (1993) in an *in vitro* experimental study on a Swiss-albino mouse strain which express an uniform DBA-cell surface glycoprotein on its endothelial cells showed a dramatic increase in this cell surface-associated lectin-binding site(s) in the course of long term tissue culture. This increase was exclusive to the endothelial cells of the myocardium and neither brain nor aorta-derived endothelial cells showed a comparable increase.This alteration in DBA binding was not paralleled by an increased labelling intensity for other lectins,.

This further indicates that endothelial cells represent a highly heterogeneous population reflected in organ-associated variation (Auerbach et al., 1985; Belloni &

Nicolson, 1988); variable lectin affinities have been reported for different organ sites for subpopulations of endothelial cells within the same general tissue site and allow for different developmental stages.

The DBA lectin recognises N-actyl-D-galactosamine residues, and has been used to identify adhesion-mediating molecules (Fujimoto & Singer, 1988). Age-related changes in vascular endothelial cells have been reported for different organs (Tokunaga et al., 1991), and there is increasing interest in determining how such changes correlate with progressive normal as well as disease-associated functional changes within the vascular system (Simionescue et al., 1985). Within the heart a stage-dependent alteration in the expression of glycoconjugates during embryogenesis and into adulthood has been previously described in frozen and paraffin sections (Fujimoto & Singer, 1988; Fazel et al., 1989a & 1989b) (Plendl et al., 1993).

CHAPTER 6.

MATERIALS AND METHODS.

All the technical work involved in the production of the sections in the immunohistochemical and lectin staining procedures was performed by myself. I gratefully acknowledge the assistance of the technical staff in the more routine tinctorial staining.

6.A. Scope of the study

The intention of this study was to stain serial sections taken from blocks from the human (autopsy) hearts which had been carefully selected. The methods used were tinctorial and commercially available antibodies against specific components of the cardiac myocytes, and with lectins. The findings obtained by all these methods would then be compared to identify which of these methods was able to show more consistently and easily the earlier changes of myocardial damage resulting from a deleted blood supply.

Hearts were collected from autopsies performed on behalf of the Procurator Fiscal by pathologists working with The Forensic Medicine Unit, Department of Pathology, University of Edinburgh. The cases were obtained from 3 mortuaries; the City Mortuary (Edinburgh), St. John's Hospital Mortuary (Livingston) and The Borders Hospital Mortuary (Melrose). The hearts were collected during the period 1990-1993.

6.B. Pilot study

A pilot study was carried out to determine the best fixative, the aim being to find a fixative which could serve all staining methods i.e. the tinctorial, the immunohistochemical and the lectin staining procedures. A summary of the pilot study is presented below.

Design of Pilot Study

Experiment 1

Aim: to determine the best fixatives for the fresh heart blocks.

Experiment: 8 hearts (4 MI, 4 non-ischaemic)
↓
Left ventricular block taken
↓
subdivided into 5 adjacent blocks and fixed in different fixatives (= 40 blocks)
↓
from each block a section stained with H & E, Masson's Trichrome, PTAH, anti-myoglobin antibody and seven lectins (Table 6.7) (40x11=440 sections)

Conclusion: Little to choose between NBF and PLPD

Experiment 2

Aim: to distinguish further between NBF and PLPD

Experiment: 4 of the 8 cases re-chosen. Oneblock previously fixed in NBF and another in PLPD
↓
Sections stained with H & E, Masson's Trichrome, PTAH, anti-actin, anti-myoglobin and seven lectins (8x12=96 slides)

Conclusion: NBF chosen as fixative.

Experiment 3

Aim: to choose the best case of MI and non-ischaemia

Experiment: on the basis of the overall staining pattern, preservation and fixation, one case of MI and another from a non-ischaemic heart were selected.

6.B.1. Fixation methods-General considerations.

"Fixation is always a compromise and the requirements of a fixative vary according to the different techniques employed in visualising the structure of the cells or tissues" (Boenisch, 1989). Fixation in immunohistochemistry has to provide an adequate preservation of morphology of the tissue, and the retention of the antigenic epitopes without altering the tissue structure to the extent that antigenicity is lost. Immunohistochemistry using monoclonal antibodies presents special problems since only one antigenic determinant is available for reactivity with the primary antibody. Therefore, considerable care is required to maintain antigenic reactivity during any fixation procedures. A number of fixatives have been reported to be superior to others and results cannot be generalized to all antibodies.

Various factors could affect the quality of results attained by immunohistochemical staining, these include: 1) the type of tissues, 2) methods and duration of fixation, 3) methods of processing and embedding, 4) the immunohistochemical procedures and reagents used, and 5) the day to day variation in staining techniques. Other additional factors are 1) length of exposure to reagents used, 2) dilution of primary or secondary antibodies and other reagents, and 3) any un-masking techniques.

A few studies have considered the use of lectins in routinely, fixed and embedded tissue (Yonezawa et al., 1982; Walker, 1983 & 1984a). Even relatively fewer lectin studies of the musculoskeletal system have been performed (Alroy et al., 1988), accordingly lectin histochemistry is limited by the lack of uniform baseline data on conventionally processed normal cells and tissues.

The results of lectin staining may be profoundly affected by fixation. Lectin staining of glycoconjugates in paraffin sections can identify only some glycoproteins and glycopeptides that are retained in the tissue sections following paraffin processing. The glycoconjugates visualised by lectin staining in frozen sections will be similar to those still retained in paraffin sections, however, additional glycolipids and low molecular weight oligosaccharides, which preparation for paraffin processing have extracted, will also be identified, other cellular glycoconjugates which may be masked by lipids in frozen sections, will only be apparent after extraction with xylene or chloroform-methanol solution, or by paraffin processing of the tissue.

Most retrospective studies are performed using tissues fixed in 10% formalin, which has been reported to preserve many lectin receptors (Alroy et al., 1988). As in immunohistochemistry, pre-treatment with enzymes enhances the configuration and staining of certain chemical grouping. Cryptic sugar residues are glycoproteins and glycolipids which in mature cells are masked by terminal residues. The identification of masked lectin receptors can be accomplished by cleaving the terminal residues with corresponding hydrolytic enzymes. Neuramindase is one such enzyme which specifically hydrolyses the a-glycosidic bonds between sialic acid and cell surface glycoconjugates. Sialic acid (N-acetyl neuraminic acid and its derivatives), an anionic sugar residue found at most peripheral positions of membrane glycoconjugates is responsible for a portion of the negative cell surface charge. The physiologic role of surface sugars, including sialic acid, is not completely clear: the siting of these carbohydrates, their abundance and negative charge may suggest important cellular functions. Presumably neuraminidase treatment leads to an increase in the free sialic acid concentration in the vicinity of the cardiac sarcolemma (Yee et al., 1991).

6.B.2. Evaluation of fixative.

To evaluate the influence of fixatives on the staining outcome, samples consisting of rectangular cardiac blocks measuring 2.0 x 1.5 cm and 0.2-0.3 cm thick from the normal controls and samples from the grossly infarcted areas which included adjacent non-infarcted areas (to serve as built-in controls; 'built-in' controls, are ideal because the variables of tissue preparation, processing, and staining are eliminated), were fixed seperately in different fixatives.

These fixatives comprised 10% buffered formalin (NBF), Bouin's, methacarn, paraformaldehyde, periodate-lysine-paraformaldehyde (PLPD) fixatives for a period ranging from 16 to 24 hours. The average period of fixation was 18 hours.

Bouin's fixative

Picric acid	60 ml
Formaldehyde 40%	20 ml
Glacial acetic acid	4 ml

Methacarn fixative

Chloroform	90 ml
Methanol	180 ml
Acetic Acid	30 ml

Make up fresh each time (Puchtler et al., 1970).

Paraformaldehyde fixative

Four grams of paraformaldehyde in 100 ml. of distilled water (Robertson et al., 1963).

Periodate-lysine-paraformaldehyde (PLPD) fixative

This fixative contains periodate 0.01 M; lysine-HCL 0.073 M; paraformaldehyde 2% and 0.05 M phosphate buffer pH 7.2

Disodium hydrogen orthophosphate 12.5 g

Sodium hydrogen orthophosphate 2.0 g

Preparation of fixative:

Paraformaldehyde	8 g
5 M NaoH	1 ml
Distilled water	100 ml

Mix the sodium hydroxide and the distilled water. Bring the solution up to 56°C on a hot plate in the fume cupboard. Add the paraformaldehyde a gram at a time until it is all dissolved and allow to cool before use.

Phosphate buffer	37.5 ml
Paraformaldehyde	12.5 ml
Sodium Metaperiodate	0.108 g
Lysine Hydrochloride	0.685 g

In a separate 50 ml conical add 2.5 g potassium dichromate to 50 mls of distilled water. Once all the reagents are dissolved, the contents of the conical flasks are

mixed together. Each time this is used the solution has to be made up fresh (Mclean & Nakane, 1974).

Sectioning

Specimens were embedded in paraffin, cut on a Leitz 1512 microtome at 3 μm and floated onto a warm water bath at 45°C. The slides used were coated with a 1% solution of Poly-L-Lysine (Sigma) as a tissue adhesive. The sections were dried at 75°C for 1 hour prior to staining.

In the first experiment, one block of heart was divided into 5 pieces: one of each was put into one of the following fixatives:

1. 10% Neutral buffered formalin (NBF).
2. Bouin's fixative.
3. Methacarn.
4. Paraformaldehyde.
5. Periodate lysine paraformaldeyde (PLPD).

The causes of death in these 8 cases were:

1. Case No. F100-92: inhalation of car exhaust fumes.
2. Case No. F102-92: MI.
3. Case No. F120-92: MI.
4. Case No. F139-92: MI.
5. Case No. F141-92: asphyxia due to hanging.
6. Case No. F142-92: MI.
7. Case No. F143-92: road traffic accident.
8. Case No. F144-92: inhalation of car exhaust fumes.

The other details of these cases are shown in table 6.6.

All fixed tissues were processed in the same manner and to ensure uniform staining, all sections were stained simultaneously using the same reagents. Thus, the differences in results cannot be attributed to the dehydration, clearing or the staining procedures.

Forty blocks (8 cases x 5 fixatives = 40) were then serially sectioned and each was stained with H&E, Masson's Trichrome, PTAH, anti-myoglobin antibody (ab) and seven lectins (Table 6.7) using a standard titre used in ordinary diagnostic histopathology and, in case of the lectins the dilutions recommended by the manufacturer (40x11=440 slides).

As already stated by Langeron 1921: "*Nous savons que le fixateur ideal n'existe pas encore*" (Puchtler et al., 1970). The conclusion reached was that NBF and PLPD were the best fixatives for all the purposes. Bouin's fixative was unsuitable since it produced marked shattering of the sections, lifting up of parts of the tissue and foldings. Of these 8 cases, to cut down on unnecessary cutting and staining with the inherent high financial outlay involved, only 4 cases were retained at the end of the initial work which had already taken up about 12 weeks of laboratory work. These were the four cases which were used in the second phase of the pilot study:

1. Case No. F100-92: inhalation of car exhaust fumes.
2. Case No. F120-92: MI.
3. Case No. F142-92: MI.
4. Case No. F143-92: road traffic accident..

To examine in more detail the suitability of the fixatives 8 sections (one section each from these 4 cases selected which were fixed with either of the 2 fixatives i.e. 10% NBF and PLPD: 4x2=8) were stained with H & E, Masson's Trichrome, PTAH, anti-actin, anti-myoglobin abs and seven lectins (8x12=96 slides).

On the basis of the experiment it was decided that case (F143/92) would be used in all subsequent staining as a positive control on which the working titre for antibodies would be carried out for each individual antibody and lectin, and standardisation of Lie's and Arnold's stains.

The same case (F143/92) was chosen as a negative control for the staining of complement $C5_b$-9 and CD-59 (Protectin) and a case of recent MI (F142/92) was selected as a positive control (10x2=20 extra slides than the rest of the reagents).

Neutral buffered formalin (NBF) fixative was chosen in preference to PLPD because PLPD fixative:

1. is expensive.
2. requires to be made up every morning.
3. is very time-consuming in its preparation because the individual chemicals have to be weighed into tiny vials.
4. offers no significant advantages in the staining procedure.

6.C. Collection and sampling of hearts.

The criteria for the selection of cases were based on the information which was available to the pathologists in the medical reports as well as police reports which included the full circumstances of death and autopsy findings.

Initially all the cases coming to autopsy which appeared to match by their history and gross autopsy finding, the criteria selected for inclusion in the study were collected. A total of 292 hearts were originally placed into formalin fixative after dissection, left ventricular blocks were taken from the areas which thought to be the more appropriate, this produced about 3-4 blocks per heart. Each block was then sectioned and stained with H &E. The basis for discarding blocks included the presence of autolytic change in the myocytes and excessive drying artefacts from fixation and the presence of microscopical abnormalities such as prominent focal or interstitial fibrosis.

In total, the final study involved 54 human hearts. These were collected at autopsy from cases in which myocardial ischaemia was considered to be the immediate cause of death. The test group comprised 46 cases: 30 males and 16 females. Their ages ranged from 38 years to 90 years with a mean age of 64.13 years (Table 6.1)

	Male	Female	Total	Mean Age
Group A	2	1	3	70.66
Group B	5	-	5	67.60
Group C	30	16	46	64.13

Table 6.1. Age and Sex of Study Cases.

Postmortem interval

All corpses were refrigerated at +4°C and autopsied as soon as possible given the circumstances of the case. The mean interval between death and autopsy was 53 hours and the range was from 14 hours to 109 hours.

Hearts were weighed fresh. All blood from within the heart was washed away and no significant portions of the main vessels were attached. Reweighing after being fixed in formalin was not performed because fixation in formalin for periods up to 3 months has been shown not to significantly affect the total heart weight (Hangartner et al., 1985).

A control sample of hearts were chosen from persons dying acutely and unexpectedly from conditions in which disease of the cardiovascular system was not a contributory factor. All the hearts were dissected, weighed and examined in a fashion identical to that of the experimental series. The autopsy technique employed was either the Rokitansky method or by standard dissection method with separate body cavity dissection.

The transverse sinus of the pericardium was the site at which the aorta and pulmonary trunk were severed. The pulmonary veins were cut as close to the heart as possible. The coronary arteries were dissected by transverse cuts along their entire course at about 0.2-0.3 cm. intervals.

6.D. Subdivision of cases.

The cases studied were divided into 3 main sub-groups, as indicated by Table 6.2.

Group-A: These hearts showed good macroscopical evidence of a well established myocardial infarction (MI) with coagulation necrosis (Table 6.3).

Group-B: These hearts also showed macroscopical evidence of a well established MI. The blocks of the heart muscle which were selected from these were confined to the areas immediately adjacent to the area of infarction as observed by the naked eye. In most of the blocks chosen it was insured that the edge of the MI was included in the periphery of the block subjected to the specialised staining techniques. This

group was intended to demonstrate areas of myocardial ischaemic damage at a sub-infarction level i.e. ischaemic areas in which necrosis was not demonstrable grossly or microscopically or 'peri-infarct ischaemic zones' (Table 6.4).

Group	Description	Blocks Taken	No. cases
A	Infarcts shown by microscopical & macroscopical examination	From infarcted area only	3
B	Infarcts shown by microscopical & macroscopical examination/peri-infarct zones	From infarcts and peri-infarct zone	5
C	Pre-infarcts/ischaemic zones	From microscopical area most likely to be ischaemically damaged	46
Total			54

Table 6.2. Myocardial tissue samples taken in the case groups.

Group-C: These hearts were collected from persons who although showing no macroscopical or microscopical evidence of MI, had demonstrable acute coronary arterial thrombi or very gross occlusive atheromatous disease to the extent that the cause of death was attributed to such findings, after an extensive autopsy inclusive of histology has been carried out in all cases as well as toxicological studies as required (Table 6.5).

After gross sectioning the blocks chosen from these hearts were taken from the areas in which it was expected from the anatomical distribution of the individual coronary occlusive changes to be maximally affected by ischaemia. These specimens therefore should represent areas of ischaemic damage in which no gross or microscopical findings could be specifically identified to confirm the ischaemic damage.

The blocks which were chosen specifically for specialised stains were further selected because they demonstrated very minor histological changes such as interfascicular oedema, occasional and focal pavementation of polymorph neutrophils in blood vessels, some localised extravasation of RBCs and oedema in between cardiac myocytes, or combinations of these findings which although not

diagnostic of ischaemia, suggested that these were the likeliest blocks to show pre-infarction ischaemia-induced abnormalities.

6.E. Serial sections

In order to ensure comparability between the tinctorial stains and the individual immunohistochemical and lectin stains, serial sections were obtained from each of the blocks selected.

6.F. Tinctorial staining methods.

In addition to an H&E; the following stains were carried out on each case chosen for the study: Masson's Trichrome, PTAH, HBFP and mod-LFB.

Haematoxylin and eosin stain

1. Dewax sections in xylene	5 minutes
2. Rinse in absolute alcohol	2 minutes
3. Rinse in 95 % alcohol	2 minutes
4. Rinse in 70% alcohol	2 minutes
5. Wash in water	
6. Place in haematoxylin	4 minutes
7. Wash in water	
8. Differentiate in acid alcohol	10 seconds
9. Wash in water	
10. Blue up in Scott's tap water	1 minute
11. Wash in water	
12. Stain in Eosin	1 minute
13. Wash in water	
14. Rinse briefly in 70% alcohol	
15. Rinse well in 95% alcohol	
16. Rinse well in absolute alcohol	
17. Place in xylene	

Results:

Nuclei	blue to blue-black
Cytoplasm	shades of pink

Muscle fibres	deep pink
RBCs, eosinophil granules	orange-red.

Masson's Trichrome stain

1. Dewax and rehydrate sections in alcohols	
2. Wash in water	
3. Stain in haematoxylin	4 minutes
4. Differentiate and blue up	
5. Stain in 1% Ponceau fuchsin	5 minutes
6. Rinse in water	
7. 1% Phosophotungstic acid	5 minutes
8. Rinse in water	
9. Counterstain in 0.15 Light green	1 minute
10. Rinse in water	
11. Apply 0.1% acetic acid	1minute
12. Rinse in water	
13. Dehydrate in alcohol	
14. Clear in xylene	

Results:

Nuclei	Blue
Collagen	Green
Muscle, RBCs & fibrin	Red.

Phosphotungstic acid haematoxylin (PTAH) stain

1. Section to water.	
2. Place in acid dichromate solution	30 minutes
3. Wash in tap water	
4. Treat with acid permanganate	1 minute
5. Wash in water.	
6. Treat with 1% oxalic acid.	
7. Rinse in tap water.	
8. Stain in PTAH solution	overnight
9. Dehydrate, clear and mount.	

Results:

Muscle Striation, fibres, fibrin	Dark blue
Nuclei & RBCs	Blue.
Collagen	Deep brownish red.
Cytoplasm	Pale pinkish brown.

Luxol-fast blue (LFB)-modified stain

I. Staining solutions

Solution A: dissolve 1 g of Luxol Fast Blue in 1000 ml of 96% alcohol, add 5 ml of 10% acetic acid. This solution may be prepared as stock solution; it is very stable and keeps well in a closed brown bottle for more than a year. Filter before use.

Solution B: prepare a 0.05% aqueous lithium carbonate solution. This solution keeps well up to 10 days.

Solution C: dissolve 0.1 g of Nuclear Fast Red in 100 ml of a hot 5% aqueous solution of aluminium sulphate. Filter after cooling. The solution can be used as stock solution.

II. Staining procedure

1. Remove paraffin with xylene and run sections through absolute alcohol and several changes of 96% alcohol.
2. Stain for 5 hours in solution A at 60°C.
3. Place slides in 75% alcohol for 10 minutes.
4. Rinse in distilled water for 2 minutes.
5. Place slides in solution B for 5 minutes (with occasional agitation).
6. Rinse in distilled water for 2 minutes.
7. Differentiate for 1-3 minutes in several changes of 75% alcohol to a faint dove coloured blue. If differentiation is not sufficient repeat steps 4-7.
8. Place in distilled water for 5 minutes.
9. Stain for 10-30 minutes in solution C.
10. Rinse in distilled water, dehydrate, clear in xylene and mount in mounting mediums.

Results:

Degenerating myocytes	Blue.
Normal myocytes & nuclei	Red.
Elastic fibres	Deep blue.

Arnold et al. (1985) described two basic staining features in myocytes: (A) myocytes with irregular blue transverse bands, (B) myocytes with diffuse blue colour.

Haematoxylin-basic fuchsin picric acid (HBFP) stain

The HBFP technique uses three staining and colour-differentiation solutions:
Solution A: Alum haematoxylin, prepared as follows: mix six grams of aluminium ammonium sulfate, 0.5 gram of haematoxylin, and 0.25 gram of yellow mercuric oxide in 70 ml. of distilled water; boil for 10 minutes; cool; add 30 ml. of glycerin and four ml. of glacial acetic acid; filter before use.
Solution B: Basic fuchsin, 0.1% in distilled water.
Solution C: Picric acid, 0.1% in absolute acetone.
Staining can be preformed conveniently in Coplin jars at room temperature.

1. Cut sections at five to six μm, deparaffinize, and hydrate to water.
2. Stain in solution A for 10 seconds.
3. Wash in running cold tap water for five minutes.
4. Stain in solution B for three minutes.
5. Rinse briefly (five to 10 seconds) in distilled water.
6. Rinse briefly (five to 10 seconds) in absolute acetone.
7. Differentiate in solution C until the red (basic fuchsin) colour ceases to run off the sections; usually about 20 seconds for human tissue and about 15 seconds for animal tissue.
8. Rinse briefly (five to 10 seconds) in absolute acetone.
9. Clear in xylol and mount.

(Solution C and all rinsing fluids should be renewed after each batch of five to six tissue sections).

Inclusion of both positive and negative control sections with each batch of unknown sections during the staining procedure is essential until complete familiarisation with the technique, especially the correct timing of decolorization (step 7) has been achieved.

Results:

Normal myocardium	Light brown.
Nuclei	Purple hue of haematoxylin.

Ischaemic myocardium Bright crimson-red colour.

"Other structures which were also stained by basic fuchsin included erythrocytes, fibrin and plasma proteins, elastic fibres, and collagen" (Lie et al., 1971; Lie et al., 1972).

6.G. Immunohistochemistry methods.

6.G.1. Enzymatic pre-digestion.

The value of prior fixation in immunohistochemical methods, helps to ensure that antigens do not diffuse away from their sites within the tissues and by these preserving the structure and integrity of the tissue is made easier detailed histological study. Fixation however, often renders a tissue immunologically unreactive and this may lead to the assumption that the fixative has either destroyed the antigen or allowed it to diffuse away. The employment of digestion with proteolytic enzymes, such as pronase, on sections obtained from formalin fixed and paraffin embedded material was first introduced by Huang (1975). Later on trypsin (Huang et al., 1976; Curran & Gregory, 1977) was shown to be useful in paraffin sections which were stained for either by immunofluorescence or by an immunoperoxidase technique. The treatment has been described to result in un-masking of immuno-reactive sites, and suppression of background staining to some degree.

Trypsin has been shown to be capable of demasking antigens. This suggests that when carefully-fixed tissue fails to react immunologically, the cause is more likely to be fixative-induced impermeability of the tissue rather than actual loss or destruction of the antigen by the fixative. Trypsin presumably acts by causing some disruption of tissue structure but this appears to be at the ultrastructural or molecular levels since it is not apparent in the light microscope in those sections optimally exposed to the enzyme.

Whatever the action of such proteolytic enzymes on the tissues, it is undoubtedly facilitated by the fact that the reactions in the immunostaining sequence takes place on the surface of the tissue section. Trypsin treatment is very efficient in unmasking antigens in tissue sections and it is recommended that it should be employed routinely when fixed tissues are processed for immunohistochemical procedures

(Curran & Gregory, 1977). Another possibility that trypsin increases the permeability of the cells or causes cleavage of cross links between fixative and antigenic determinants (Huang et al., 1976; Jacobsen et al., 1980).

Trypsin digestion solution pH 7.6-7.8

Trypsin (ICN Biomedicals INC-Cat. No.150213)	100	mg
Calcium Chloride-dihydrate (Sigma Code No. C-3881)	100	mg
Distilled water.	100	ml

Procedure for proteolytic digestion of tissue section

1. Deparaffinize and rehydrate sections.
2. Incubate sections in trypsin solution for 20 minutes at 37°C.
3. Stop digestion by rinsing slides under gently running cold tap water for 5-10 minutes.

Staining

Successful diagnostic use of an antibody depends heavily on the availability of an optimal staining technique and an adequate database on sensitivity and specificity that is usually unique for the antibody in question. In reality these data, essential for avoiding diagnostic disasters, are often not available for antibodies obtained from a commercial source.

The optimal staining condition for each commercially available antibody should be determined before actual use (Truong et al., 1990). Antigenic preservation was considered adequate when there was positive staining of built-in controls. Specificity of staining was validated by the use of both the negative and built-in positive controls.

Specificity

When specificity of antibodies is considered in immunohistochemical staining, this refers to the ability of that antibody to detect only one specific antigenic determinant,

to the exclusion of all others. In practice, if an immunohistochemical reaction is to be regarded as specific, it must satisfy two sets of criteria, 'method specificity' and 'antibody specificity' (Petrusz et al., 1976)

For 'method specificity' positive staining must result entirely from the immunochemical reactions between the primary antibody and the tissue antigen. All subsequent reagents which are used in the staining procedure, must be selected and standardised not to react directly with any other tissue component. Method specificity is best obtained by simultaneously staining section with increasing dilutions of the primary antibody, up to such dilutions at which all staining disappears – a situation comparable to omission of the primary antibody. Any positive staining which does not appear to depend on the titre of the primary antibody probably represent 'method non-specificity'. Simple changes such as (dilution, time, temperature etc.), in the conditions of application of some of the subsequent reagents should be tried solve the problem on these occasions, one has to resort to special non-specific 'blocking' agents (e.g. serum proteins) before applying one or each of the antibodies. The commonest source of 'method non-specificity' is the second (bridge or link) antibody; its 'non specific' binding to the tissue can be eliminated by using a dilute solution of normal serum which has been derived from the species which the second antibody has been produced (Petrusz et al., 1980). Once problems of method specificity have been solved, the antibody specificity is the next matter to be addressed (Petrusz et al., 1980).

6.G.2. Antibodies for staining.

The proper storage temperature as recommended by the manufacturer should be observed with all antibodies. Refrigerators and freezers used for storage of immunochemicals should be monitored for accurate and consistent temperatures. Proper reagent care also reduce problems stemming from contamination, heat, excessive light exposure. Reagent contamination can be avoided by the use of clean pipettes (Boenisch, 1989).

Antibody titres:

An antibody titre is defined as the highest dilution of an antibody which results in optimal specific staining with the least amount of background. Usually the manufacturer offers pre-diluted reagents ready for use, or recommends dilution

ranges to be made compatible with other variables such as method, incubation time and temperature.

To determine the optimal titre for each ab 10 serial sections from each case selected (5 dilutions of 1:10, 1: 25, 1: 50, 1: 100, 1: 200 for trypsin digestion and 5 similar dilutions without trypsin digestion) for 25 different abs: 10x25=250 slides.

Incubation time.

Incubation times for the primary antibody with 20-30 minutes as this probably the most widely used.

Endogenous enzyme activities.

Peroxidase activity is a common property of all haemoproteins. The most frequently used procedure for the suppression of endogenous peroxidase activity in formalin-fixed tissue entails the incubation of sections in 3% H_2O_2 for 10 minutes as follows:

Methanol/hydrogen peroxide solution.

Hydrogen Peroxide 100 vols	9	ml
Methanol	291	ml

Avidin biotin complex staining (ABC) method:

Hsu et al. (1981) developed the immunohistochemical procedure which employs an avidin-biotin-peroxidase complex (ABC). They employed the avidin/biotin system because of unique features of these substances. Avidin is a glycoprotein a protein from egg white with a molecular weight of 68,000. Avidin has an unusually high affinity for biotin (a vitamin). The extent of this affinity is approximately 10^6 higher than that of most antibodies for their specific antigens; in contrast to reversible antigen-antibody interaction it leads to a union that is essentially irreversible, and can be employed to improve sensitivity of staining at sites where biotin-labelled antibodies are localised. Biotin had the further capacity of being able to conjugate with many proteins including enzymes (Lewis et al., 1983).

Their procedure employs primary antibody, biotinylated secondary antibody and a pre-formed avidin-biotinylated horseradish peroxidase complex (ABC). After incubating the tissue section with primary antibody specific for the antigen being sought, biotin-labelled secondary antibody is applied. This is followed by avidin-biotinylated horseradish peroxidase complex which then binds to the biotinylated secondary antibody.

The following antibodies were tested:

Monoclonal mouse anti-cell adhesion molecule (A-CAM) antibody
Sigma Immuno Chemicals. Product No. C-2542. Clone GC-4

It is also known as N-Cadherin. The antibody is a mouse IgG1 isotype, derived from hybridoma cells. Affinity purified chicken heart A-CAM was used as an immunogen. Monoclonal anti-A-CAM is specific for a polypeptide (M.W.=135kD) isolated from a freshly prepared extract of rat cardiac muscle. The antibody reacts with the N-terminal half of the extracellular domain of A-CAM. It reacts with the A-CAM molecule from chicken, rat, mouse, rabbit tissue and cells. It stains a large variety of intercellular junctions.

Monoclonal mouse anti-human muscle actin antibody
DAKO-Muscle Actin. Product No. HHF35. Code No. M 635

The antibody is a IgG1, kappa isotype produced from hybridoma cells immunized with extracts from human myocardium. It recognizes muscle specific actin isomers but fails to react with non-muscle actin; it recognises the cardiac actin, and the actin from smooth muscle sources. HHF35 labels myocardial, skeletal muscle, and smooth muscle cells as well as myoepithelial cells, pericytes of small vessels, and capsular cells of several parenchymal organs, including liver, kidney and spleen. Other non-muscle cells are non-reactive, including vascular endothelial cells, lymphoid cells, macrophages and connective tissue cells.

Monoclonal mouse anti-α-actinin (sarcomeric) antibody
Sigma Immuno Chemicals. Product No. A-7811. Clone EA-53

Monoclonal anti-α-actinin (sarcomeric) is a mouse IgG1 isotype derived from hybridoma mouse immunized with purified rabbit skeletal α-actinin, although in

isotyping tests of this product an IgG2b background is observed. It is specific for α-skeletal muscle actinin and α-cardiac muscle actinin. It stains Z lines in myocytes in skeletal and cardiac muscle but not in non-sarcomeric muscle elements (connective tissue, epithelium, nerves, smooth muscle). It shows wide reactivity with human and animal muscle tissue.

Monoclonal mouse anti-human amyloid-A antibody

DAKO-Amyloid A. Code No. M 759

The antibody is of the subclass IgG2a, kappa produced from hybridoma cells immunized with equal mixture of human amyloid protein A coupled to horseradish peroxidase and of human amyloid protein A coupled to high molecular weight kininogen. Amyloid-A antibody reacts with native and fixed amyloid fibrils in paraffin-embedded tissue sections. Cross-reactivity with the serum precursor of protein amyloid-A has been observed (Linke, 1983 & 1984). Amyloid-A antibody binds specifically to isolated protein amyloid-A in both the native fibrils form as well as denatured form.

Monoclonal anti-atrial natriuretic peptide antibody

Cymbus Bioscience Limited. Product Code: CBL 66. Clone 23/1

Specificity: anti-atrial natriuretic peptide (ANP-whole molecule)-Isotype IgG1
It works on formalin-fixed, paraffin-embedded tissue.

Monoclonal anti creatine kinase (BB & MB iso-enzymes) antibody

Serotec. Code No: MCA 488. Clone No: B005

The antibody is of isotype mouse lgG1. It is a concentrated tissue culture supernatant. CK-BB and CK-MB are used as immunogens. Specificity: brain isoenzyme (CK-BB) and the Creatine kinase heart hybrid iso-enzyme (CK-MB: M for muscle and B for brain).

Monoclonal mouse anti-human $C5_b$-9 antibody

DAKO-$C5_b$-9, aE11. Code No. M777

The antibody is a Mouse IgG of isotype: IgG2a, kappa. The immunogen is a purified membrane-bound terminal complement complex. It reacts with a neoepitope in activated C9 complement.

Rabbit anti human C5-9 antibody

Calbiochem-Novabiochem Corporation. Catalog No. 204903

Specificity: This product has been adsorbed against the individual C5 through C9 complement components and shows reactivity only against the C5-9 complex as a whole. This product reacts equally well with both membrane-bound and fluid phase C5-9.

Monoclonal mouse anti human endothelial cells antibody

Serotec. CD No. CD34. Clone No. QBEND/10

The antibody is IgG1 isotype, recognises the CD34 antigen but shows some cross reactions with vascular associated adventitia and some basement membranes. The immunogen is human endothelial vesicles. It is associated to a degree with the baso-luminal surfaces of endothelium and shows a slight reaction with an intra-cytoplasmic vesicular compartment. Predominant staining is on the endothelial cell membrane.

Monoclonal rat anti human GPI-antigen antibody

Serotec. Code no: MCA 715. CD No: CD 59. Clone No: YTH53.1

The antibody is IgG2b isotype from a hybridoma mouse cells immunized with human peripheral blood T cells.

Monoclonal mouse anti-swine desmin antibody

DAKO-Desmin, DE-R-11. Code No. M 724

The antibody is mouse IgG of Isotype IgG1 produced from a hybridoma cells immunized with desmin purified porcine stomach. It reacts with the 53 kD intermediate filament protein desmin in muscle cells and shows a broad inter-species cross-reactivity, reacting with human desmin as well as desmin from other species, e.g. rat, chicken and hamster. It does not appear to recognize other filament proteins (Debus et al., 1983).

Reactivity: in normal tissues both striated (skeletal and cardiac) and smooth muscle cells are labelled. The labelling is confined to the Z bands in skeletal and cardiac muscle, giving a characteristic striated appearance.

Monoclonal mouse anti-human desmin antibody

DAKO-Desmin D33. Code No. M 760. Clone: D33

The antibody is a mouse lgG, subclass: lgG1, kappa produced from a hybridoma cells immunized with desmin purified from human muscle. The antibody labels both striated (skeletal and cardiac) and smooth muscle cells. The labelling is confined to the Z bands in skeletal and cardiac muscle. It reacts with the 53 kD intermediate filament protein desmin in muscle cells. It shows a broad interspecies cross-reactivity reacting with human desmin as well as desmin from other species, e.g. rat, chicken, and hamster and does not recognize other filament proteins.

Monoclonal mouse anti-Endothelin-1 antibody

Cymbus Bioscience Limited. Product code: CBL 85.Clone IC4

The antibody is lgG1 isotype from mouse ascitic fluid. It specifically recognises endothelin ET-1. Endothelin-1 is a 21-amino acid endothelium-derived peptide first described by Yanagisawa et al. (1988). It is the most potent vasoconstrictor known, the vasoconstrictor action of endothelin-1 is about 100 times more potent than that of norepinephrine (Miyauchi et al., 1990)

Monoclonal mouse anti-human myoglobin antibody

Sigma Immuno Chemicals. Product No. M7773. Clone No. MG-1

The antibody is lgG1 subclass derived from hybridoma mouse cells immunized with purified human skeletal muscle myoglobin. No cross-reaction is observed with haemoglobin.

Rabbit anti-human myoglobin antibody

DAKO PATTS. Code No. A 324

The antibody is a rabbit anti-human myoglobin i.e. a polyclonal immunoglobin fraction of rabbit immunized with myoglobin isolated from human heart.

Rabbit anti-human moysin (skeletal) antibody
Sigma Immuno Chemicals. Product No. M-7523

The antibody is developed in rabbit using purified human skeletal myosin (heavy and light chain) as the immunogen. It is specific for the A band of human skeletal myosin. The product does not react with human smooth muscle myosin. Myosin contains two identical heavy chains and four light chains. It is of use to study the cytoskeletal architecture of thick filaments.

Rabbit anti-human moysin (smooth and skeletal) antibody
Sigma Immuno Chemicals. Product No. M-7648

Specificity: the antibody specifically stains the A band of skeletal muscle.

Monoclonal mouse anti-tropomyosin antibody
Sigma Immuno Chemicals. Product No. T 2780. Clone No. TM311

The antibody is of the mouse IgG1 isotype which is derived from mouse hybridoma immunized with chicken gizzard tropomyosin. It stains cultured human and chicken fibroblasts as well as rat muscle cells.

Monoclonal mouse anti-troponin-T antibody
Serotec. Code No. MCA 470. Clone No. T1/61

The antibody is of IgG1 isotype produced from a hybridoma mouse cells immunized with chicken breast muscle troponin T. It cross-reacts with chicken cardiac but not slow muscle troponin T. It cross-reacts strongly with human fast muscle troponin T but shows no reaction with the rabbit protein.

Monoclonal mouse anti -troponin-T antibody
Sigma Immuno Chemicals. Code No. T6277. Clone JLT-12

Monoclonal anti-troponin-T is a mouse IgG produced from a hybridoma cells immunized with purified troponin-T from rabbit skeletal muscle. Troponin-T is a microfilament protein closely associated with actin and its microfilament accessory proteins. Monoclonal anti-troponin-T is specific for troponin-T from various species.

Rabbit anti-tubulin antibody

Sigma Immuno Chemicals. Code No. T3526

The antibody is developed in rabbit using tubulin purified from 13 days old chicken embryos as the immunogen. The microtubules are composed of tubulin and several additional proteins designated microtubule associated proteins (MAPs). Tubulin is the major building block of microtubules. This intracellular cylindrical filamentous structure is present in almost all eukaryotic cells. The antibody is specific for tubulin and it has not been adsorbed on microtubule associated proteins (MAP's) and therefore may contain antibodies to high and low molecular weight MAP's.

Monoclonal mouse anti-β-tubulin antibody

Sigma Immuno Chemicals. Code No. T4026. Clone TUB 2.1

Monoclonal anti-β-tubulin is a mouse IgG1 presented in the form of specially processed ascites fluid obtained from hybridoma mice immunized with purified rat brain tubulin.

Monoclonal anti-human vinculin antibody

Sigma Immuno Chemicals. Product No. V-9131. Clone hVIN-1

The antibody is a mouse IgG1 isotype derived from hybridoma cells. Vinculin, purified from human uterus was used as the immunogen. It stains vinculin at cell-cell and cell-substrate contacts in tissue and cultured cells (using indirect immunofluorescent labelling).The antibody reacts with vinculin band applying an immunoblotting technique. The antibody shows cross reactivity with the smooth muscle meta-vinculin.

Monoclonal anti-mouse vinculin antibody

Sigma Immuno Chemicals. Product No. V-4505. Clone VIN-11-5

The antibody is a mouse IgG1 isotype derived from hybridoma cells. Purified vinculin from chicken gizzard smooth muscle was used as the immunogen. It is immunospecific for vinculin (as determined by indirect immunofluorescent staining or immunoblotting). Specific staining occurs at cell-substrate focal contacts on membranes of cultured cells.

Monoclonal mouse to human vinculin antibody

Serotec. Code No. MCA 465- S. Clone No: V 284

The antibody is a mouse Isotype IgG1 produced from hybridoma cells immunized with purified human platelet vinculin. The antibody reacts with leucocytes, muscle cells, epithelial cells and fibroblasts.

The optimal titres for the individual antibodies which were selected for the study were as follows:

Antibody	Titre
Anti-A-CAM/N-Cadherin	1:50 + T*
Anti-actin	1:100
α-actinin	1:100 + T
$C5_b$-9	1:50 + T
CD59	1:50 + T
CD34	1:50
Desmin	1:20 + T
Myoglobin	1:20 + T
Myosin	1:10 + T
Tropomysin	1:200 + T
Troponin-T	1:50 + T
Tubulin	1:10 + T
Vinculin	1:100 + T

*T=trypsin.

6.G.3. Antibodies tried & discarded.

The following antibodies have been discarded due to failure to react despite repeated attempts to stain with varying range of dilutions (e.g. dilution of 1: 5), enzymatic digestion of different timings, and increasing the primary antibody incubation periods e.g. overnight incubation. The probable reason for this is that these antibodies have been prepared specifically for immunofluorescent studies on cryostat sections. It is often the case that such antibodies will not react with formalin-fixed, paraffin-embedded sections.

Monoclonal mouse anti-human amyloid-A antibody

DAKO-Amyloid A. Code No. M 759

Monoclonal anti-atrial natriuretic peptide antibody

Cymbus Bioscience Limited. Product Code: CBL 66. Clone 23/1

Monoclonal anti creatine kinase (BB & MB iso-enzymes) antibody

Serotec. Code No: MCA 488. Clone No: B005

Rabbit anti human C5-9 antibody

Calbiochem-Novabiochem Corporation. Catalog No. 204903

Monoclonal mouse anti-human desmin antibody

DAKO-Desmin D33. Code No. M 760. Clone: D33

Monoclonal mouse anti-Endothelin-1 antibody

Cymbus Bioscience Limited. Product code: CBL 85. Clone IC4

Rabbit anti-human myoglobin antibody

DAKO PATTS. Code No. A 324

Rabbit anti-human moysin (skeletal) antibody

Sigma Immuno Chemicals. Product No. M-7523

Monoclonal mouse anti-troponin-T antibody

Sigma Immuno Chemicals. Code No. T6277. Clone JLT-12

Monoclonal mouse anti-β-tubulin antibody

Sigma Immuno Chemicals. Code No. T4026. Clone TUB 2.1

Monoclonal anti-human vinculin antibody

Sigma Immuno Chemicals. Product No. V-9131. Clone hVIN-1

Monoclonal anti-mouse vinculin antibody

Sigma Immuno Chemicals. Product No. V-4505. Clone VIN-11-5

6.H. Lectin staining methods.

The techniques commonly used for demonstration of lectin binding sites include direct and indirect methods. In the direct staining, the visualants are conjugated directly to the lectin. Indirect staining methods employ multi-stage procedures in which the first stage is the reaction of biotinylated lectin with the tissue, followed by reaction with an enzyme. The major differences in results between the direct and indirect lectin staining techniques are in their sensitivities and in the extent of background staining. Indirect techniques require lower concentrations of lectins than direct methods.

Currently two major indirect methods are used: the first is the peroxidase-anti-peroxidase (PAP) method, the disadvantage of this method is that it requires a large battery of anti-lectin antibodies. In the second method, biotinylated lectins, as probes, and the avidin-biotin-peroxidase complex (ABC) are used (Alroy et al, 1988).

Avidin biotin complex (ABC) procedure for lectin staining:

1. dewax sections in xylene and take to 70% ethanol	
2. place in hydrogen peroxide in distilled water	10 mins
3. wash in running tap water H_20	3-5 mins
4. wash in 10 mM HEPES-buffer pH 7.5 in 0.15 M NaCl	3 x 5 mins
5. place in 1% BSA in HEPES buffer	10 mins
6. incubate in biotinylated lectin at optimal dilution in HEPES buffer	60 mins
7. wash in HEPES buffer	3 x 5 mins
8. incubate in avidin/biotin complex HRP	30 mins
9. wash in TBS buffer	3 x 5 mins
10. visualise in DAB	3 mins
11. wash in running tap water	5 mins
12. counterstain in haematoxylin	10 seconds
13. wash in water	1 min
14. dehydrate, clear and mount in Pertex.	

Optional: required for those lectins which the neuraminidase pre-treatment after step-3 produces better morphology. 60 minutes.

HEPES buffer

HEPES (Sigma code No. H-3375)

10 mM HEPES, MWt 283.3 g/L 2.383g

NaCl 8.766 g Distilled water 1000 Ml

Neuraminidase

Sigma Chemical Company. Product No. N-2876.

Sialidase receptor destroying enzyme; N-acetyl-neuraminate glycohydrolase.

Isolated from Clostridium perfringens.

Neuraminidase

Sigma Chemical Company. Product No. N-7885.

Sialidase receptor destroying enzyme, Acylneuraminyl hydrolase.

Isolated from *Vibrio cholerae*

Inhibition for the lectin staining

Specific blockers/inhibitors for each lectin are known. By incubating each lectin with solution of its specific blocking sugar for 20 minutes before application of the lectin solution to the tissue section it is possible to test the specificity of the lectin. Table 3 lists the lectins commonly used and the corresponding carbohydrates that are employed to inhibit their binding.

To determine the optimal titre for the different lectins 15 serial sections (5 different dilutions, as with the antibody, for 3 patches: the first patch without any prior treatment, the second batch with trypsin digestion and the third patch with neuraminidase pre-treatment): 7x15=105 slides. The optimal titres for the individual lectins used were as follows:

Lectin	**Ratio**
Con A, UEA 1	1:100
DBA, SBA	1:20+Nm*
PNA, RCA 1, WGA	1:100+Nm

*Nm=neuraminidase.

It is noted that with the majority of the lectins pre-treatment with neurominidase was found to be essential to enhance the intensity and sharpness of the staining. The attached photographs (figures 6.1-6.6) demonstrate this phenomenon.

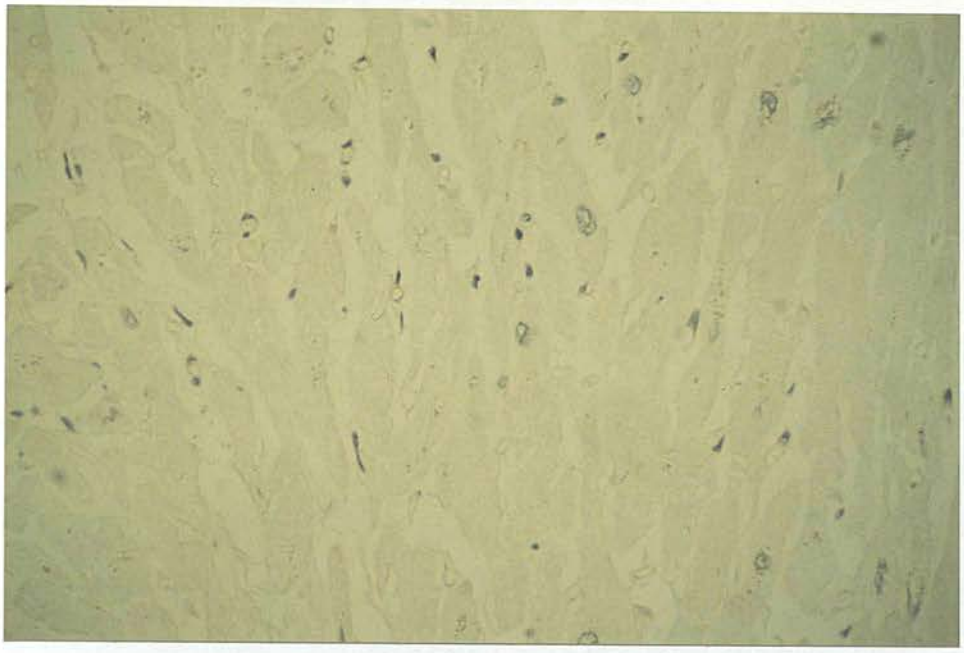


Figure 6.1 X 25. Normal myocardium stained with PNA.

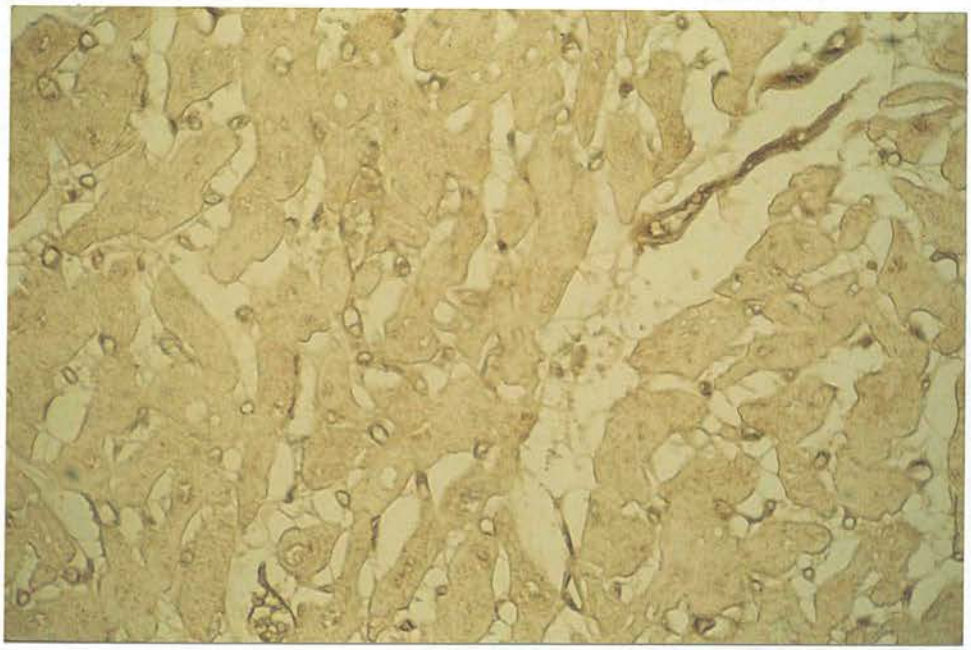


Figure 6.2 X 25. Normal myocardium stained with PNA after pre-treatment with neuraminidase.

Figure 6.3 X 25. Normal myocardium stained with RCA 1.

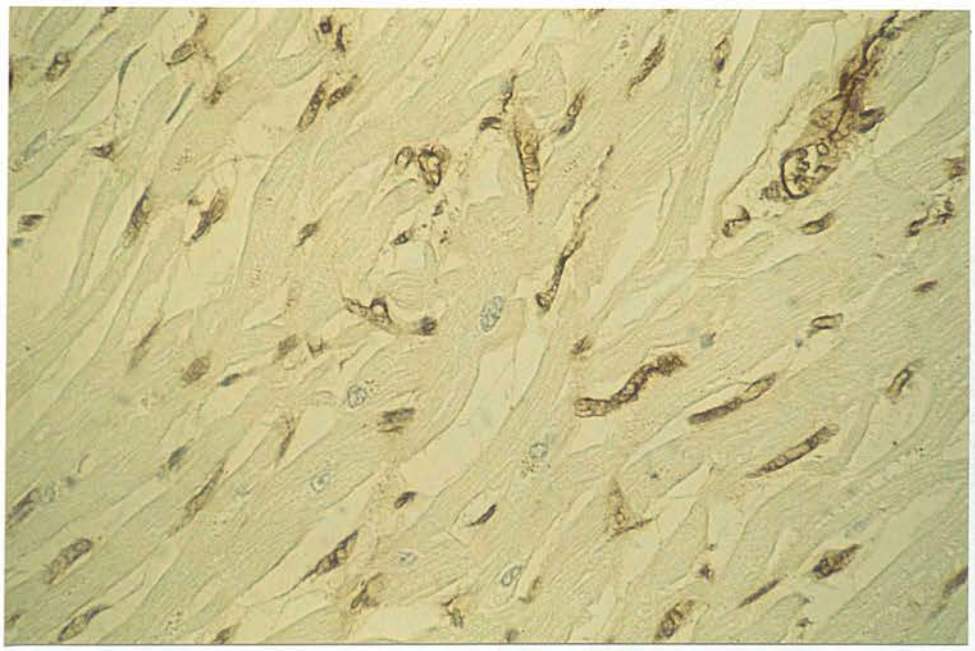


Figure 6.4 X 25. Normal myocardium stained with RCA 1 after pre-treatment with neuraminidase.

Figure 6.5 X 25. Normal myocardium stained with SBA.

Figure 6.6 X 25. Normal myocardium stained with SBA after pre-treatment with neuraminidase.

With each lectin pre-treatment with the same trypsin solution as made use of for the immunohistochemical studies. Trypsin removed almost completely the lectin staining (see figures 6.7 and 6.8):

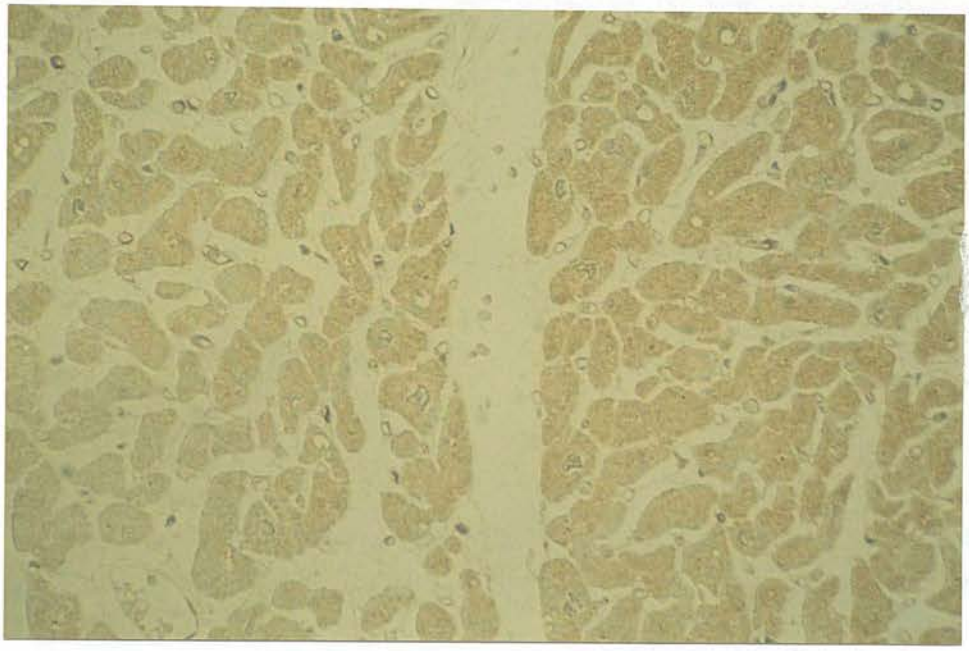


Figure 6.7 X 25. Normal myocardium stained with DBA.

Figure 6.8 X 25. Normal myocardium stained with DBA after pre-treatment with trypsin.

The lectins used in this study comprised:-

Concanavalin A (Con A)

Con A is isolated from *Canavalia ensiformis* (Jack bean) recognises α-linked mannose. Since a wide variety of serum and membrane glycoproteins have a 'core oligosaccharide' structure which includes α-linked mannose residues, many glycoproteins can be examined with Con A and its conjugates.

Con A requires calcium or manganese ions at its saccharide binding sites. Buffers which can bind calcium (such as phosphate) generally are avoided in diluting Con A, since a gradual loss in activity may occur (Vector Laboratories, 1991).

Dolichos Biflorus Agglutinin (DBA)

DBA is isolated from *Dolichos biflorus* (horse gram) seeds. It has a carbohydrate specificity toward α-linked N-acetylgalactosamine which is a glycoprotein associated with surface and intracellular membranes.

Peanut Agglutinin (PNA)

PNA is isolated from *Arachis hypogaea* (peanut). PNA binds preferentially to a commonly occurring structure, galactosyl (β-1,3) N-acetylgalactosamine. Sialic acid which is not bound directly to the receptor sugars may inhibit binding. The presence of calcium ions in dilutents can enhance the binding of PNA to receptors, possibly by neutralizing the negative charges on sialic acid residues adjacent to the receptor sequence.

Ricinus Communis Agglutinin (RCA 1)

RCA 1 is isolated from *Ricinus communis* (castor bean) seeds. It binds preferentially to oligosaccharides ending in galactose but may also interact with N-acetylgalactosamine. Often, desialylation of the glycoproteins have terminal sialic acid residues which can block lectin binding.

Soybean Agglutinin (SBA)

SBA is isolated from *Glycine max* (soybean) seeds. It preferentially binds to oligosaccharide structures with terminal α- or β–linked N-acetylgalactosamine, and to a lesser extent, galactose residues.

Ulex Europaeus Agglutinin (UEA 1)

UEA 1 is isolated from *Ulex europaeus* (Furze gorse) seeds. It binds to many glycoproteins and glycolipids containing α-linked fucose residues. It has been established as an excellent marker for human endothelial cells.

Wheat Germ Agglutinin (WGA)

WGA is isolated from *Triticum vulgaris* (wheat germ). The receptor sugar for WGA is N-acetylglucosamine. WGA can bind oligosaccharides containing terminal N-acetylglucosamine. It can interact with some glycoproteins via sialic acid residues

Product Name	Inhibiting/Eluting Sugar
Concanavalin A (Con A)	200 mM = methyl mannoside/ 200 mM = methyl glucoside mix.
Dolichos biflorus agglutinin (DBA)	200 mM N-acetylgalactosamine
Peanut agglutinin (PNA)	200 mM galactose
Ricinus communis agglutinin I (RCA 1)	200 mM galactose or lactose
Soybean agglutinin (SBA)	200 mM N-acetylgalactosamine
Ulex europaeus agglutinin 1 (UEA 1)	50 mM L-fucose
Wheat germ agglutinin (WGA)	500mM N-acetylglucosamine

Table 6.7 Lectins used and their inhibiting sugars.

Biotinylated Lectin Kit-1

Vector Laboratories Ltd. 16 Wulfric Square, Bretton, Peterborough, PE3 8RF.

Catalog No. BK-1000

Biotinylated Lectin	Amount	Lot	Active Conjugate	Lectin Biotinylated
Con A	1 mg	80718	96%	99%
SBA	1 mg	70326	98%	99%
WGA	1 mg	B1009	96%	96%
DBA	1 mg	80705	98%	99%
UEA 1	1 mg	80713	98%	99%
RCA 1	1 mg	80721	96%	98%
PNA	1 mg	80629	99%	99%

Product Specifications Biotinylated Lectin Kit-1 (issued by Vector Laboratories.)

Reconstitution of the lyophilized biotinylated lectins was done by addition of 0.5 ml of water; the resulting solutions having the following composition: 10 mM HEPES, 0.15 M NaC1, 0.1 mM, Ca^{++}, 0.04% sodium azide, pH 7.5.

Reagents used in immunohistochemical & lectin staining:

TRIS l0X-buffer

To 500 ml 0.5 M TRIS add NHCl until pH is 7.6 (approx. 200 ml).
Make up to 2000 ml with distilled water and recheck pH.
For use dilute 1:10 with normal saline.
0.5M Tris–30.25g Trisma Base (hydroxymethyl) methylamine (FISONS Scientific Equipment-England) in 500 ml H_20.
N HCl-85 ml Concentrated HCl in 1000 ml H_20.
Normal saline-42.5 gms Sodium Chloride (Sigma Code No. S-9625) in 5000 ml H_20.

Biotinylated swine anti-rabbit immunoglobulins
Code No. E 353-DAKO

Biotinylated rabbit anti-mouse immunoglobulins
Code NO. E 354-DAKO

ABC complex/HRP

Code No. K. 355

Working solution:

1. Place 5 ml of 0.05 M Tris/HC1 pH 7.6
2. Add 1 drop* of avidin (reagent A).
3. Add 1 drop* of biotin (reagent B).
4. Leave for 30 minutes before use.

*1 drop corresponds to approximately 45 µl.

ABC buffer (Tris/HCl buffer pH 7.6)

0.2 M Tris-24.228 g/L)	24 ml
0. lN HCL-8.5 ml concentrated HCl/l000 ml	38 ml
Distilled water	38 ml
	100 ml

DAB (di-aminobenzidine) buffer

As with ABC buffer with the addition of 0.0l M Imidazole (Sigma Code No. 1-0125). Recheck pH (7.6)

0.0l M Imidazole - 0.068 g per l00 ml

DAB solution

DAB	5 mg
DAB buffer	5 ml
30 Vols H_2O_2	1 drop

Avidin - Biotin Method for Monoclonal Antibodies.

Step	1	2	3	4	5	6	7	8	9	10	11	12	13
Reagent	WASH	WASH	NRS	PRIM Ab.	WASH	RAMB	WASH	ABC (Prepare 30 mins Before use)	WASH	DAB	WASH	HAEM	DCM
Dilution			1:5			1:400		as per kit		5mg/5ml			
Diluant	WATER	TBS	TBS	1:5 NRS/TBS	TBS	1:5 NRS/TBS	TBS	TRIS/HCL BUFFER pH 7.6	TBS	TRIS/HCL BUFFER pH 7.6 Imidazole	WATER		
Time (MINS)	5	5	10	30	5 X 2	30	5 X 2	30	5 X 2	3	5	10 SECS	

ABBREVIATIONS:

TBS – TRIS BUFFERED SALINE pH 7.6

NRS – NORMAL RABBIT SERUM

RAMB – RABBIT ANTI-MOUSE IMMUNOGLOBULINS, BIOTINYLATED

ABC – AVIDIN/BIOTIN/PEROXIDASE COMPLEX

DAB – DIAMINO BENZIDINE TETRAHYDROCHLORIDE

D.C.M. – DEWAX – CLEAR – MOUNT

NOTES – OPTIONAL PRE-TREATMENTS:

3% H_2O_2 METHANOL – 10 MINUTES TO BLOCK ENDOGENOUS PEROXIDASE

0.1% TRYPSIN IN 0.1% $CaCl_2$ pH 7.6-7.8 at 37°C (IF NECESSARY) - 20 MINUTES

Avidin - Biotin Method for Polyclonal Antibodies.

Step	1	2	3	4	5	6	7	8	9	10	11	12	13
Reagent	W A S H	W A S H	NSS	PRIM Ab.	W A S H	SARB	W A S H	ABC (Prepare 30 mins Before use)	W A S H	DAB	W A S H	HAEM	D C M
Dilution			1:5			1:400		as per kit		5mg/5ml			
Diluant	WATER	TBS	TBS	1:5 NRS/TBS	TBS	1:5 NRS/TBS	TBS	TRIS/HCL BUFFER pH 7.6	TBS	TRIS/HCL BUFFER pH 7.6 Imidazole	WATER		
Time (MINS)	5	5	10	30	5 X 2	30	5 X 2	30	5 X 2	3	5	10 SECS	

ABBREVIATIONS:

TBS – TRIS BUFFERED SALINE pH 7.6

NSS – NORMAL SWINE SERUM

RASB – RABBIT ANTI-SWINE IMMUNOGLOBULINS, BIOTINYLATED

ABC – AVIDIN/BIOTIN/PEROXIDASE COMPLEX

DAB – DIAMINO BENZIDINE TETRAHYDROCHLORIDE

D.C.M. – DEWAX – CLEAR – MOUNT

NOTES – OPTIONAL PRE-TREATMENTS:

3% H_2O_2 METHANOL – 10 MINUTES TO BLOCK ENDOGENOUS PEROXIDASE

0.1% TRYPSIN IN 0.1% $CaCl_2$ pH 7.6-7.8 at 37°C (IF NECESSARY) - 20 MINUTES

Tables of cases.

Key:

RCA	Right Coronary Artery
LAD	Left Anterior Descending Artery.
LCXA	Left Circumflex Artery
FDIB	Found Dead In Bed
COAD	Congestive Obstructive Airway Disease
MI	Myocardial Infarction
IHD	Ischaemic Heart Disease.

Table 6.3 Group A (Positive Controls, MI cases)

Case No.	Age (in years)	Sex M/F	Postmortem Interval (in hours)	Cause of Death	Heart Weight (in grams)	Coronary Arteries	Medical History	Events Relating to the Time of the Sudden Death:
653/91	54	M	61	MI	560	All 3 coronary arteries show 60%-90% narrowing.	Angina pectoris-1987 Varicose veins Lt. leg Medication: Adalat retard Atenolol Heparin	Cough with haemoptysis and occasional heartburn
529/92	76	M	40	MI	340	Moderately severe complicated atheroma with reduction of the posterior part of RCA. Both main Lt. branches with pinhole bore at various points.	Bilateral inguinal hernia-1987	Breathlessness Cardiac arrest.
543/93	82	F	44	Ruptured MI.	350	Widely patent	Rheumatoid arthritis-1985 COAD-1985 Medication: Quinine, Piroxicam, Temazepam, Co-Codamol, Bendrofluazide.	FDIB by her son.

Table 6.4 Group B (Infarct and peri-infarct)

Case No.	Age (in years)	Sex M/F	Postmortem Interval (in hours)	Cause of Death	Heart Weight (in grams)	Coronary Arteries	Medical History	Events Relating to the Time of the Sudden Death:
769/92	64	M	69	Thrombus-RCA	400	RCA: gross atheromatous changes with marked narrowing. 6cms from the origin, recent thrombus extending 3cms. Two other main branches of LCA are atheromatous up to 70-80% of normality at several sites.	Unstable angina-1986 "Heart attack"-1989 Arthritis-1990 Duodenal ulcer-1990	Found dead by wife lying in the garden.
770/92	56	M	81	Thrombus-LAD	400	RCA: numerous plaques with calcification and narrowing up to 60%. LAD: occlusion by thrombus. LCXA: atheroma with narrowing up to 70%.	Depression-occasional	Breathless, found by ambulance crew to be pulseless and in cardiac arrest.
129/93	84	M	63	Thrombus-LAD	440	All three main coronary arteries: show gross atheroma and reduced to a pinhole. LAD: shows recent thrombus.	Prostrate operation-1985	Chest pain followed by sudden fall in the kitchen and collapse.

248/93	68	M	92	Coronary Atheroma	480	RCA & LCXA: show 90% stenosis due to atheroma.	NIL	Found dead lying on his left side unconscious in the golf club.
473/93	66	M	100	Thrombus-LAD	380	LAD: recent thrombus All three coronary arteries contain atheroma with reductions to pinhole lumen at several sites.	Chronic bronchitis & emphysema-1970 Severe claudication-1980 Cervical operation-1989 Recent chest infection Medication: Antibiotic & Co-codamol.	Chest pain and shortness of breath later sweating heavily, stopped breathing and died.

Table 6.5 Group C (Ischaemic cases)

Case Ref. (No.)	Age (in years)	Sex M/F	Postmortem Interval (in hours)	Cause of Death	Heart Weight In grams	Coronary Arteries	Medical History	Events Relating to the Time of the Sudden Death:
338/92 (1)	56	M	56	Thrombosis	360	RCA: completely occluded by a combination of thrombus & atheroma. LAD: very gross occlusion by plaque up to 90% of normality. LCXA: small but patent.	NIL	Collapsed at work, breathless and sick.
342/92 (2)	86	F	60	Thrombosis	580	RCA: 80% narrowing by plaque. LAD: numerous plaques.	Blind (lt. eye)-childhood Glaucoma-childhood Cataract (rt. eye) Hyperthyroidism	Collapsed at home.
353/92 (3)	79	F	96	Coronary occlusion	550	Complete occlusion of RCA by atheroma and old thrombus. Lumen reduced to a pinhole size at a distance of about 6cms. Two main trunks of LCA: calcified atheromatous changes.	Rheumatoid arthritis-1963 IHD-1980 Hiatus Hernia-1987 Vertebrobasilar insufficiency-1987	FDIB

467/92 (4)	52	M	40	Coronary Atheroma	540	70% atheromatous narrowing of main RCA. LAD reduced to a pinhole.	Subdural haematoma-1987 Grand Mal fit-1991	Found dead lying face down in the kitchen floor
570/92 (5)	64	M	20	Thrombosis	400	RCA completely occluded by recent thrombus at about 4cm from its origin for a length of 4cm.	"Heart attack"-1976	Found dead lying face down in street.
591/92 (6)	54	F	48	Thrombosis	350	Recent thrombus 1.5 cm in length involving LAD at a distance of 4cm from bifurcation. Minimal atheroma throughout.	Epilepsy-early 1970s Thyroidectomy-1962 Medication: Epilim-200 Mg Epanutin-100 Mg Thyroxine-100 Mg	Found dead in bed by her husband.
712/92 (7)	47	M	70	Coronary occlusion	410	Patchy complicated and calcified atheroma. Narrowing to pinhole bore of RCA and within the origin of two diagonal branches of LAD.	NIL	Felt unwell, sweating profusely became pale, vomiting, Defibrillated and died.
723/92 (8)	81	M	69	Atheroma	400	RCA completely occluded for 8cm by calcific atheroma and old thrombus. LAD completely occluded for entire length. LCXA small and patent.	Arthritis Coronary artery disease-1990. Medication: Moduretic Slow-K Co-Proxamol	Collapsed while walking on footpath and died.

737/92 (9)	41	M	92	Atheroma	290	RCA narrowed to a pinhole by calcified atheroma. LAD occluded by soft atheromatous deposits and old thrombus of a few days duration. The LCXA small and grossly atheromatous.	Migraine-1988 Depression-1988 Strange sexual tendencies-1990	Collapsed while having tea at home and died.
741/92 (10)	38	M	42	Atheroma	460	RCA completely blocked at about 6 cm from its origin by old thrombus superimposed on calcified plaques. LAD reduced to a pinhole sized lumen by calcified atheroma and old thrombus.	"Heart attack"-1986 Heart failure-1987 UTI-1992 "Heart attack"-1992	Collapsed in bed. Emergency cardiac massage by ambulance crew and failed to respond.
743/92 (11)	64	M	51	Atheroma	350	Diffuse atheromatous change with marked narrowing throughout. 50% blockage of RCA and about 70% or 80% of both LCA branches.	Arthritis	Found by wife dead lying on bathroom floor.
788/92 (12)	76	M	96	Thrombosis	520	RCA: completely occluded by organised and fresh propagated thrombus.	"Heart attack"-1990 Breathlessness-1990	Collapsed on bus and died.

737/92 (9)	41	M	92	Atheroma	290	RCA narrowed to a pinhole by calcified atheroma. LAD occluded by soft atheromatous deposits and old thrombus of a few days duration. The LCXA small and grossly atheromatous.	Migraine-1988 Depression-1988 Strange sexual tendencies-1990	Collapsed while having tea at home and died.
741/92 (10)	38	M	42	Atheroma	460	RCA completely blocked at about 6 cm from its origin by old thrombus superimposed on calcified plaques. LAD reduced to a pinhole sized lumen by calcified atheroma and old thrombus.	"Heart attack"-1986 Heart failure-1987 UTI-1992 "Heart attack"-1992	Collapsed in bed. Emergency cardiac massage by ambulance crew and failed to respond.
743/92 (11)	64	M	51	Atheroma	350	Diffuse atheromatous change with marked narrowing throughout. 50% blockage of RCA and about 70% or 80% of both LCA branches.	Arthritis	Found by wife dead lying on bathroom floor.
788/92 (12)	76	M	96	Thrombosis	520	RCA: completely occluded by organised and fresh propagated thrombus.	"Heart attack"-1990 Breathlessness-1990	Collapsed on bus and died.

803/92 (13)	55	M	38	Coronary Thrombosis	410	Thrombus in LAD and its diagonal branch.	Sarcoidosis & TB lung-1974 Fitted with heart pace maker-1990	Suffered a 'heart attack' at the wheel within the vehicle crashed into a wall.
816/92 (14)	51	F	35	Thrombosis	270	Thrombus in distal part of LAD.		Collapsed while arguing with her husband.
824/92 (15)	49	M	48	Coronary occlusion	380	RCA completely occluded by a plaque. LAD occluded to about 10% of normality at two separate sites.	Hernia operation-1990	Found lying on his back in the street.
859/92 (16)	65	F	14	Thrombosis	340	RCA contains recent thrombus for about 3cm, commencing about 3cm from its origin.	Subtotal thyroidectomy-1970 Otitis media-1973	Chest pains and sickness.
860/92 (17)	80	M	14	Atheroma	380	All 3 coronary arteries show 80%-90% narrowing by atheroma	Angina-1989	Found dead face down on the living room floor of his home.
864/92 (18)	63	F	38	Thrombus	350	LAD: moderately severe atheroma with complete occlusion below its origin by fresh thrombus.	"Heart attack"-1990 Medication: Glyceryl trinitrate Isosorbide mononitrate.	Collapsed on bus deck and died.

868/92 (19)	57	M	44	Atheroma	310	Complete occlusion of LAD by combined atheroma and old thrombus. RCA markedly atheromatous with narrowing at several sites up to 20%. LCXA very small.	Mentally retarded-1956	Found lying at the road side frothing at the mouth.
897/92 (20)	45	M	89	Coronary Occlusion	380	Coronary arteries show patchy atheroma with severe narrowing of LCA. Foci of severe narrowing within LAD.	NIL	Collapsed shortly after playing football match and breathless.
900/92 (21)	60	M	22	Atheroma	560	LCA: shows atheromatous changes with narrowing up to 60% Both branches show numerous foci of atheroma and plaques reducing the lumen to about 40%.	Mild hypertension Hiatus hernia-Mid 1980s	Complained to wife of malaise. Later found dead sitting in the chair.
909/92 (22)	57	M	22	Atheroma	430	LAD and LCXA show very marked atheroma and narrowing. RCA only minimally atheromatous with narrowing at the site of atheromatous plaques to about 40% of normality.	Enjoyed good health	Left work feeling unwell. Collapsed at home while climbing stairs.

1088/92 (23)	73	F	91	Atheroma	380	RCA & LAD: completely occluded to pinhole by atheroma. LCXA: very markedly narrowed by atheroma.	Hypertension	Found dead by her son sitting on the chair in the living room.
1116/92 (24)	56	M	85	Thrombus	500	RCA & LCXA: complete occlusion by thrombi. LAD: moderate to severe atheroma.	"Heart attack"-2 weeks prior to death.	Found dead by his friend sitting in the living room.
150/93 (25)	61	F	96	MI	540	RCA: completely occluded by a thrombus.	NIL	Breathless, died at hospital.
250/93 (26)	87	M	45	Atheroma	510	All three coronary arteries show gross calcified atheromatous changes reducing the lumen to 5-10% of normality.	NIL	Found dead in the living room lying on his back in his night clothes.
421/93 (27)	65	M	94	Atheroma	380	LAD: shows 90% stenosis by atheroma.	Headaches-1990 Arthritis-1990 Heavy smoker (50 a day) for most of his life.	Slumped back in the chair throwing both his arms upwards after having breakfast with his wife. Unsuccessful attempts to resuscitate.

429/93 (28)	63	F	109	Hypertensive heart disease	430	LAD: shows 90% stenosis by atheroma	Hepatitis -several years ago	Breathless while on the phone then stopped talking and found in bed in a seated position slouched over to one side.
440/93 (29)	72	M	43	Thrombosis	530	All 3 coronary arteries contain severe calcified atheroma with multiple foci of organised thrombi and plaque haemorrhage and multiple narrowing to a pinhole bore.	Spermatocele-1990 Depression-1992 Medication: Sinequan (anti-depressant)	FDIB by his daughter.
441/93 (30)	90	M	46	Atheroma	550	All 3 coronary arteries are severely narrowed by atheroma with old calcified plaques.	Angina-1970 Adenocarcinoma of the rectum (removed by operation)-1977 Medication: Glyceryl trinitrate	FDIB by his GP at home.
477/93 (31)	69	M	24	Thrombosis	550	LAD: thrombus	Hypertension and "Stroke"-1984 "Prostrate trouble". Medication: Oxybutin for prostate Adalat Retard	Found dead sitting in a chair in his garden.
481/93 (32)	70	M	18	Atheroma	580	RCA: 80% narrowed LAD: 90% narrowed LCXA: 70%. All by atheromas.	NIL	FDIB at home by his wife.

483/93 (33)	70	F	89	Thrombosis	480	LCXA: completely occluded by thrombus. RCA & LAD: show 70%-80% narrowing by atherosclerotic change.	Blind-13 years Arthritis "Stomach problems" "Stroke" 18 months prior to her death.	FDIB by her neighbour.
485/93 (34)	73	F	94	Thrombosis	360	RCA & LAD: 90% narrowing. Occlusive thrombus in the RCA. LCXA: 70% narrowing by atheroma.	NIL	Found dead sitting in living room.
511/93 (35)	80	F	90	Thrombosis	400	LAD: shows acute thrombosis at about 2cm from its origin.	Arterial disease - long standing. "stroke"-1984	Feeling dizzy and breathless and immediately expired.
513/93 (36)	86	F	46	Atheroma	430	LAD: reduced to a pinhole size lumen by atheroma.	Hip replacement-1990 Late onset asthma-two months prior to her death.	FDIB
515/93 (37)	75	M	46	Atheroma	470	RCA: shows 80%-90% narrowing.	Stroke-1989 Anxiety-1993 Medication: Molipaxin & Nitrazepam.	Pain in Rt. groin and later found unconscious on the bathroom floor.
524/93 (38)	83	M	21	Atheroma	400	LAD&LCXA: show 90% narrowing by calcified atheroma.	Chronic Bronchitis-1968	Chest pain Sudden fall onto the ground while walking, followed by vomiting. Died after unsuccessful resuscitation attempts.

531/93 (39)	80	M	91	Atheroma	630	LAD: 90% narrowing by atheroma.	Hypertension Angina many years prior to death. Medication: Frusemide, Metprolol, Isosorbide mononitrate, Frumil & Nitrolingual spray.	Thought to be asleep in the taxi while going home.
555/93 (40)	64	F	55	Atheroma	580	RCA & LAD: show 80%-90% narrowing by atheroma.	Angina-1991 "Heart attack"-1991 Bronchitis-1993	Breathless, turned a 'funny colour' and started gasping for breath; died at home.
557/93 (41)	75	M	70	Atheroma	430	RCA & LAD: show 90% narrowing LCXA shows 80% narrowing by atheroma.	Rheumatic fever-1930 MI-1986 Polymyalgia Rheumatica-1992 Medication: Prednisolone, Amiloride, Lasix and Glyceryl trinitrate.	Sore back the night before death. Found dead lying on the floor of the living room.
560/93 (42)	61	F	62	Atheroma	500	RCA: 90% narrowing by calcified atheroma.	Duodenal ulcer-1974 Vagotomy-1974 COAD-1978 Partial gastrectomy-1982 Iron deficiency anaemia-1988 Pelvic fracture-1992 Pulmonary embolism-1993	Breathless then speechless later cardiac arrest upon arrival in hospital

572/93 (43)	69	M	48	Thrombosis	410	RCA & LAD: show 90% narrowing LCXA: show 80% narrowing LAD is also occluded by a thrombus.	Hiatus hernia	Chest pain (thought by his wife to be due to his hiatus hernia) later fell down and seen by wife collapsing dead in the living room.
573/93 (44)	71	F	61	Atheroma	350	LAD: shows 90% narrowing. LCXA: shows 80% narrowing.	"Fluid in Knee"-1989	Dizzy, sick and chest pains one day before death. Found dead in bathroom by husband.
574/93 (45)	58	M	89	Atheroma	580	RCA & LCXA: show 90% & 80% narrowing by atheroma respectively.	Angina & IHD-1973 "Blocked arteries" in back & buttock-1990 Hypothyroidism-1992	Indigestion & heartburn then drove his car and collapsed on the wheel.
577/93 (46)	53	M	34	Atheroma	530	All 3 coronary arteries show 90% narrowing by atheroma.	Angina & stroke-1989 <u>Medication:</u> Adalat Retard "Angina spray"	Found by his son slumped in the chair while watching TV and died.

Table 6.6 Pilot Cases

Case No.	Age (in years)	Sex M/F	Post mortem interval (in hours)	Cause Of Death	Heart Weight In grams	Coronary Arteries	Medical History	Events Relating To The Time of the Sudden death:
100/92	39	M	47	Inhalation of car exhaust fumes	530	All coronary arteries are widely patent.	Polyarthropathy-1984 Renal colic-1986 Pain Rt. testicle-1992	Seen inside his car unconscious with engine running and a hose from the exhaust into the vehicle.
102/92	47	M	34	MI	400	RCA: shows 70% narrowing.	Mental illness-11yrs Overdose1970 & 1977	Found dead lying face down in the bathroom.
120/92	88	M	48	Atheroma	520	LAD & RCA: pinhole bore at various points by foci of haemorrhage and organised thrombi.	Intermittent Claudication-1990 Angina-1991 Breathlessness-1992	Collapsed on the foot -path, blue in the face and failed to respond to resuscitation.
139/92	66	M	48	Thrombus	440	All 3 coronary arteries: show severe atheroma reduced to pin hole lumen. In addition LAD: show fresh thrombus.	Stomach cancer (Operated)-1992	FDIB
141/92	23	M	52	Hanging	380	All coronary arteries widely patent	knee injury–1992 Depression-1992	Found dead lying on with an electric flex wrapped twice around his neck and knotted into a tight noose.

142/92	80	F	50	Atheroma	360	RCA & LAD: contain severe complicated atheroma, reduced to pinhole bore.	Hypertension MI-1967 Chronic Bronchitis-1979 <u>Medication:</u> Nifedepine Ventolin tablets Aspirin Mefanemic acid	Intermittently breathless, few minute later stopped breathing and collapsed dead.
143/92	25	M	55	Road traffic accident	340	All coronary arteries widely patent	Manic depressive illness-1988 <u>Medication:</u> Carbamazepine	Found dead by flat mate lying on his back on the floor.
144/92	45	M	35	Inhalation of car exhaust fumes	350	All coronary arteries are widely patent	NIL	Found lying on the ground at the entrance to his garage, his face was close to the car exhaust pipe with car engine running.

CHAPTER 7.

RESULTS.

7.1. Controls.

The case chosen as a non ischaemic control showed no abnormalities such as contraction bands, loss of striations etc., which indicated the lack of intrinsic pathology. Furthermore, in all the sections of the hearts examined with the exception of group A (MI) cases, normal cardiac myocytes were present and these acted as an in-built control with an identical staining pattern. All the immunoperoxidase labels antibodies performed adequately; this was also confirmed in the sections from the test cases in that normal areas of myocardium within these sections stained in a similar fashion to that of the normal controls. It was observed that over 95% of the normal myocytes showed the expected staining reaction; with the other staining procedures the other myocytes showed a slightly less pronounced intensity of staining. The peroxidase-labelled lectins gave a similar, appropriate and universal staining of myocytes.

In the sections taken from the hearts of those dying of well-established myocardial infarcts, over 90% of the myocytes within the infarcted areas showed the expected changes in staining reaction to both the panel of antibodies and of the lectins. In those myocardial fibres in which the staining pattern was not completely altered, the residual staining pattern of these remaining fibres was still abnormal. These fibres, which were invariably closely admixed with the appropriately staining myocytes, still showed a variation in the expression of their staining response; some of these fibres showed a patchy loss of their intra-cytoplasmic staining, and in others the staining was focally depleted or less sharp and clearly delineated, producing a ghost-like outline of the cell.

At the edges of the infarcted area there was a gradation from normality to the fibres which were depleted of staining indicating that in this area for a variable distance some damage due to ischaemia was occurring. The changed staining pattern in these particular muscle fibres was altered, but the damage which appears to be taking place was not of the same degree as that in the infarcted zones.

The microscopic findings for each of the antibodies used and for each of the lectins are described and illustrated below.

7.2. Serial sectioning.

It was possible to match the areas of abnormal staining between individual antibodies and lectins. An attempt was made to assess this further by attaching a drawing arm to the microscope and sketching out the abnormal areas. This method was however abandoned as proved to be laborious and gave no additional information other than that could be assertained by simple microscopical examinations. No attempts were made to analyse the material further by morphometric methods. Comparison of sections with a comparison microscope confirmed that there was a close match between abnormally stained areas as identified by individual antibodies and lectins.

7.3. Antibodies: Qualitative findings in each of the antibodies.

7.3.A. A-CAM/N-Cadherin (antibody to cell adhesion molecules).

In the normal myocytes there is specific staining of the intercalated discs which appear as dense regular slightly serrated bands extending across the myocytes. The striated cytoplasmic structures of the myocytes can be identified but there is no nuclear or membranous staining and no other cytoplasmic staining. Staining of connective tissue cells or blood vessels is not present.

With the development of infarction complete loss of the specific staining at the intercalated disc sites is observed even though in some of the fibres transverse striations can still be observed. In the 'intermediate' ischaemic areas, the intercalated discs staining is no longer well demarcated; it diffuses out into the cytoplasm producing a wider more irregular and less deeply stained bands with complete dissipation of the staining and complete disappearance in frankly ischaemic fibres. In some of the fibres there appear to be diffusion of the staining material into other portions of the cytoplasm at some distance from the intercalated disc often accumulating as particulate material within the cytoplasm; on occasions these foci of

staining are immediately related to (and adjacent to) the sarcolemmal outer membrane.

7.3.B. Actin.

Cardiac muscle cells are uniformly stained with the staining localised specifically to the cytoplasm. The staining is diffuse and granular with a specific arrangement into both transverse and longitudinal striations in a large majority of fibres. There appear to be no staining of the nuclear membrane. Under high magnification the staining within the cytoplasm shows a range of multi-tubular pattern. There is sparing from staining of the peri-nuclear zone. Focal linear staining of the outer cell membrane is identified in a number of the fibres. The intercalated discs are not staining and there is also no staining of the connective tissue cells. As is to be expected some of the smooth muscle cells in the smaller venules and arterioles are also positively stained with some staining of the cytoplasm of occasional endothelial cells. The inter-fascicular connective tissue fibres are not staining and the capillary walls are also not stained.

In the frankly infarcted areas there is very marked diminution in the intensity of staining within the damaged myocytes. Any semblance of myofibrils and striations are lost with the cytoplasm which then show irregular and non descript staining, which is also to a large extent granular rather than tubular. The membrane on the surface of the damaged myocytes shows focal linear accentuation of the staining.

In the ischaemic areas the changes seen appear to reflect an alteration in cytoplasmic architecture. Some residual features of striations remain but they are completely obliterated as the ischaemic damage becomes more pronounced with loss of the nucleus and with peri-fibre infiltration with polymorphs. The membrane staining is retained but it is less obvious and continuous in comparison with normal fibres. The membrane staining also shows a tendency to become irregular and undulating, in some areas the staining of it is more pronounced than the rest of the cytoplasm.

The distribution of the changes in the fibres indicate that there is a variation in the actual changes observed within individual muscle fibres from cell to cell in that normal fibres and fibres showing only a little diminution in their staining intensity, are intimately intermixed with fibres which are showing more frank ischaemic

damage with loss of the staining pattern suggesting that varying stages and degrees of ischaemia are reflected in the staining pattern for actin.

7.3.C. α-Actinin.

In the normal myocytes there is specific staining 1) of intercalated discs, 2) of the cross striations-which appear as dense regular bands extending across the myocytes, and 3) of linear outer membranous staining. There is no myocyte nuclear staining and of no other cytoplasmic components; blood vessels or connective tissue are not stained.

In the cases of developed infarcts with necrosis there is complete loss of the specific staining at the intercalated disc sites and there is also no staining of the cross striation. A very faint and patchy diffuse cytoplasmic staining remains and in some places the striations can still be observed.

In the "ischaemic areas" there is linear discontinuous fine staining of the sarcolemma. The cross striations appear as 'ghosts' or are absent. The intercalated discs showed fine particulate staining with focal accumulation of the stain along them.

7.3.D. Desmin.

Normal cardiac myocytes stained with desmin show a very prominent pattern of staining and both the longitudinal and the transverse striations are easily identifiable giving the fibre a very pronounced striated pattern. The nuclear membrane is not stained and in the peri-nuclear zone an area full of staining is identifiable. In a number of the fibres the intercalated discs are prominently and deeply stained, appearing as dense wide bands in which some focal accentuation of the stain along the man axis of the fibres is obvious. The surface membrane of the cells does not appear to stain except very focally and in these areas the staining shows a very thin flimsy linear pattern. There is no staining of blood vessel walls and of the connective tissue cells.

In the frankly infarcted areas there is gross diminution in the intensity of the staining in a large proportion of the fibres. The fibres which are stained also show a marked

irregularity in the staining pattern which although still granular, has a pronounced vacoulated floccular appearance. The intercalated discs also lose their staining pattern.

This loss of the staining shows a gradation between fibres with almost intact staining, to fibres which have completely lost any staining whatsoever within them. The striation pattern is also lost and there also appears to be loss of staining of the outer cell membrane.

In the area of early ischaemia fibres show similar changes to those exhibited towards the periphery of the infarcted zones. Some fibres in these cases (ischaemic group) appear to retain at least focally the striation pattern and it appears that this gradually fades out with staining retained in the intercalated discs but of lesser degree of intensity. Focally within some smooth muscle cells there is some staining but there is no endothelial staining.

7.3.E. & 7.3.F. Myoglobin & Myosin.

The staining for these two protein constituents proved to be problematic for the following reasons:

1. in the first instance it was difficult to find a commercially available antibody which reacted specifically and consistently with the formalin-fixed tissue sections.
2. once the sections were stained there appeared to be no specific pattern by which one could identify the three areas of major interest namely: frankly infarcted, frankly normal and the ischaemic zones.
3. the delineation of membranes and cytoplasmic contents was very non-discriminatory with diffuse intra-cytoplasmic staining and no ability to distinguish intra-cytoplasmic components.

7.3.E. Myoglobin.

The damaged myofibres show very irregular edges with fraying of their membranes, releasing of intra-cytoplasmic content into the adjacent interfasicular connective tissue; as a consequence the muscle fibres showed a variegated appearance with decreased staining and overall moth-eaten appearance.

7.3.F. Myosin.

In the positively stained myocytes there is patchy linear staining of the membrane and a meshwork representing striations staining as a fine linear pattern with accentuation at the points where the striation overlapped, an in-built control is the staining of the smooth muscle cells with arterioles and venules. The pattern of change with ischaemia is that the striation pattern is lost, the level of staining is decreased, and the cell membrane staining is less pronounced. In isolated cells there are vacuoles with no positive staining within them occupying various proportion of the cytoplasmic volume occasionally up to half of the volume, in a few cells the vacuoles are multiple. One possible explanation for these may be inadequate penetration of the fixative in these the change is not uniform or in any way related with specific areas within the myocardium or a specific foci of cellular infiltration. It is suggested that this change is likely to be artefactual.

7.3.G. Tropomyosin.

The normal myocytes show a very pronounced intra-cytoplasmic, regular, reticulated staining pattern represented as thick transverse striations and thinner longitudinal striations throughout the fibres, with some accentuation at the cross-over points of the striations. There is no staining of the peri-nuclear membrane or of the nuclei; thus the nuclei appeared to be suspended within a basket weave pattern of the fibrils in some of the cells.

There appears to be no specific staining of the cell membrane or of the nuclear membrane. The connective tissue cells i.e. the fibroblasts also show diffuse intra-cytoplasmic staining and similarly the smooth muscle forming blood vessel walls. There also appears to be some staining of the endothelial cells of all blood vessels, including capillaries. Occasional mononuclear inflammatory cells, probably macrophages, also show some positivity. No staining of intercalated discs was observed.

In the frankly infarcted area there is loss of the patterned staining which in some cells residual fibrils remaining as 'ghosts' with only very minimal and diffuse non-discript staining within the cytoplasm.

This loss of staining intensity and pattern can also be identified in ischaemic fibres in areas where frank infarction cannot be seen. In some of these fibres focal but less well stained patterned striations are identifiable, in others, the loss of staining is complete with loss of delineation as well as of intensity.

7.3.H. Troponin T.

The staining of the myocytes in contrast to smooth muscle staining is generally less pronounced and intense in these sections. In normal fibres with the exception of the peri-nuclear area, there is diffuse particulate staining involving the entire cytoplasm. A tendency to alignment of the staining pattern into ill defined broad bands was noted but true striations are only identifiable in a few of the fibres and when striations are present these are mostly arranged in a longitudinal direction. There are spaces within the cytoplasm which also appeared to be devoid of stain. Focal linear accentuation of the stain is observed at the cell membrane but this continues in most normal fibres and there is no staining of intercalated discs. The endothelium of blood vessels including capillaries, is not stained, but some of fibroblasts and inflammatory cells show some intra-cytoplasmic staining.

In the frankly infarcted areas the degree of staining intensity is diminished and the staining pattern becomes irregular and disorganised with large areas of myocytic cytoplasm appearing to be bereft of staining. Some of the muscle fibres show a diffuse peppering of positively staining particles. Membrane staining is however to a large extent retained.

In the ischaemic zones the ischaemic fibres show a gradation from normality to infarct appearances and there is admixture of fibres which are almost normal, with fibres showing varying degrees of depletion of positive staining.

7.3.I. Tubulin.

The staining pattern is not very specific in that there is positive staining of blood vessels, connective tissue, nerve bundles as well as of the myocytes. In the blood vessels both the endothelial lining (including that of the smaller blood vessels and capillaries) and smooth muscle cells are staining positively. Neural bundles show diffuse staining throughout. The connective tissue elements show the presence of positively staining inflammatory cells as well as the occasional fibroblasts.

Penetration of the antibody into normal muscle fibres appears to be limited with the peripheral fibres showing a much more accentuated staining pattern than elsewhere. The nuclei within the muscle fibres are not staining and the staining pattern is to a large extent is not patterned and diffuse. Few fibres show a semblance of striation. The staining is diffuse throughout the cytoplasm with a focal accentuation into particulate staining with some tendency to focal staining in a linear fashion of the surface membrane. Red blood cells are also positively stained.

In the infarcted zones although a diffuse non-patterned staining of the cytoplasm persists together with a linear membranous staining on the surface, there is focal accentuation and accumulation of the stain into parts of the cytoplasm with no consistent pattern of striation in relation to the nuclei or other aspects of the cell. Frankly necrotic fibres still retain this pattern but show a glassy almost hyalinised appearance of the cytoplasm.

In the areas of ischaemia there is no specific and recurrent alteration identified. The changes present indicate that some damage has taken place but adequate discrimination of these areas from adjacent, presumably normal, fibres is largely impossible.

7.3.J. Vinculin.

The normal non-ischaemic myocytes show three foci of staining. The area of the myocytes which is picking up most of the stain is the intercalated disc, which shows up as a band of closely related, densely packed, speckled, punctate staining arranged transversely across the muscle fibre. There is also faint almost exclusively transverse, striation staining within the myocytes; this is not a universal finding. The outer cell membrane also shows focally pronounced discontinuous linear staining. The nuclei and the nuclear membrane do not stain. In the connective tissue in-between the muscle fibres there is positive staining of the blood vessels which appears to involve both the endothelium and muscle. Some of the fibroblasts and the macrophages also show positive cytoplasmic staining.

Occasional myocytes contain within their cytoplasm portions of staining composed of accumulation of stain. Only one such fibre of every 30 or 40 fibres is noted in the controls. The significance of this is not apparent, in that frankly infarcted and

ischaemic fibres do not show a similar change. This may represent a non-specific agonal phenomenon.

The changes seen in the infarcted areas are loss of discrete staining of the intercalated discs with hardly any, or no, staining, and diffuse fine particulate staining throughout the cytoplasm with focal accentuation, mostly beneath the cell membrane. The staining of the cell membrane is less pronounced and more discontinuous and irregular. Some of the material in between the muscle fibres which are damaged show punctate extra-cellular staining. This is difficult to interpret and may to some extent be an artefact. The vascular staining is also less pronounced and more irregular and diffuse.

7.3.K. $C5_b$-9 (antibody to membrane attack complex).

The normal myocardium shows no positive staining except in the walls of the larger blood vessels and this is mostly seen in the small muscular arteries and arterioles within the myocardium. A few capillaries and venules show patchy staining of the endothelium and similarly some histiocytes. The connective tissue cells and fibres do not stain.

In the frankly infarcted areas where polymorph neutrophils can be seen in the immediate vicinity of the damaged muscle cells, these fibres show diffuse intra-cytoplasmic staining of a punctate type with focal membrane staining. The cytoplasm of some of these fibres show a gradation of staining from very patchy irregularly distributed staining to one involving the entire cytoplasm.

By and large in the ischaemic zones with no other features of infarction, very occasional muscle fibres show similar changes with the same changes in gradation of the staining pattern. These fibres are isolated and are rarely seen in clumps, and as such their value at discriminating totally normal areas from partially ischaemic areas is not found to be very high.

The blood vessels in the infarcted areas and to a lesser extent in the ischaemic areas show some diminution in the intensity and the distribution of staining of their endothelial lining. The capillaries remained unstained throughout.

7.3.L. CD59 (antibody to human Protectin).

The normal muscle fibres show a striated or a finely particulate staining pattern which is exhibited throughout the cytoplasm with no cell membrane or nuclear staining. There is no vascular or connective tissue staining.

In the frankly infarcted areas most of the intra-cytoplasmic staining is lost, and if any staining is retained, this is diffuse and composed of very fine particles diffusely distributed with no striation pattern and a much decreased intensity than in normal fibres. In the fibres which are totally necrotic the staining appears to have faded completely and in these areas occasional inflammatory cells stain positively.

In the ischaemic zones there is an intermediate pattern of staining with a decreased intensity and loss of the striation pattern. There is no staining of capillaries or other blood vessels.

7.3.M. CD34 (antibody to human endothelial cells).

This marker appears to pick out specifically the endothelial lining of any blood vessels particularly capillaries and shows up as diffuse endothelial cytoplasmic staining. The smooth muscle cells in the larger vessels do not stain and similarly the cytoplasm of the cardiac myocytes. As a consequence the intricate plexiform network of blood vessels in between the myocardial cells shows a very pronounced staining pattern. In some areas it is difficult to distinguish between the surface membrane of the myocytes and the closely adherent extensions of the endothelium of capillaries in the close vicinity of the muscle fibre. No nuclear membranes are staining but the collagen fibres in some areas appear to be picking up the stain. The red blood cells do not stain.

In the infarcted areas the capillary network is, as expected, disrupted and in quite a number of areas the endothelial staining looses its specific alignment. It also becomes more dense and particulate with extrusion of positive particles into the adherent connective tissue. In some capillaries there appears to be collapse of the endothelial lining of the vascular wall to produce the reticulated staining pattern to represent where the capillary had been with loss of its lumen and extrusion of the particulate material in what may remain of the lumen.

In the ischaemic zones the staining of the capillaries is less well pronounced and some tendency to irregularity of staining with distortion of the lining is noted. The loss of endothelial particulate staining into the surrounding connective tissue is only observable in frankly infarcted zones and not in the ischaemic zones.

7.4. Semi-quantitative assessment of the staining response.

The qualitative changes which have been described above were assessed in a semi-quantitative manner in all the 46 ischaemic (Group C) cases. There was some variation in the staining pattern between individual fibres in many of the sections but an overall picture could be readily obtained in all cases in that most of the abnormal fibres within the abnormally staining areas showed showed an identical variation.

7.4.A. Method of grading the observations.

The changes that could be identified in the ischaemic zones were graded as follows:

if the section contained fibres which stained *identically to normal fibres* , the section was graded 0,

if the section contained fibres which stained with *minimal variation from normality* , the section was graded 1,

if the section showed *more pronounced variation* from normality, in the staining pattern the section was graded 2,

if the section showed *very pronounced variations* from normal but *not to a level of alteration as seen in an infarct*, the section was graded 3.

The results of these studies are shown in table 7.1.

7.4.B. Interpretation of the grading of the antibody staining.

Overall variability.

There was a significant variation in the extent of the staining reaction to antibodies directed at cardiac myocytic components between the 13 antibodies used and between the 46 ischaemic cases. This analysis is summarised in table 7.2.

	Friedman Statistic	d.f	
Antibodies	49.8	12	$p< 0.001$
Cases	233.5	45	$p< 0.001$

Table 7.2. Test for variability in Antibody Staining.

The method by which the 46 ischaemic cases were selected may possibly account for this in that although there was an expectation that ischaemic changes were to be observed in each one of these test cases, the degree of ischaemic damage-if any-was an unknown quantity and could not be delineated in any way. The duration and severity of the ischaemic period experienced by the muscle fibres tested could not be measured in any of the cases. The degree of staining variation from normality may be a function of this ischaemic duration but in the human situation, the completeness and length of the ischaemic episode cannot be objectively measured, as it is not even possible to identify the time when it had commenced let alone whether ischaemia operated throughout this period. The severity and duration of myocardial ischaemic episode are also not measurable by any biochemical or other retrospective means. The damage caused by a period of ischaemia is a function of the severity and completeness of the occlusion, the rapidity of onset of the episode, associated cardiac tachy-arrythmias and also the presence of arterial collaterals to prevent.

In the various animal models which have been used it is possible to time accurately the ischaemic episode and to carefully monitor it; it may be possible to correlate the changes in the immunoperoxidase staining reactions with the duration and severity of the ischaemic interval in such experimental models however work of this type was outwith the scope of this study.

Comparison of individual antibodies.

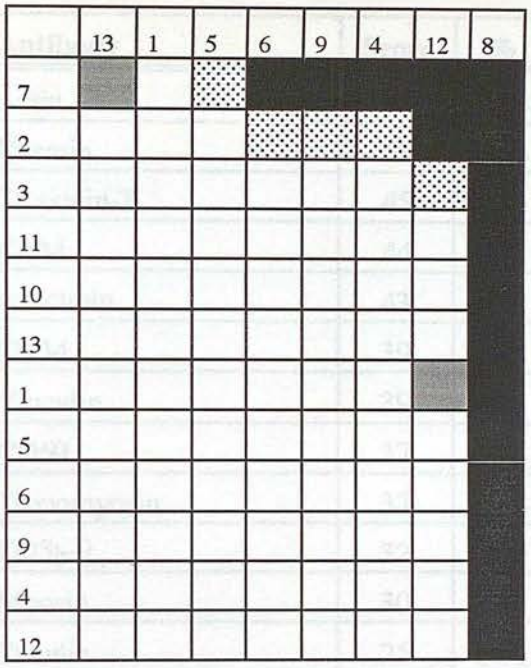
The data in table 7.2 indicate that there was a significant difference in the performance of the individual antibodies used in this study. The matched pair sign test statistic was calculated for these data in order to establish whether the observed differences reached statistical significance. A series of t tests (using the Friedman mean ranks) were also performed, these tests are less robust because of the non-parametric nature of the data.

It is evident from table 7.3 (and the associated key) that antibody 8 (myoglobin) was significantly less effective in highlighting ischaemic change than all the other antibodies tested. However, only desmin (7) and actin (2) produced results significantly better than other antibodies.

These data indicate that an antibody panel rather than a single antibody is more likely to produce significant assistance in actual cases and that the choice of antibodies to form such a panel has to be made with care.

Interpretation of the Total Grade Scores for the antibodies.

The maximum obtainable score for each case would be 13x3 = 39, and with such a score the staining pattern would have to have been very close to that expected of an area of acute infarct. Table 7.4 shows the actual scores (Σ grades) for the 13 antibodies in the study.



Test for significant difference	p<0.05
Matched pair sum test	
t test (Friedman mean ranks)	
both tests	

Table 7.3 Summary of significant differences between pairs.

1	CAM	6	CD34	11	Troponin-T
2	Actin	7	Desmin	12	Tubulin
3	α-Actinin	8	Myoglobin	13	Vinculin
4	$C5_b$-9	9	Myosin		
5	CD59	10	Tropomyosin		

Antibody	Score	%
Actin	53	38.4
Desmin	53	38.4
Troponin-T	45	32.6
CAM	44	31.9
α-Actinin	43	31.2
CD34	39	28.3
Vinculin	39	28.3
CD59	37	26.8
Tropomyosin	37	26.8
$CD5_b$-9	32	23.2
Myosin	30	21.7
Tubulin	25	18.1
Myoglobin	12	8.7

Table 7.4 Total and Percentage Scores for the Antibody Markers.

It will be observed from table 7.4 that the changes from normal staining patterns in these ischaemic cases are relatively subtle. The results of this study suggest that ischaemic damage manifests itself as a slight decrease from the staining distribution and intensity as might be expected in normal myocardial fibres rather than approaching the changes associated with acute infarction. However, as demonstrated by this study, the slight changes resulting from an ischaemic episode are identifiable. In an actual case in which these antibody studies are being performed, assuming identical laboratory conditions and reagents, a pattern of ischaemia should be identifiable if present. If no change in staining pattern is observed (as in case numbers 40 and 41) it cannot be said that no ischaemia is present but simply that the probability of its being present is reduced in an objective test.

7.5. Qualitative findings in each of the lectins.

7.5.A. Con A (Concanavalin A).

The normal myocyte shows a diffuse staining of the cytoplasm. This is mostly bland but in some fibres cross-hatching by striation is noticeable and in others there is focal particulate accumulation of stain, largely within the central part of the cell. The nucleus shows peri-nuclear staining likely to represent the nuclear membrane but this is very fine and difficult to visualise, except under oil immersion. The outer cell membrane of normal myocytes show a continuous linear staining surrounding the entire cell. In areas this has a regular wavy appearance and in some cells it has a distinct double, linear appearance that can be visualised under oil immersion. There is no staining of intercalated discs, but the blood vessels show surface and nuclear staining, and similarly the membranes of red blood cells and of inflammatory and of connective tissue cells.

In infarcted zones the membrane staining is less pronounced and for a large portion of the perimeter of the infarcted myocytes it is deficient and shows no waviness or double layering. The diffuse and striated staining within myocytes is also lost, but focally. The other parts of the sections stain as normal areas. The general intensity of staining is depleted throughout the infarcted zone.

In the ischaemic zones, although the fibres seem to be morphologically intact, they exhibit focal losses in their outer lining membranes where pore-like areas of disruption are visible under oil immersion. These areas are not specific to any portion of the cell and seem to affect individual cells differently.

7.5.B. DBA (Dolichos Biflorus Agglutinin).

Cardiac muscle fibres show both membrane and cytoplasmic staining. The membrane staining is thin and linear, and follows the contour of the cell. However not every part of the cell membrane is staining. The cytoplasm shows diffuse staining which is accentuated to produce a stippled appearance, which involves the entire area of the cell more or less uniformly. The nuclear membrane and the inner aspect of nuclei are not staining. There is prominent staining of vascular endothelial

linings and of the surface membranes of red blood cells. There is no uniform connective tissue staining.

In infarcted zones there is depletion of the cytoplasmic staining with most of the cytoplasm showing complete blanching of the staining with only a few stippled remnants of positivity. By and large the cytoplasmic membrane still stains but is much more discontinuous and well-pronounced.

Ischaemic fibres show an intermediate amount of blanching and a less prominent accumulation of staining.

Even in the infarcted areas the endothelial lining still shows well established positive staining.

7.5.C. PNA (Peanut Agglutinin).

The staining pattern in normal myocyte is rather bland but with a diffuse peppering of positivity within the cytoplasm and a rather prominent cell membrane following the outline of the myocyte. The nuclei are not staining. There is some positivity in endothelial linings, on the surface of red blood cells and in the cytoplasm of nucleated white blood cells.

In the infarcted zones, the pattern of staining is similar but is less pronounced than in normal myocytes. The infarcted areas stand out as clearer and less stained areas in the sections.

It is difficult to distinguish appropriately partially ischaemic zones from normal areas.

7.5.D. RCA 1 (Ricinus Communis Agglutinin 1).

This lectin appears to pick up specifically the cell membranes of myocytes which in some areas can be distinguished as double-layered. Cytoplasmic staining is minimal but striations are demonstrable both in longitudinal and transverse sections, in a high proportion of normal myocytes. Endothelial linings and the surface of neutrophils are also stained. No nuclear staining can be identified and there is no specific connective tissue staining.

In the infarcted areas the intra-cytoplasmic pattern of staining is lost, and the membrane staining is much more incomplete and in some areas is completely lost.

The changes in staining is only very minimally changed in the ischaemic zones and these cannot be specifically distinguished as abnormal.

7.5.E. SBA (Soybean Agglutinin).

Staining of membranes is particularly pronounced. The myocyte's cell membrane is well displayed and in some areas appears to be bi-laminar. It surrounds the larger part of the cell. The nuclear membrane is not staining but there is faint intra-cytoplasmic staining which in areas shows either a stippled pattern or in other areas a vague pattern of striation. The capillary lining throughout the myocardium shows a very pronounced linear staining pattern; a similar appearance is noted in venules and smaller arteries, but there was no connective tissue staining.

In the infarcted zones there is a tendency for focal accumulation of stain which is not uniformly or symmetrically distributed throughout the cytoplasm. The outer membrane staining is retained around the myocardial cells but is more patchy, wavy and irregular. The capillary lining is also much less distinct and in a number of areas the network of capillaries cannot be identified in between the muscle fibres. Throughout all the sections, the cytoplasm of the red blood cells is positively staining.

In ischaemic areas, the depletion of the staining that was present was of a degree which was substantially less marked than in areas of infarction.

7.5.F. UEA 1 (Ulex Europaeus Agglutinin 1).

The muscle fibres show a variegated staining pattern ranging from an accumulation of deeply staining vesicular and particulate stain throughout the cytoplasm. There is a suggestion of the vacuolation within the cells in that some rounded intra-cytoplasmic areas are free of stain. The nucleus is not staining, but there is a fine linear staining of the external cell membrane. This is also seen in the larger vessels. In longitudinally displayed fibres there is some tendency to longitudinal and much less marked transverse striations. The vascular endothelium, particularly within the

smaller blood vessels, shows some membrane positivity. Red blood cell membranes throughout are also stained.

In infarcted zones the staining pattern is much less pronounced, however normally staining fibres can be identified mainly at the edges of these zones. The cell membrane is staining patchy in these damaged areas and similarly the endothelial lining is much less pronounced in these zones.

Ischaemic areas devoid of frank necrosis show an intermediate pattern of degree of staining but are often normal and thus indistinguishable.

7.5.G. WGA (Wheat Germ Agglutinin).

The general pattern of staining with this lectin appears to be largely membrane-related with linear staining on both the myocyte's outer surface and on the endothelial lining of capillaries and larger blood vessels and of red blood cell membranes. Patchy, irregular and poorly reproduced cytoplasmic staining is identifiable with a vague striation pattern, particularly pronounced in longitudinally displayed fibres.

Although the intensity of staining appeared to be decreased in zones of infarction, the staining of membranes and the cytoplasm seem to be maintained. This stain was particularly difficult to reproduce and appeared to be very temperamental in its demonstration of myocytes, however endothelial and red blood cell membrane staining could be shown in all slides and preparations.

7.6. Semi-quantitative assessment of the staining response.

7.6.A. Method of grading the observations.

The lectin stains were assessed on the serial sections of the same cases in a manner identical to that for the antibodies and the semi-quantitative data obtained are presented in table 7.5.

7.6.B. Interpretation of the grading of the lectin staining.

Overall variability.

In contrast to the antibodies, the lectins used in this study formed a more homogenous group. There were, however, significant differences between the cases and the same considerations discussed under the antibodies section apply. These data are shown in table 7.6.

	Friedman Statistic	d.f	
Lectins	13.02	6	NS
Cases	152	45	$p< 0.001$

Table 7.6. Test for variability in Lectin Staining

Comparison of individual lectins.

As a homogenous group, the lectins exhibited few differences between the performances of the individual stains. The matched pair sign test indicated that the only significant difference in the lectins was the performance of WGA compared with Con A. Other differences were non-significant.

7.7. Comparison of lectins and antibodies.

The results from the 13 antibodies were compared with those from the 7 lectins to assess whether the results were consistent and whether one panel performed significantly better than the other. The Spearman rank coefficient for non-parametric data was calculated and indicated a significant degree of correlation between the antibodies and lectins ($p<0.001$). This result is as anticipated-the lectins and the antibodies both identified ischaemic changes in the same cases.

The Wilcoxon rank sum matched pair test was also performed, comparing the means for each group. This analysis demonstrated that the antibodies performed significantly better than the lectins (rank sum = 240, normal approximation = 3.283, $p < 0.001$) in aiding the detection of alterations in staining intensity as a result of an ischaemic episode.

7.8. Combined analysis of the ischaemic cases (Group C).

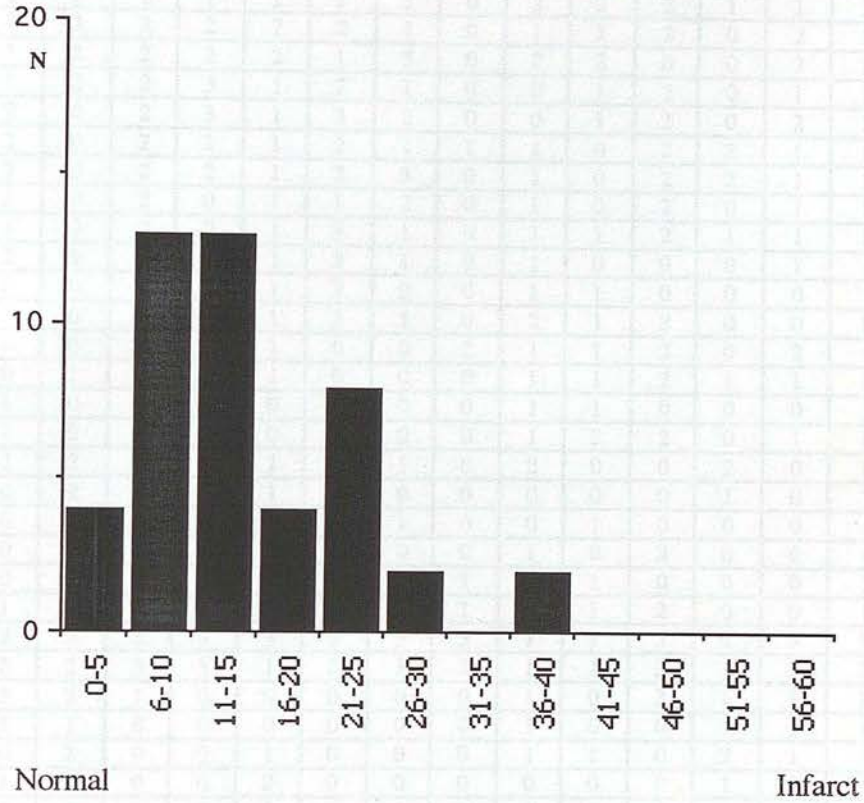


Figure 7.1. Distribution of total (antibody and lectin) scores for Ischaemic cases (Group C)

Figure 7.1 plots the total scores for the 46 ischaemic cases for the antibodies and the lectins. The maximum possible score was 60 (20 stains x 3). A score of zero indicates apparent normality whereas acute infarction is represented by the maximum score. The distribution of scores is clearly shown to be towards the lower end of the score range. These results suggest that ischaemic damage manifests itself as a slight change from the staining distribution and intensity as might be expected in normal myocardial fibres rather than approaching the changes associated with acute infarction. However, as demonstrated by this study, the changes resulting from an ischaemic episode are identifiable. In an actual case in which these studies are being performed, assuming identical laboratory conditions and reagents, a pattern of ischaemia should be identifiable if present. If no change in staining pattern is observed (as in case numbers 40 and 41) it cannot be said that no ischaemia is present but simply that the probability of its being present is reduced in an objective test.

	CAM	Actin	α-actin	C5b9	CD59	CD34	Des.	MyG	MyS	Tm	TnT	Tub	Vnc	Σ
1	1	2	2	2	2	1	2	2	2	2	2	0	1	21
2	1	2	2	1	2	2	2	0	2	2	0	0	1	17
3	1	2	2	1	2	2	2	0	2	2	2	1	1	20
4	1	2	2	2	2	2	1	0	1	2	2	0	2	19
5	1	2	2	2	2	1	3	0	2	2	0	0	2	19
6	1	2	2	3	1	2	1	0	0	1	2	0	1	16
7	2	0	2	3	1	3	2	0	0	1	2	0	2	18
8	2	0	2	2	1	2	1	1	1	0	2	2	1	17
9	2	0	2	2	1	2	3	0	1	0	2	2	1	18
10	1	0	2	0	1	1	2	0	1	0	2	0	1	11
11	3	0	2	0	1	3	1	0	1	1	2	1	1	16
12	2	0	0	0	1	3	1	0	1	0	0	0	1	9
13	0	0	0	0	1	2	0	0	1	1	0	0	0	5
14	0	0	0	0	1	3	1	0	2	1	2	0	0	10
15	0	0	0	0	1	0	0	2	1	1	2	0	2	9
16	0	1	1	2	1	0	0	0	1	1	2	1	1	11
17	1	0	2	0	0	0	0	0	1	1	0	0	0	5
18	1	2	2	2	0	0	0	0	1	2	2	0	1	13
19	2	2	2	2	1	0	1	0	2	0	0	2	0	14
20	0	2	1	2	1	0	0	0	0	0	0	1	0	7
21	0	2	0	0	0	0	1	0	0	1	0	0	0	4
22	0	0	0	0	0	0	0	0	1	0	3	0	0	4
23	0	0	0	0	0	0	1	1	0	1	0	0	0	3
24	1	2	0	0	0	0	1	1	0	1	2	0	0	8
25	2	2	3	3	3	2	3	3	1	1	3	1	3	30
26	2	2	3	3	1	2	3	2	1	1	3	1	3	27
27	2	2	1	0	0	0	0	0	1	0	3	1	1	11
28	2	1	0	0	0	0	0	0	1	0	0	1	1	6
29	0	2	0	0	1	0	0	0	1	1	0	2	1	8
30	1	2	0	0	0	0	0	0	0	0	0	1	2	6
31	0	0	0	0	0	0	2	0	0	0	0	1	1	4
32	0	0	0	0	0	0	2	0	1	0	0	1	1	5
33	0	0	2	0	1	0	3	0	0	0	0	1	1	8
34	0	2	0	0	1	0	1	0	0	0	0	0	1	5
35	2	3	0	0	0	0	2	0	0	0	2	0	0	9
36	1	3	0	0	0	0	1	0	0	0	0	0	0	5
37	3	2	0	0	1	0	1	0	0	0	0	0	0	7
38	2	3	0	0	0	2	2	0	0	0	0	0	0	9
39	2	3	0	0	1	2	1	0	0	0	0	1	0	10
40	0	0	0	0	0	0	0	0	0	1	0	1	0	2
41	0	0	0	0	0	0	0	0	0	1	0	0	1	2
42	1	0	0	0	2	0	1	0	0	2	0	1	1	8
43	0	1	0	0	0	0	1	0	0	1	0	0	0	3
44	0	0	1	0	0	0	1	0	0	2	0	0	0	4
45	1	1	1	0	2	2	2	0	0	2	2	1	2	16
46	0	1	2	0	1	0	1	0	0	2	1	1	1	10
Σ	44	53	43	32	37	39	53	12	30	37	45	25	39	

Table 7.1 Grades for Antibody Markers (Group C -Ischaemic Cases)

CAM	CAM	CD34	CD34	TnT	Troponin-T
Actin	Actin	Des	Desmin	Tub	Tubulin
α actin	α-Actinin	MyG	Myoglobin	Vinc	Vinculin
C5b9	$C5_b$-9	MyS	Myosin		
CD59	CD59	Tm	Tropomyosin		

	Con A	DBA	PNA	RCA 1	SBA	UEA 1	WGA	Σ
1	1	1	1	2	2	1	1	9
2	1	1	1	0	1	2	1	7
3	0	1	0	2	2	2	1	8
4	0	0	1	1	1	0	1	4
5	0	1	1	1	0	0	1	4
6	0	1	1	1	0	0	2	5
7	0	1	2	1	0	2	1	7
8	0	0	1	1	0	1	1	4
9	1	1	1	2	0	1	1	7
10	0	0	1	0	0	1	1	3
11	1	0	0	0	2	0	1	4
12	0	0	0	0	0	0	0	0
13	0	1	0	0	0	0	0	1
14	0	1	0	2	0	0	0	3
15	0	1	0	0	0	0	0	1
16	0	0	0	0	0	1	0	1
17	0	1	0	0	0	0	0	1
18	0	0	0	2	0	0	0	2
19	1	0	0	0	0	1	0	2
20	0	1	1	0	0	0	0	2
21	0	0	0	0	0	0	0	0
22	0	1	0	2	1	0	0	4
23	0	0	0	0	1	0	0	1
24	0	0	0	0	1	1	0	2
25	1	0	2	0	1	2	2	8
26	1	0	2	2	1	2	2	10
27	1	0	0	2	2	1	1	7
28	0	1	1	0	0	1	1	4
29	0	0	2	0	0	2	1	5
30	1	1	2	1	2	1	2	10
31	1	0	0	1	0	2	1	5
32	0	0	1	2	2	1	0	6
33	1	1	0	0	2	1	0	5
34	1	1	0	2	2	1	1	8
35	0	1	1	0	2	1	1	6
36	0	1	0	0	0	0	1	2
37	0	1	0	0	0	0	1	2
38	0	0	1	0	2	0	0	3
39	0	0	0	1	0	0	0	1
40	0	0	0	0	0	0	0	0
41	0	0	0	0	0	0	0	0
42	1	0	1	1	0	0	1	4
43	1	0	1	0	0	0	1	3
44	0	1	1	1	0	0	1	4
45	1	1	1	2	1	2	1	9
46	1	0	1	0	1	1	1	5
Σ	16	22	28	32	29	31	31	

Table 7.5 Grades for Lectin Markers (Group C -Ischaemic Cases)

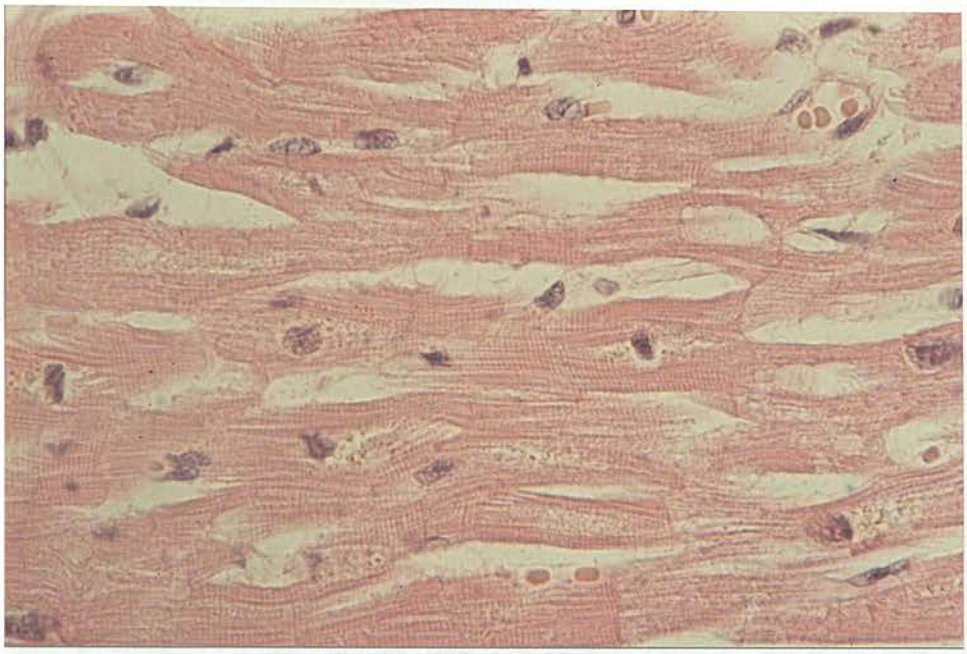


Figure 7.1 X40 H&E–Normal Myocardium.

Normal myocytes with intricate capillary network showing pinkish cytoplasm with prominent striations. Some lipofuchsin can be seen within some of the myocytes. Nuclei are central and prominent.

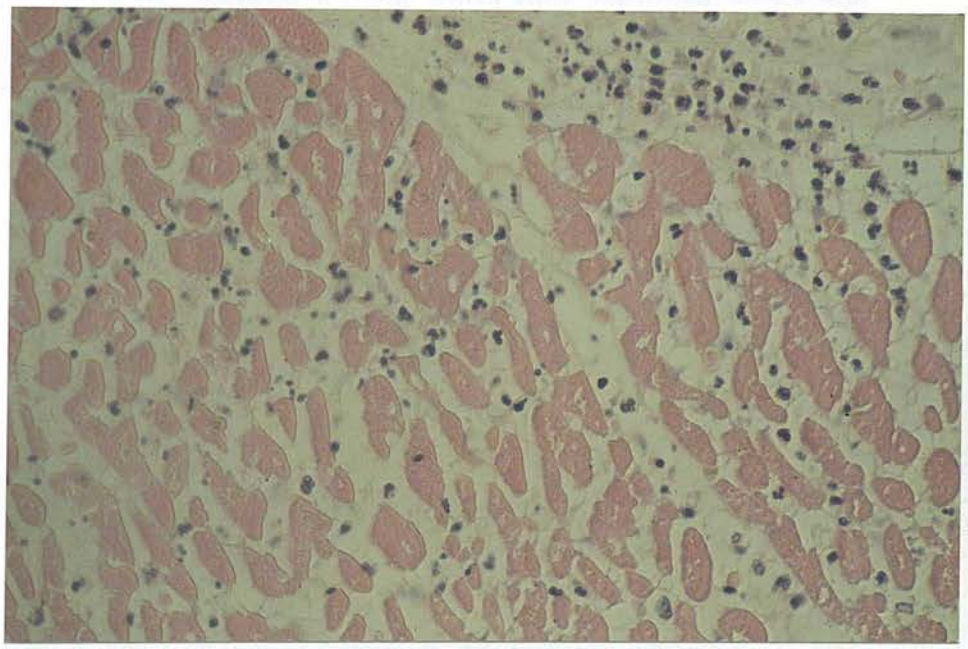


Figure 7.2 X25 H&E–Myocardial Infarction.

Necrotic eosinophilic myocytes show uniformity of staining in the cytoplasm with pyknotic or absent nuclei, inter-fascicular oedema and diffuse polymorpho-neutrophilic infiltration.

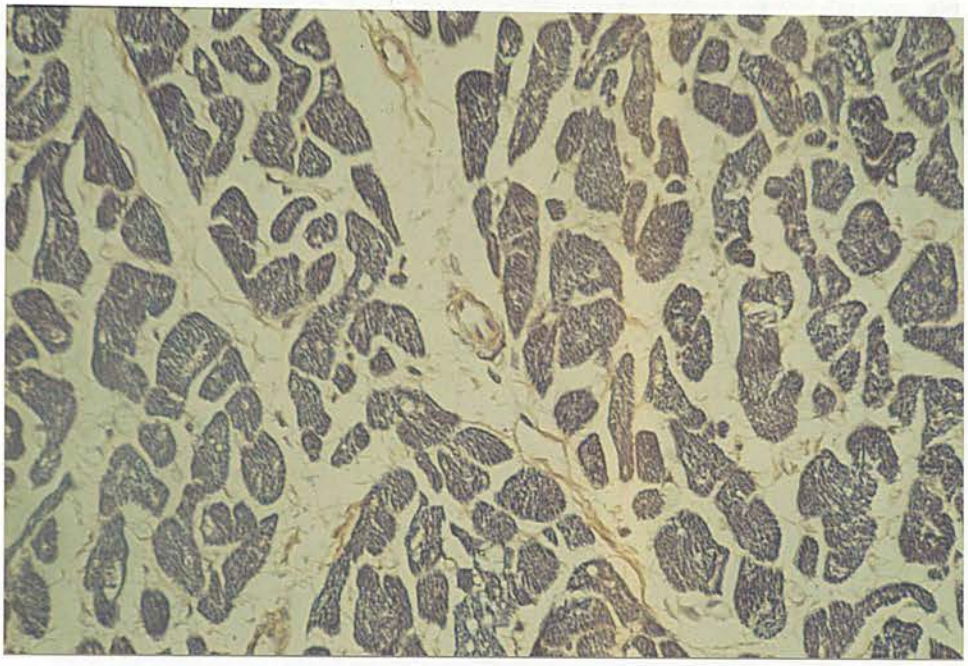


Figure 7.3 X25 PTAH–Normal Myocardium.

Normal cardiac myocytes show the dark blue striations within the cytoplasm. Connective tissue fibres stain yellowish brown and similarly the vascular walls.

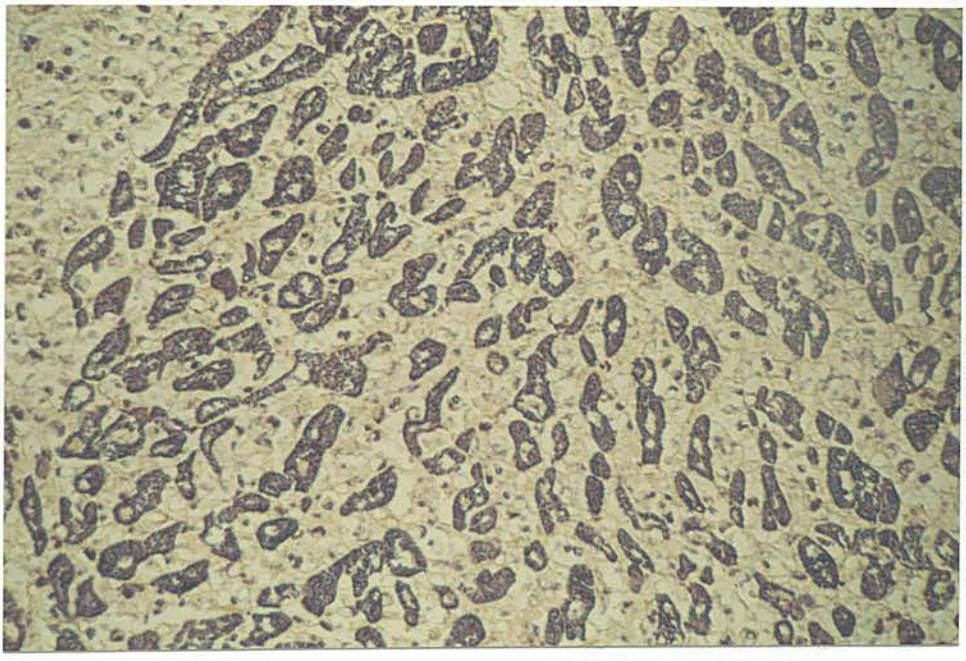


Figure 7.4 X10 PTAH-Myocardial Infarction.

The necrotic myocytes show slightly pale staining cytoplasm.

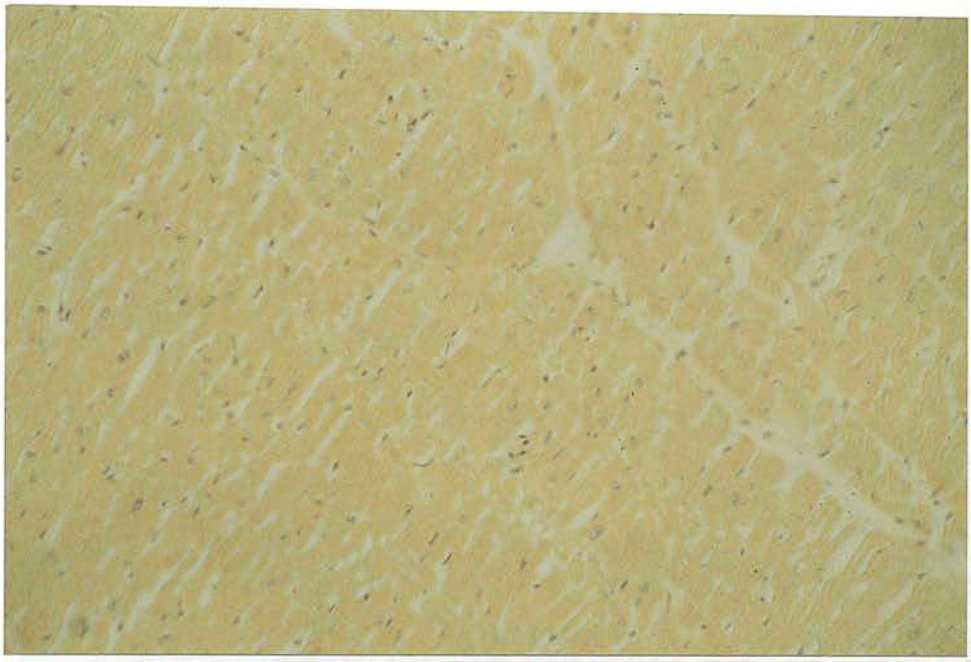


Figure 7.5 X10 Lie's stain-Normal Myocardium.

All myocytes show homogenous brownish–yellow stain. Nuclei are counterstained reddish.

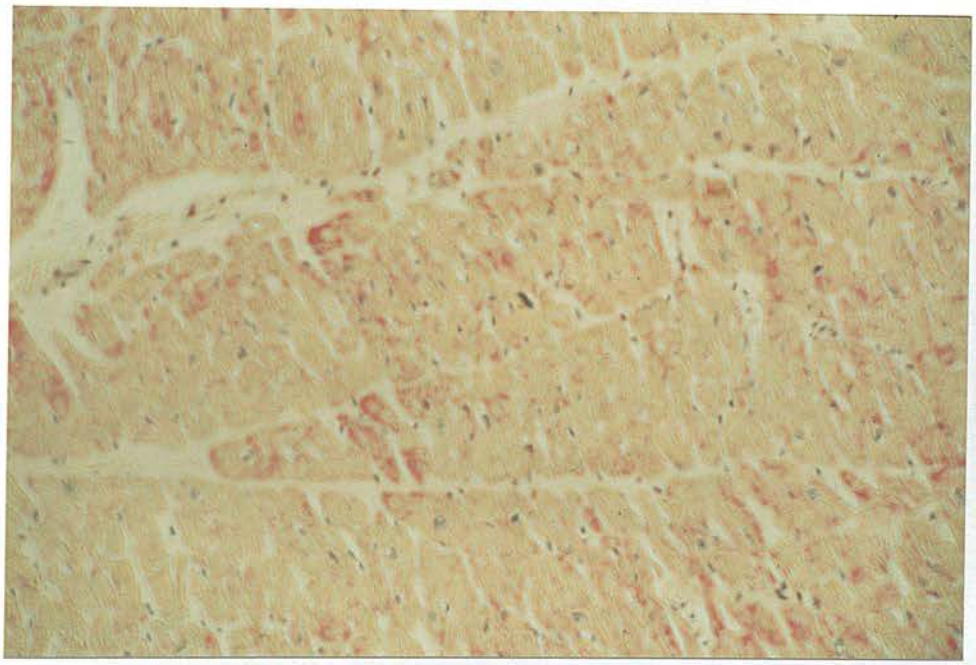


Figure 7.6 X10 Lie's stain-Normal Myocardium.

Normal cardiac myocytes are shown in this field. However there is an admixture of red-coloured (crimson) cells which is thought to be indicating coagulative necrosis. This demonstrates the lack of specificity and reproducibility of this stain.

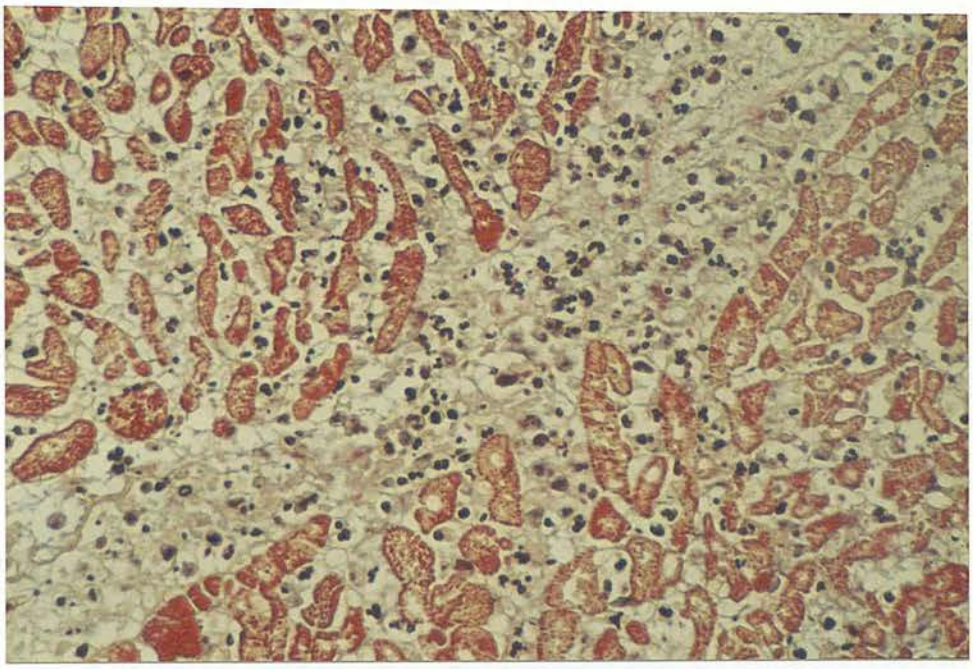


Figure 7.7 X25 Lie's stain- Myocardial Infarction.

Most of the necrotic myocytes show the red-crimson stain (as described in the original literature). Some necrotic fibres still show residual brown-yellow stained material within their cytoplasm.

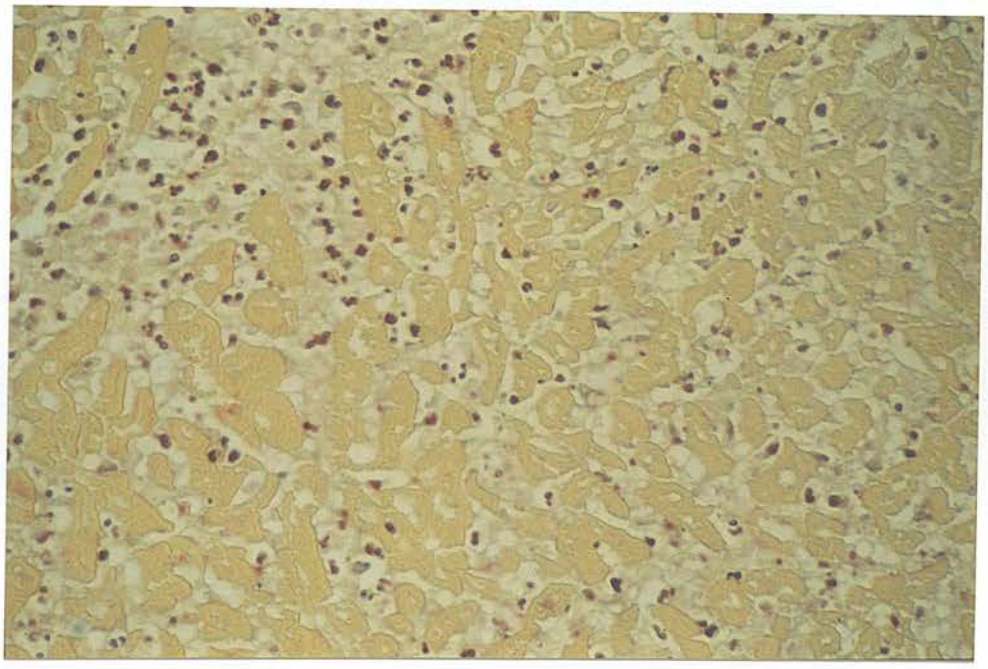


Figure 7.8 X25 Lie's stain- Myocardial Infarction.

This is the same area as in figure 7 but the duration of picric acid differentiation was different by a few seconds. Necrotic myocytes show the same colour as the normal myocytes further indicating the fallibility of this stain.

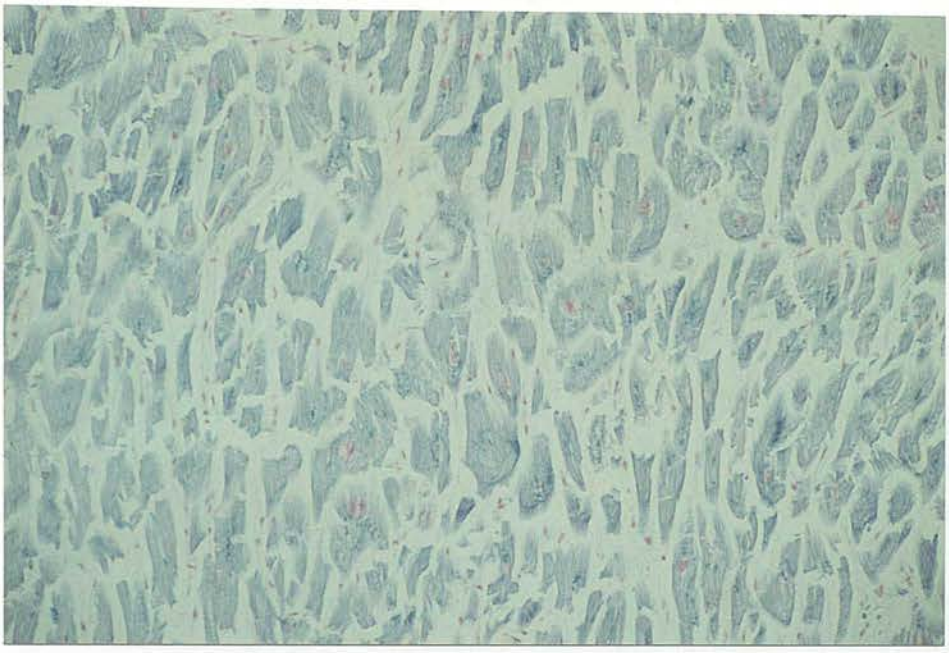


Figure 7.9 X10 Arnold's-modified Luxol Fast Blue (m-LFB) stain -Normal Myocardium.

Normal cardiac myocytes show cytoplasmic staining.

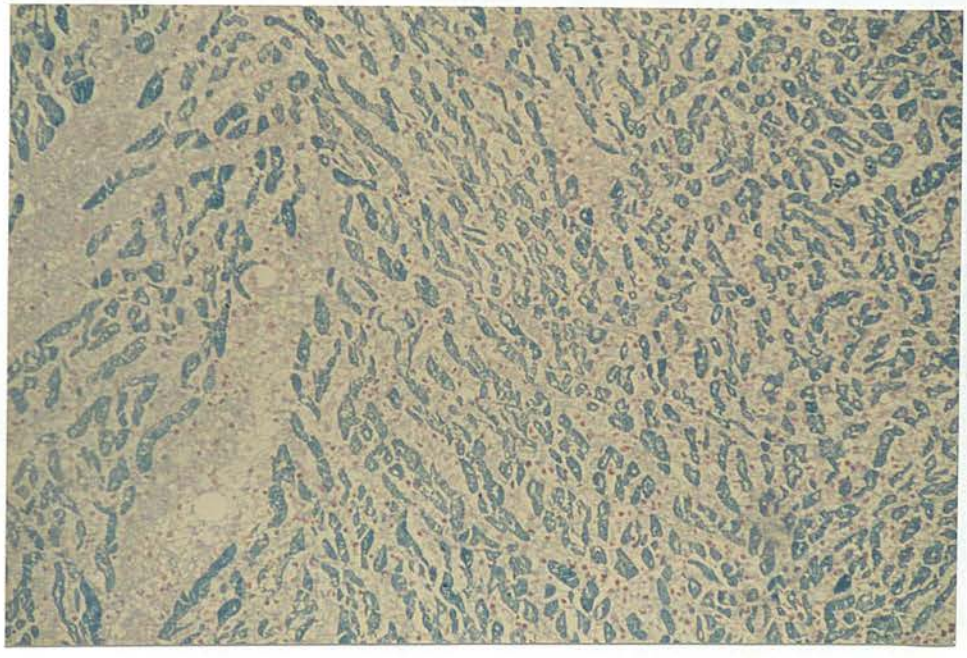


Figure 7.10 X10 m LFB-Myocardial Infarction.

Necrotic myocytes show changed to a "dove-blue" colour as described by the literature.

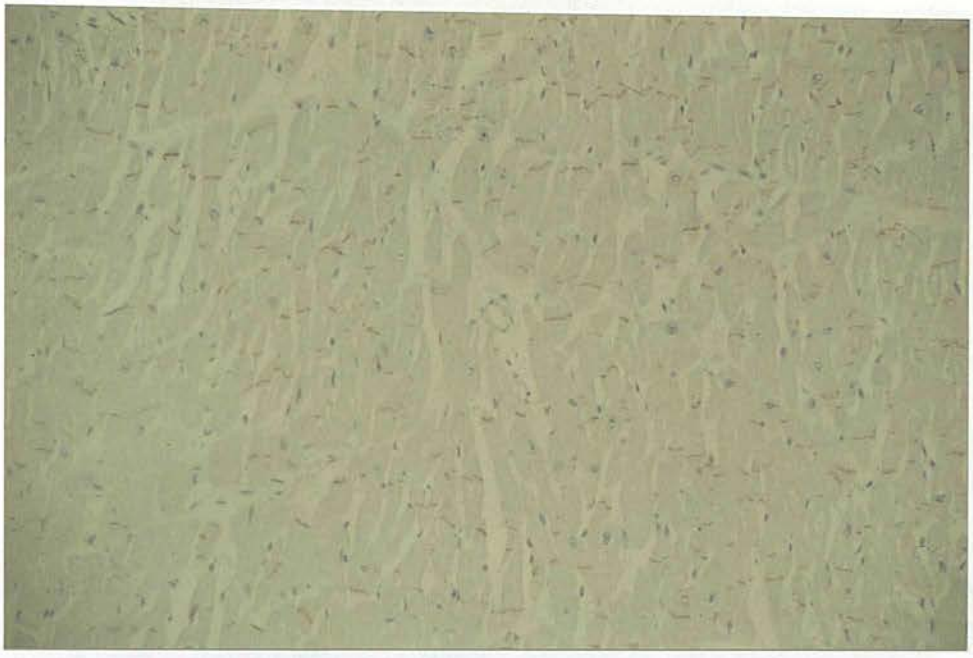


Figure 7.11 X10 A-CAM/N-Cadherin-Normal Myocardium.

Positive staining of intercalated discs (ICDs) as regular bands across the myocytes. Other components are negative.

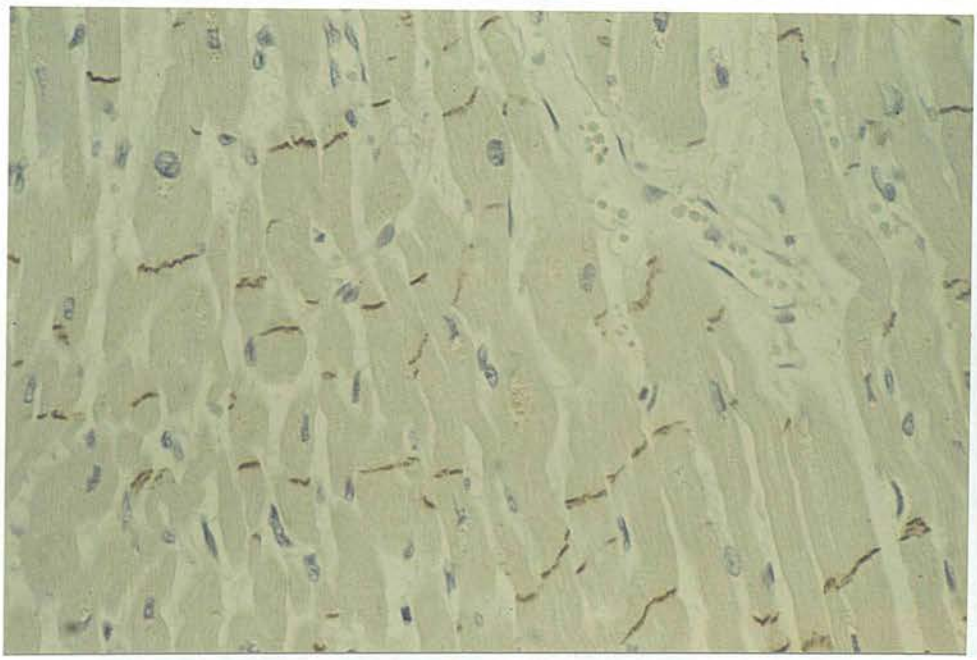


Figure 7.12 X25 A-CAM-Normal Myocardium.

The ICDs are specifically stained as regular bands. The rest of the myocyte's components and blood vessels are not stained.

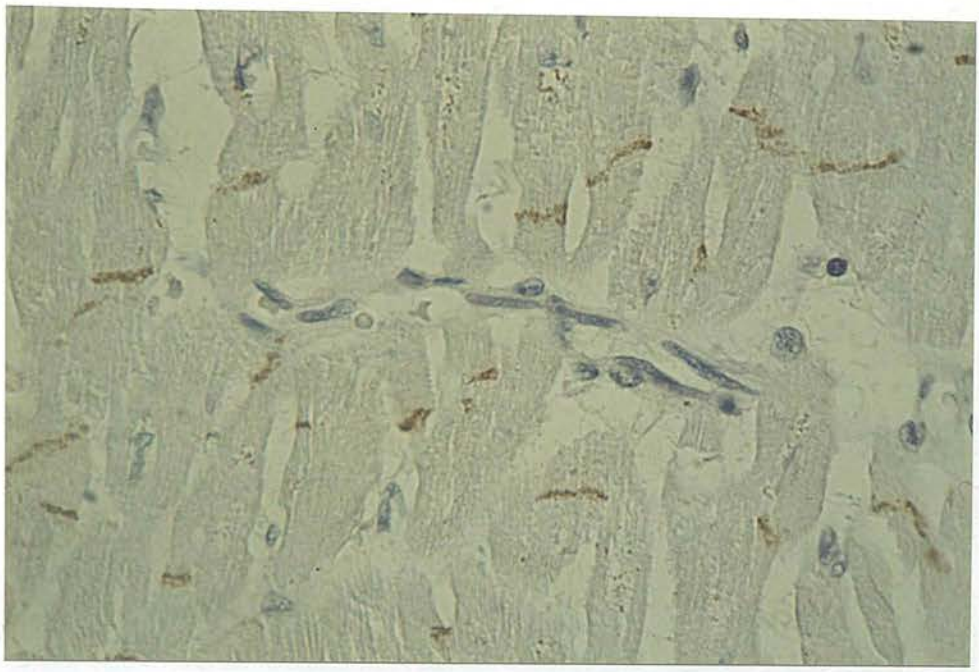


Figure 7.13 X40 A-CAM-Normal Myocardium.

The stained ICDs at this magnification show slightly serrated appearance.

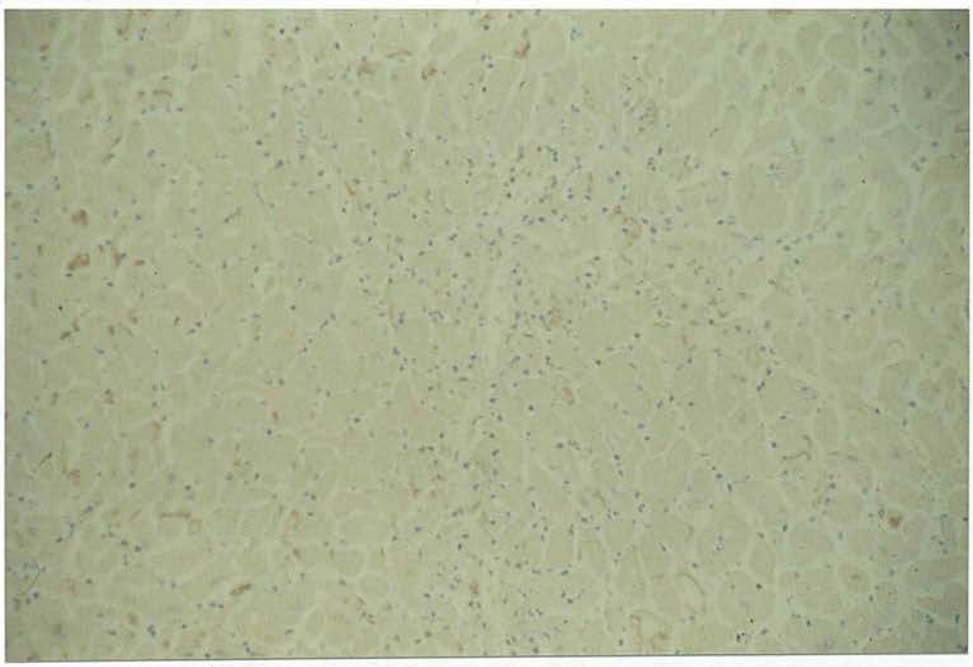


Figure 7.14X10 A-CAM-Myocardial Infarction and Ischaemia.

Necrotic myocytes admixed with acute inflammatory cells in the centre of the field showing loss of staining.

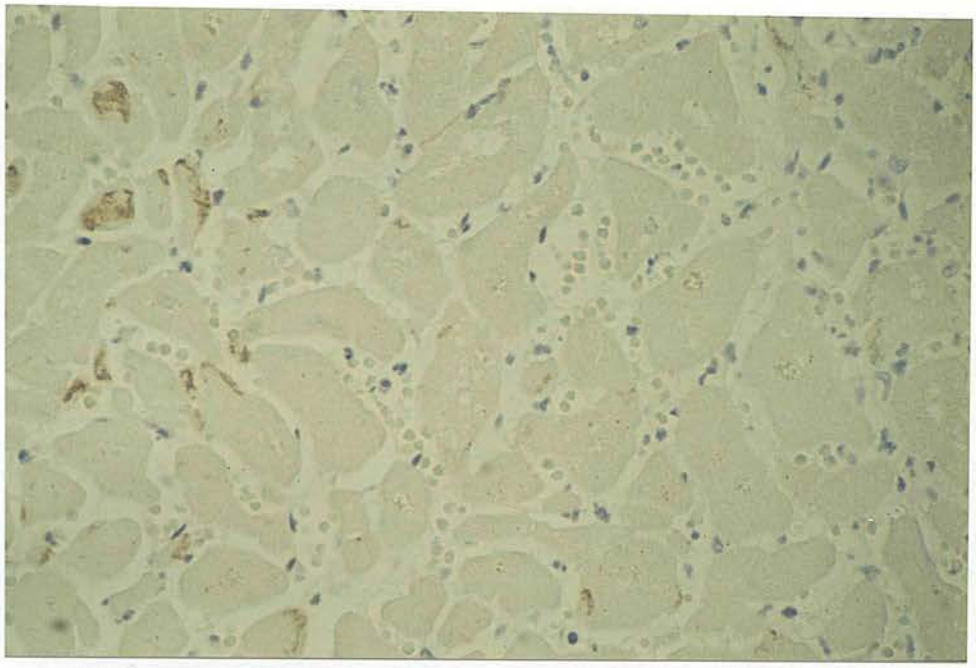


Figure 7.15 X25 A-CAM-Edge of a Myocardial Infarction.

The infarcted myocytes on the right hand side are not stained for A-CAM antibody. The "ischaemic" fibres on the left show broad irregular bands of stain which in some areas has accumulated close to the cell membranes.

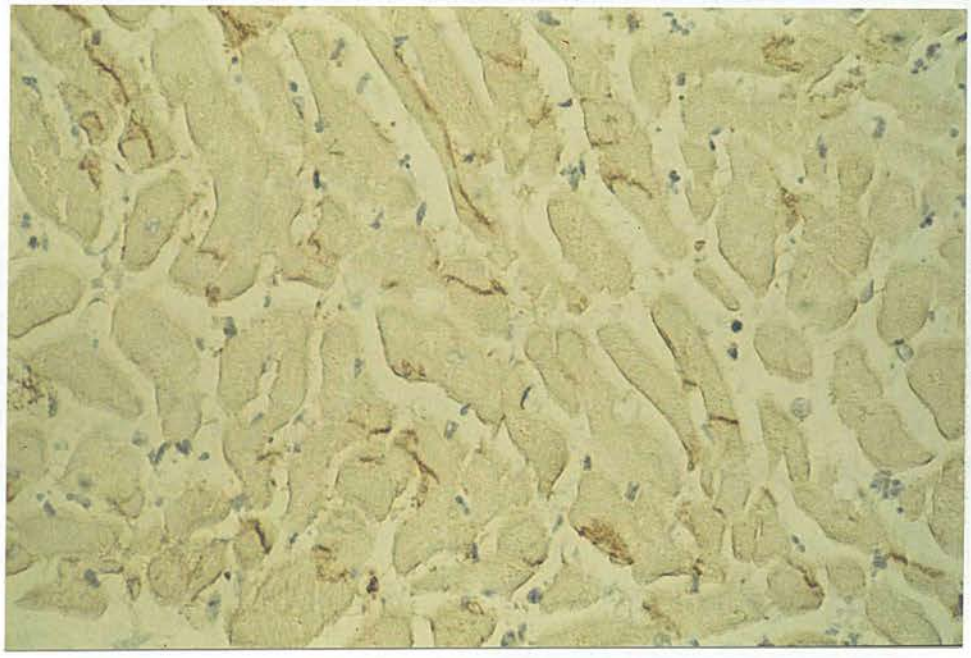


Figure 7.16 X 25 A-CAM of ischaemic fibres.

The ICDs staining is broader and either absent or of decreased intensity. Some fibres show vacuolation but this is a sporadic finding.

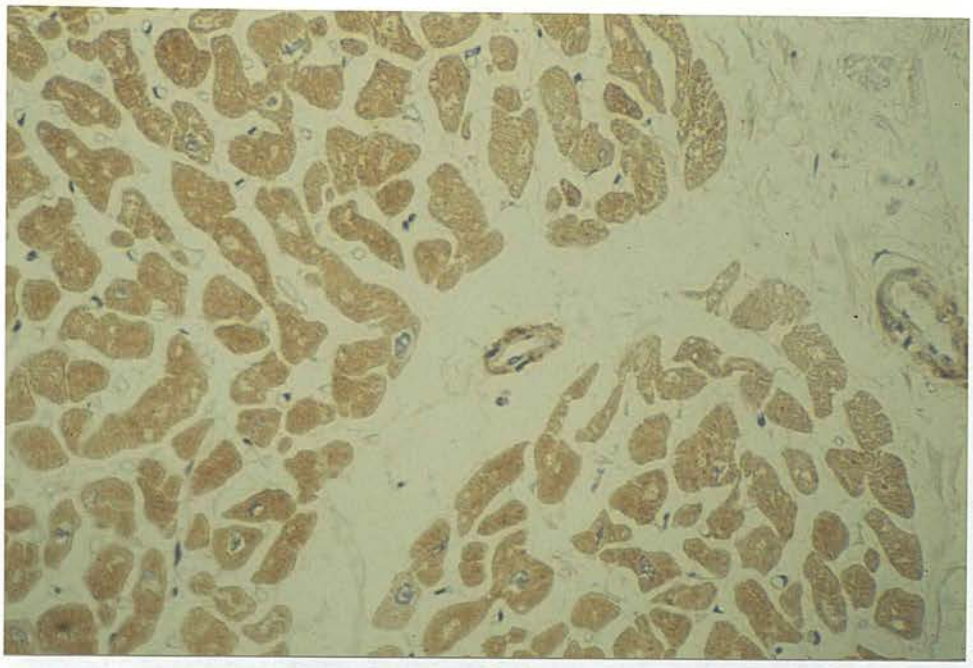


Figure 7.17 X25 Actin reaction. Normal Myocardium

Uniform diffuse cytoplasmic staining. The smooth muscle cells of the arterioles and venules, and some of the endothelial cells are also stained.

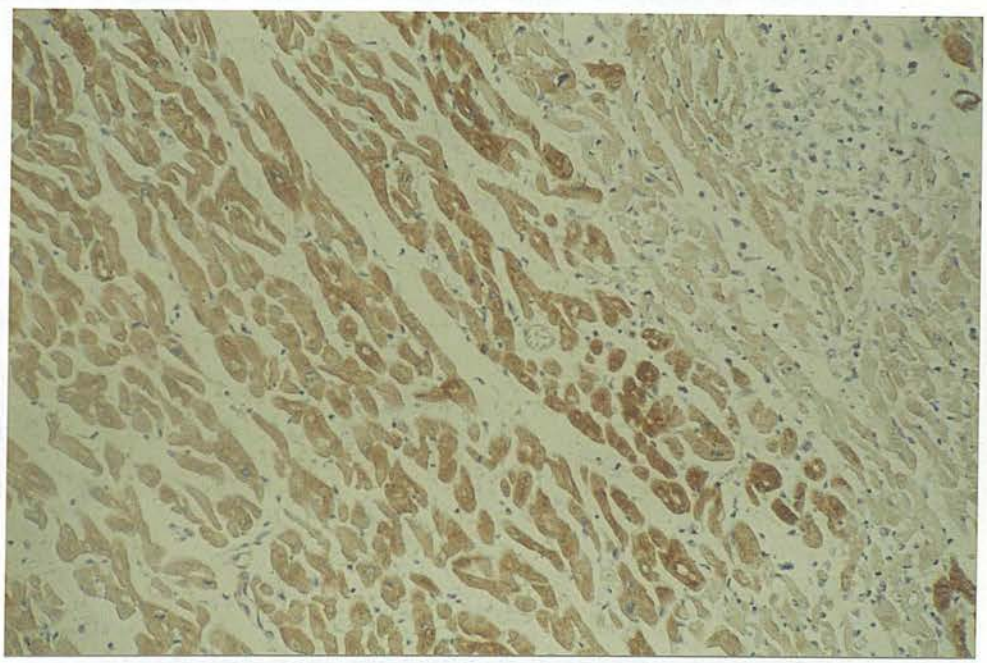


Figure 7.18 X25 Actin. Myocardial Infarction and Ischaemia.

Complete loss of staining in infarcted areas. The smooth muscle cells of the blood vessel walls still retains positivity. The ischaemic cardiac muscle cells show faint granular, vacuolated and punctate staining.

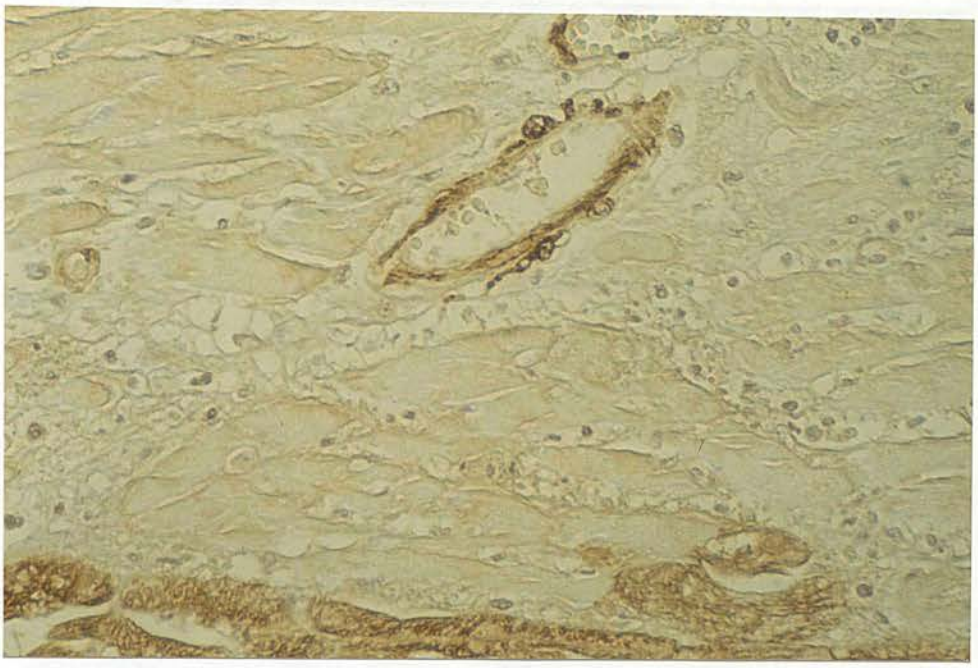


Figure 7.19 X25 Actin-Myocardial Infarction.

The necrotic myocytes show almost complete loss of staining. The ischaemic myocytes at the lower bottom rim at the edge of the infarct show patchy granular cytoplasm staining. The blood vessels in the middle of infarct still show discontinuous staining in its wall.

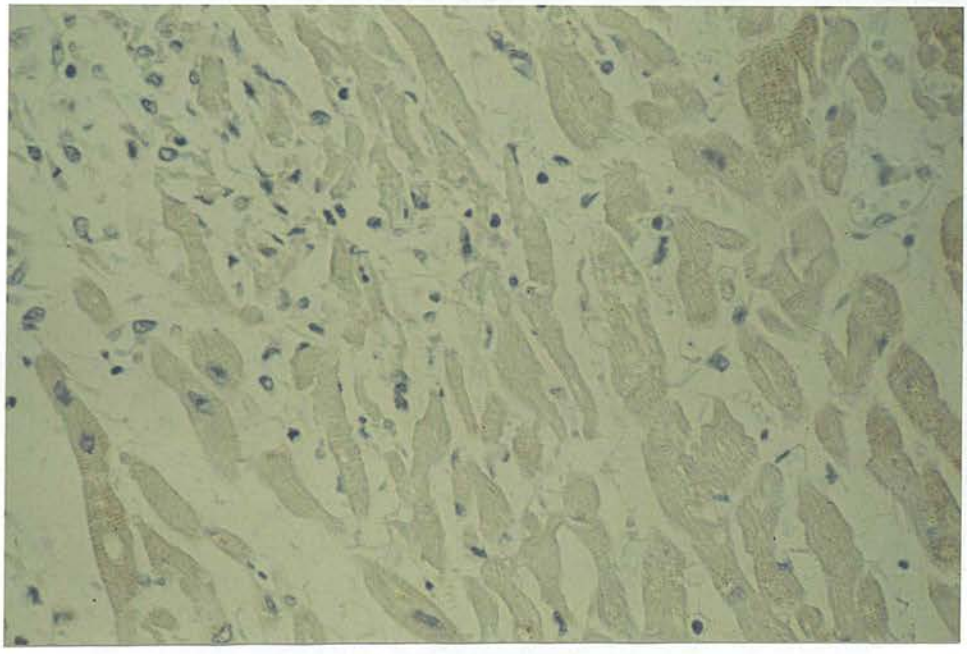


Figure 7.20 X25 Actin in Myocardial Infarction and Ischaemia.

The myocytes in the infarcted area show almost complete loss of staining. Ischaemic edges show fibres with marked depletion of staining.

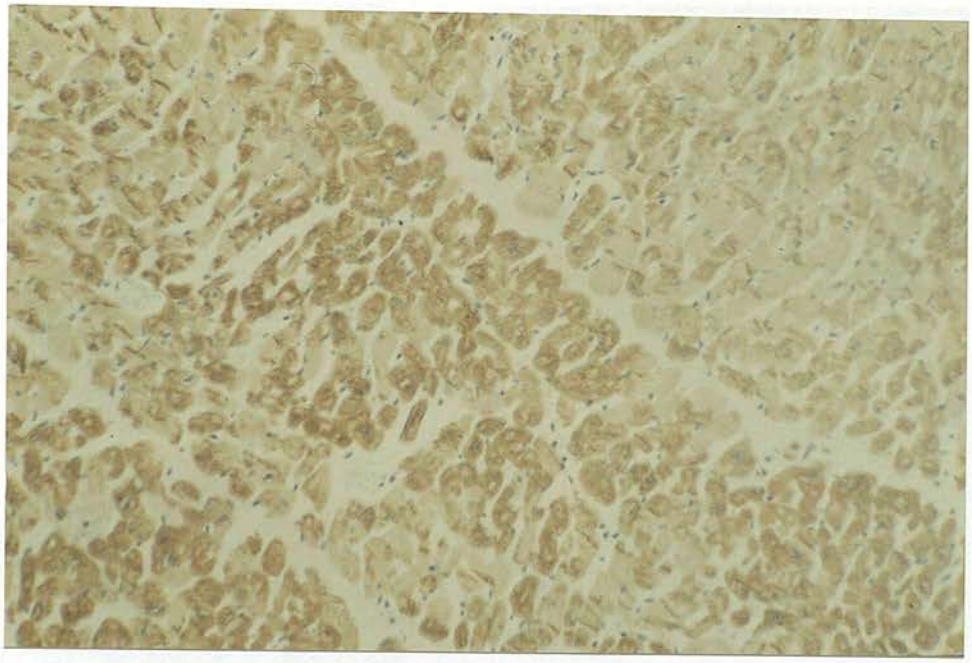


Figure 7.21 X10 α-actinin - Normal Myocardium.

Positive staining of ICDs, of cross-striations (patchily) and of the outer cell membrane.

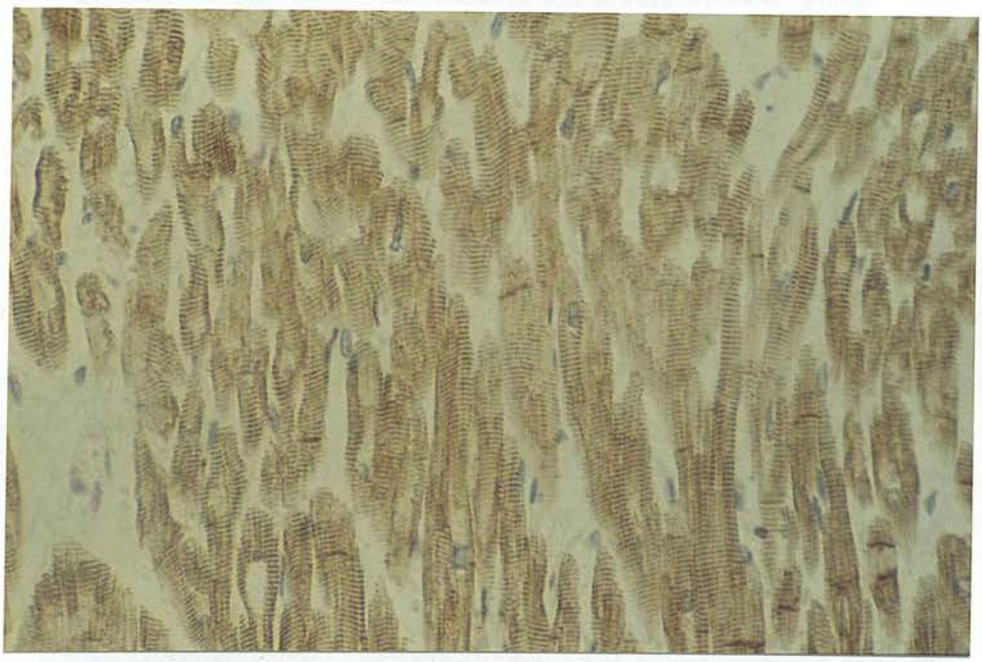


Figure 7.22 X25 α--actinin-Normal Myocardium.

The cross striations are uniformly stained. The intercalated discs show up as denser and more prominent bands across the cells. The blood vessel walls and myocyte cytoplasm are not stained.

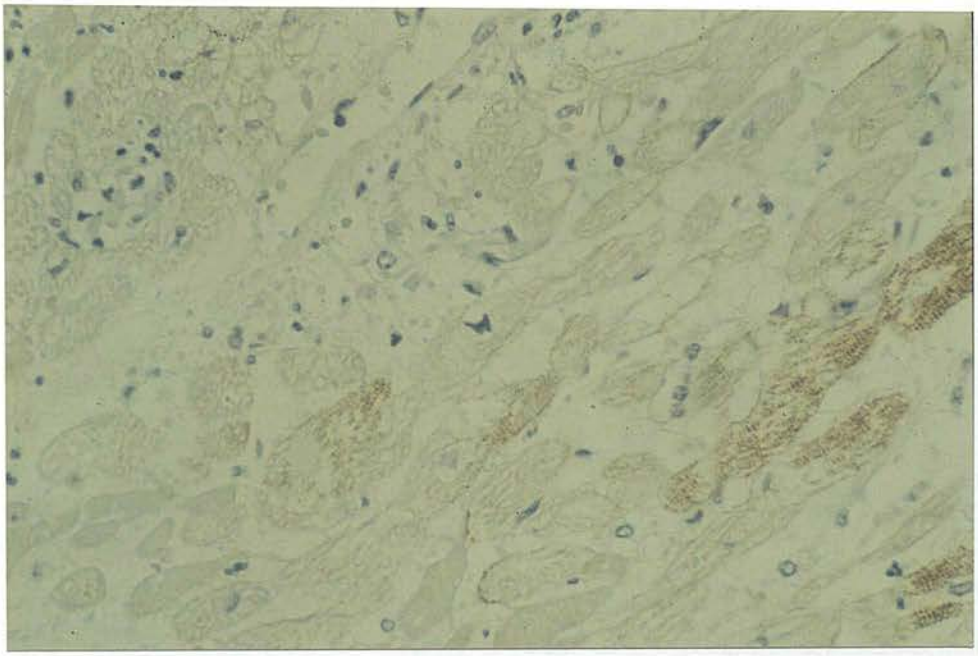


Figure 7.23 X25 α-actinin of Myocardial Infarction and Ischaemia.

The left upper part show necrotic fibres in which there is loss of staining. The lower right corner show ischaemic myocytes with discontinuous outer membrane staining and poorly defined "ghost looking" cross-striations in parts of some cells.

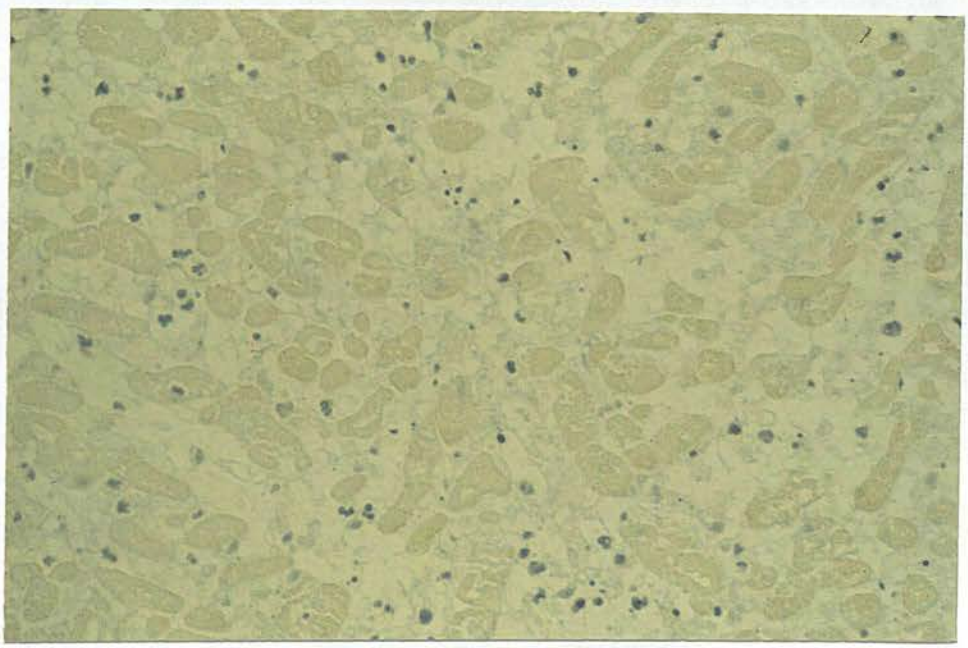


Figure 7.24 X25 α-actinin of Myocardial Infarction and ischaemia.

As figure 23.

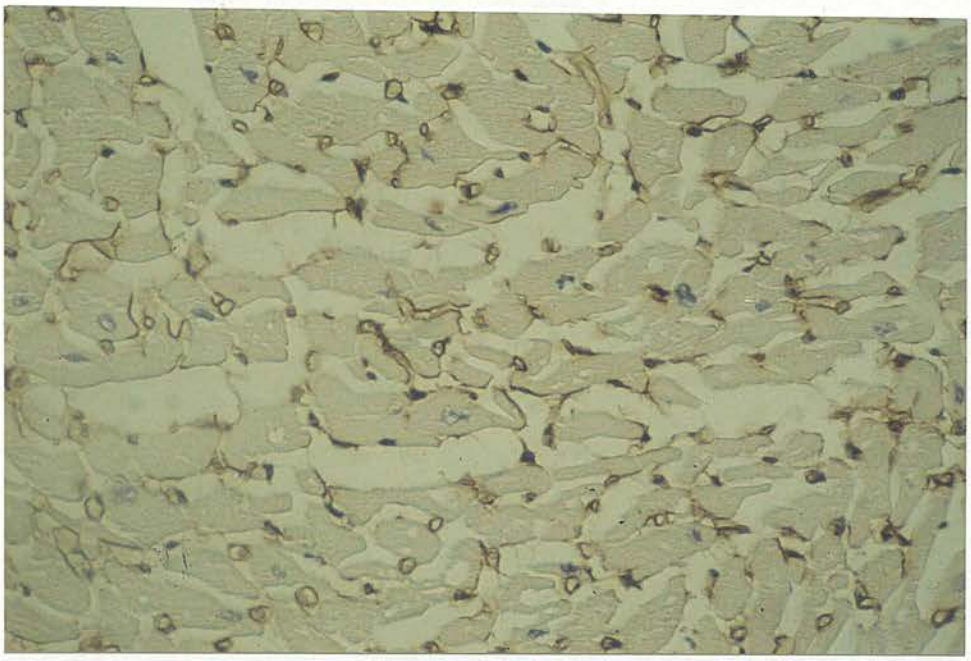


Figure 7.25 X25 CD34–Normal Myocardium.

The myocytes' cytoplasm does not stain. The endothelial lining of the capillaries in between myocytes all stain positively showing a linear reticulated pattern.

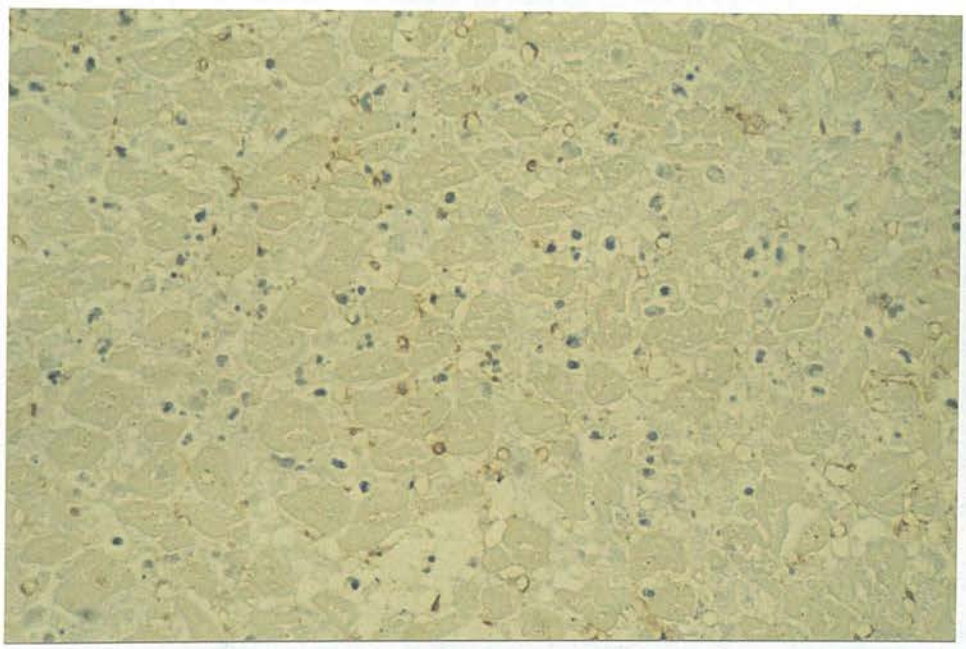


Figure 7.26 X25 CD34-Myocardial Infarction.

The capillaries in the infarcted zone show focal to complete loss of staining.

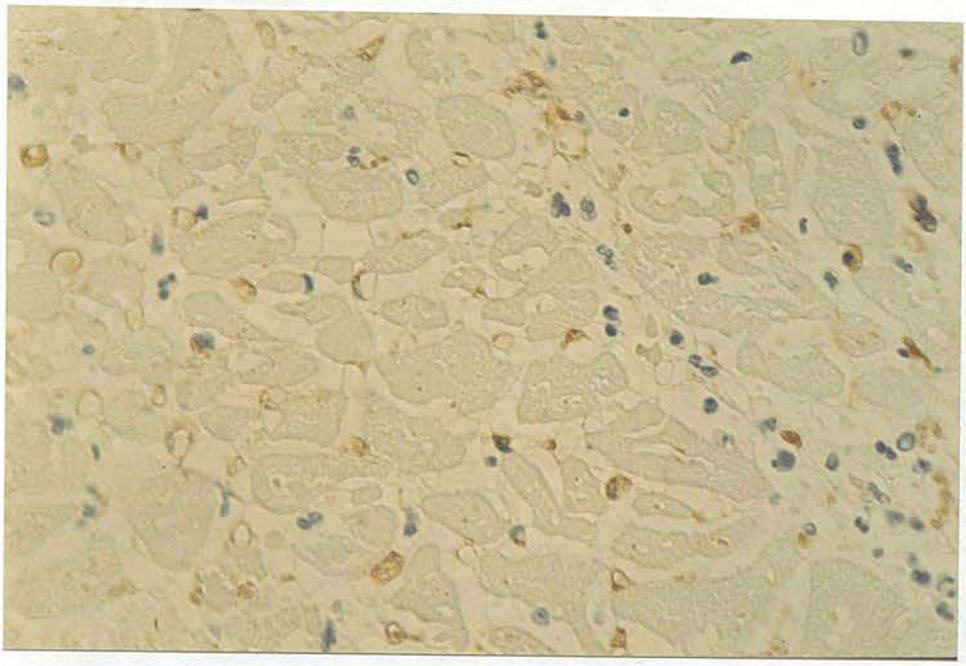


Figure 7.27 X40 CD34-Myocardial Ischaemia.

Focal capillary staining can still be observed in those ischaemic areas which show no infiltration by polymorphonuclear neutrophils.

Figure 7.28 X25 $C5_b$–9–Normal Myocardium.

Positive staining is seen in the muscular wall of blood vessels. The staining is mainly endothelial with some sub-endothelial positivity. The cardiac myocytes do not stain.

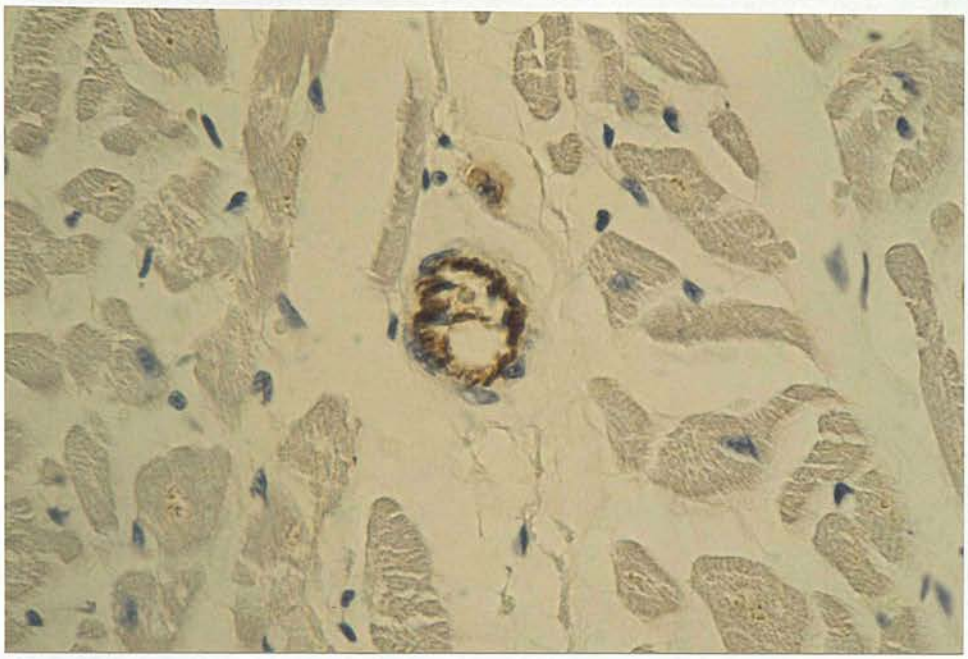


Figure 7.29 X40 $C5_b$–9–Normal Myocardium.

Blood vessels in the centre of the photograph shows endothelial staining. The myocytic and cytoplasmic fibres do not take up the stain.

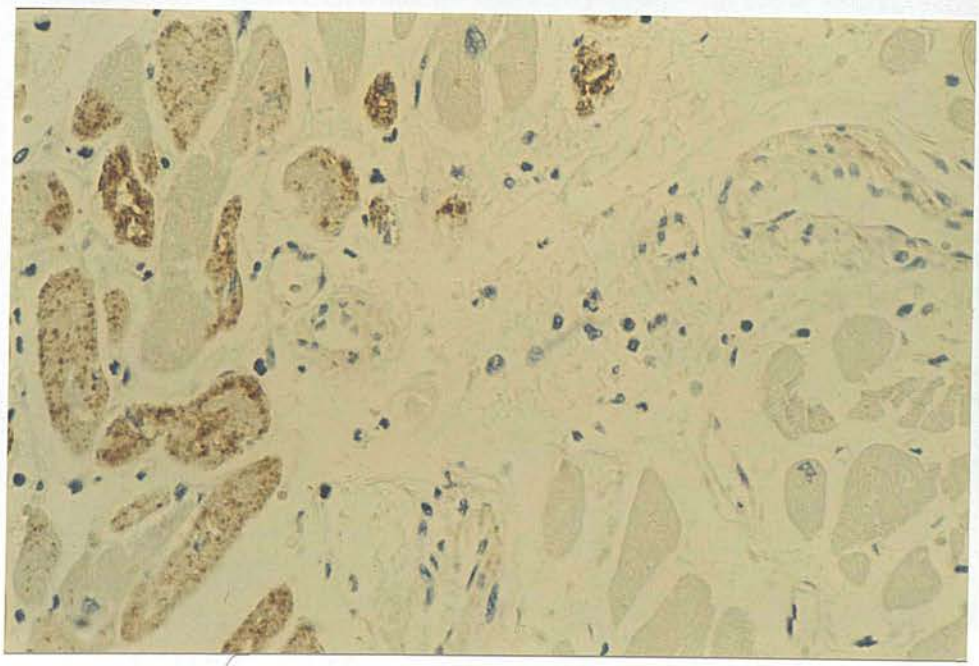


Figure 7.30 X25 $C5_b$–9–Edge of Myocardial Infarction.

The myocytes show varying degrees of positivity. Patchy and variegated, granular and vacuolated patterns are seen in the necrotic myocytes.

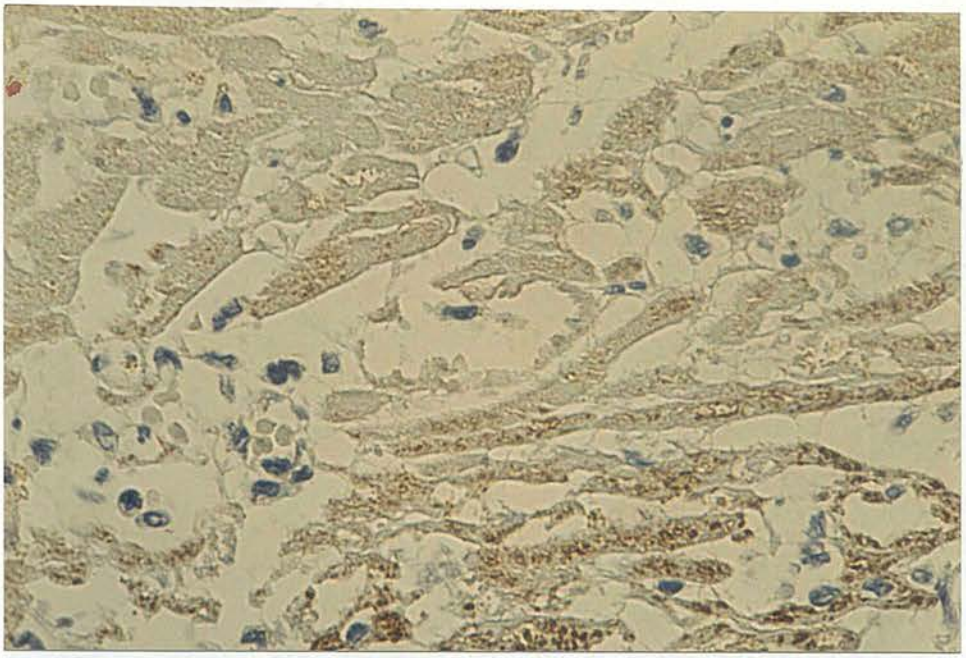


Figure 7.31 X40 $C5_b$–9-Myocardial Infarction.

The necrotic myocytes immediately next to the polymorphonuclear neutrophils show diffuse focally accentuated granular cytoplasmic staining. The myocytes in areas not infiltrated by polymorphonuclear neutrophils show focal faint cytoplasmic staining.

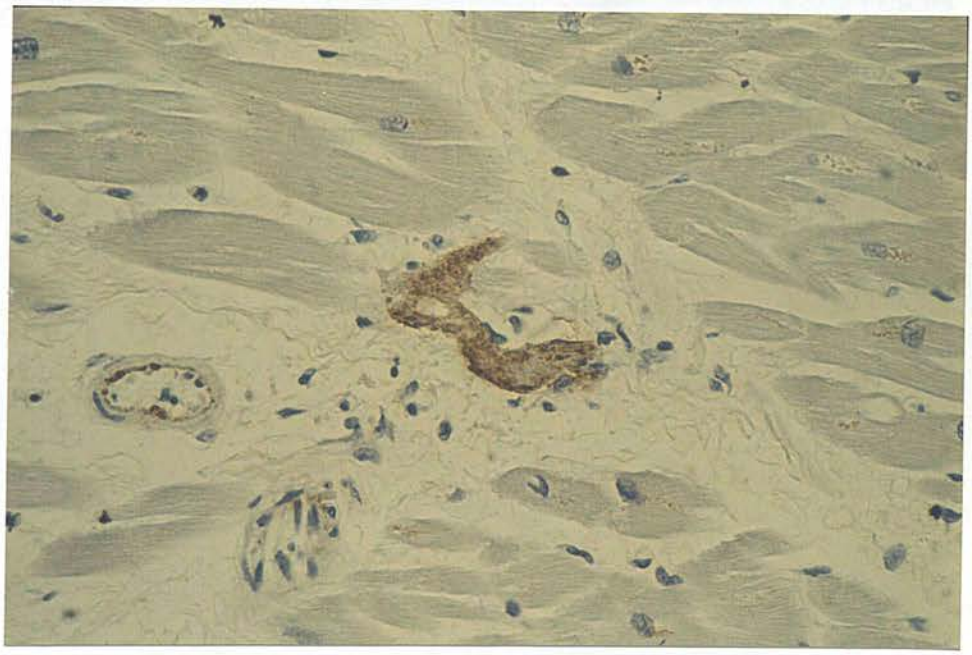


Figure 7.32 X25 $C5_b$–9–Ischaemic Myocardium.

In the ischaemic area isolated myocytes show granular cytoplasmic staining and some membranous reaction. The endothelium of blood vessels shows only patchy sub-endothelial staining.

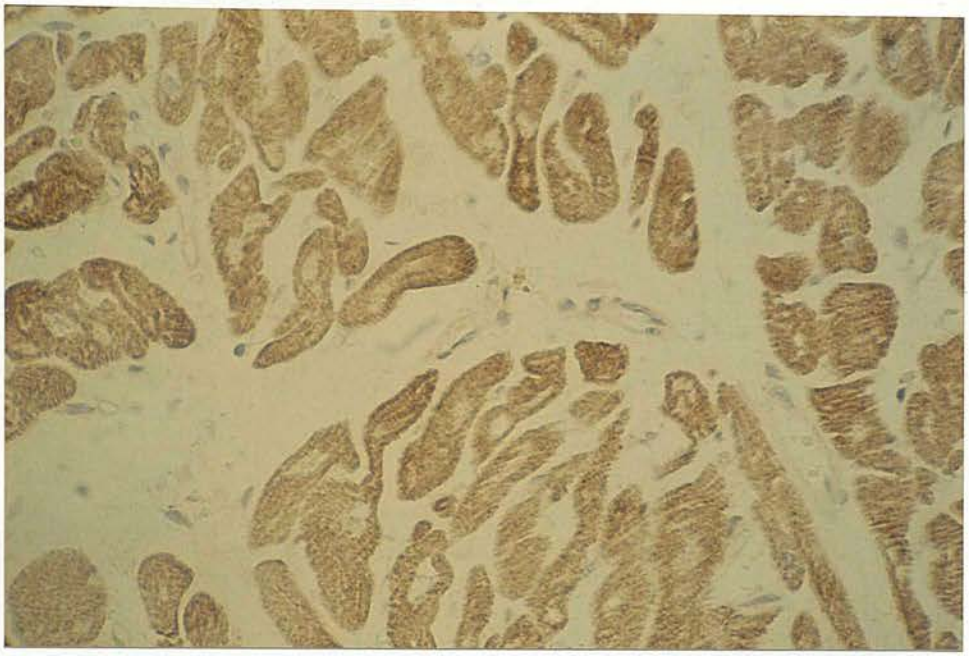


Figure 7.33 X25 CD59–Normal Myocardium.

Both striated, and diffuse, granular cytoplasmic positive reaction. The blood vessels and cytoplasm do not stain.

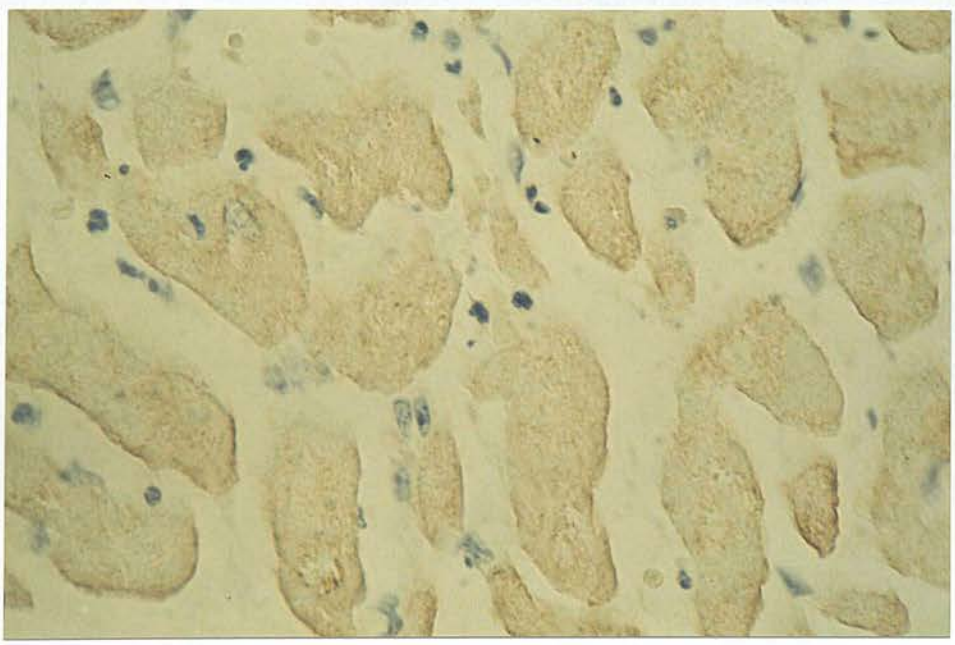


Figure 7.34 X40 CD59– Ischaemia.

The cardiac myocytes show marked depletion of their cytoplasmic staining. Disrupted discontinuous membranous staining can still be seen. There is no striation pattern identifiable.

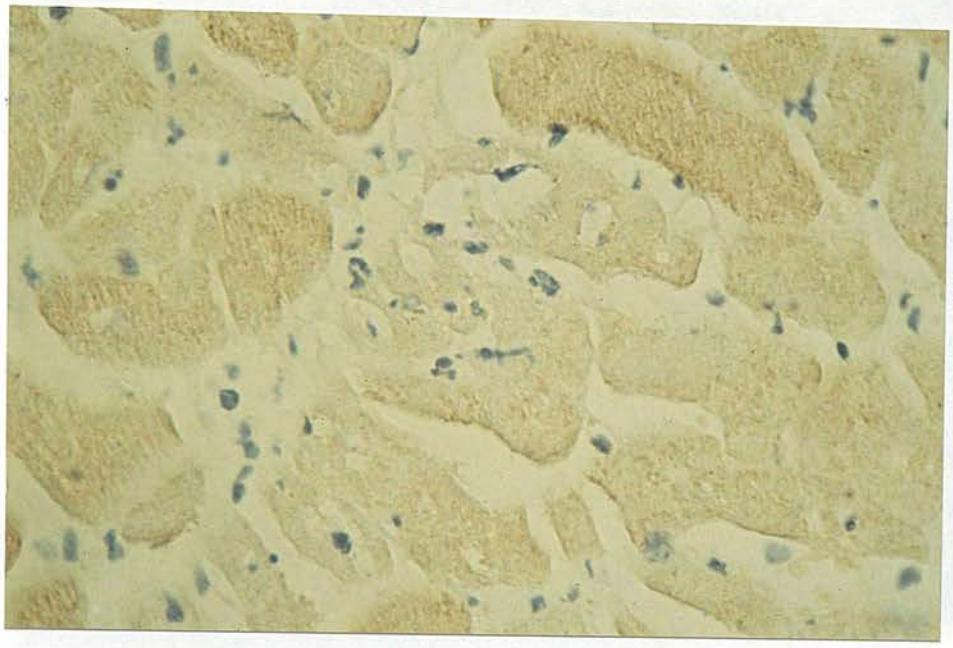


Figure 7.35 X40 CD59 - Ischaemia.

As figure 34.

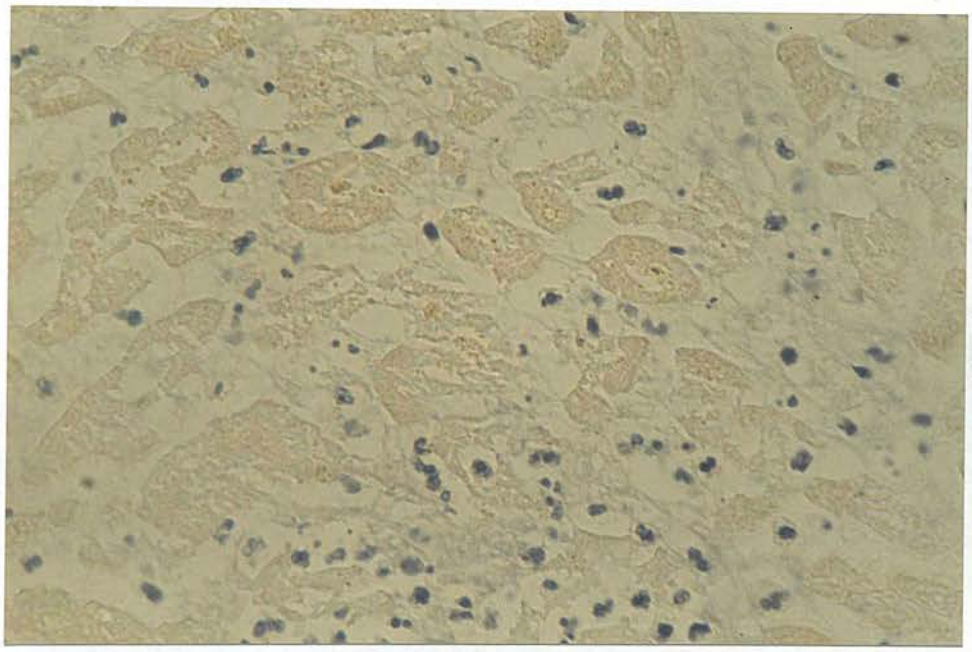


Figure 7.36 X25 CD59 - Myocardial Infarction.

The acutely infarcted myocytes show almost complete loss of cytoplasmic and membranous staining with no pattern to the residual staining.

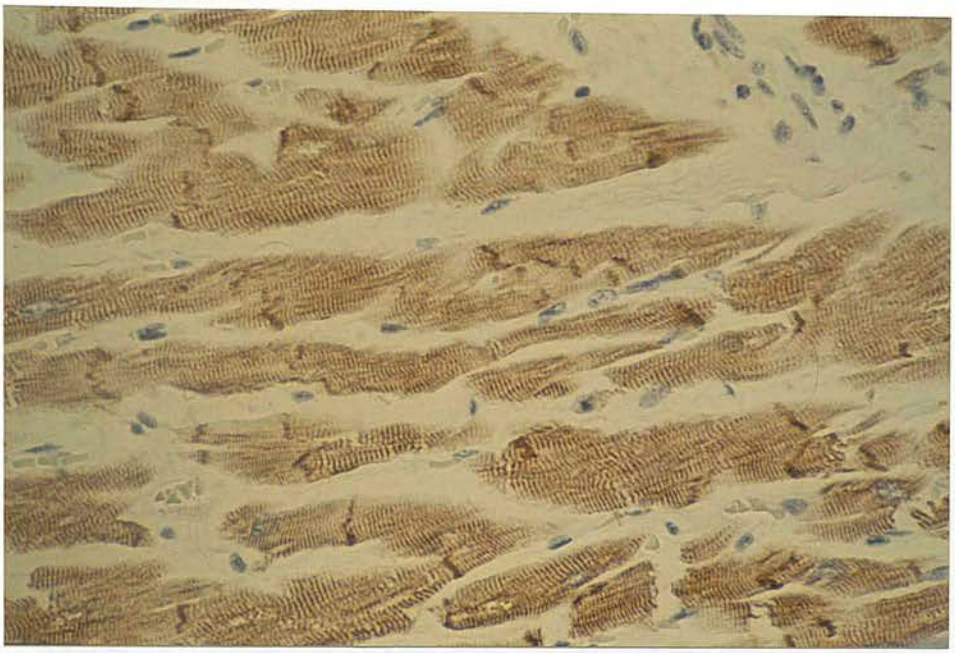


Figure 7.37 X25 Desmin-Normal Myocardium.

Prominent myocytic cross-striation staining with more prominent and denser ICD staining.

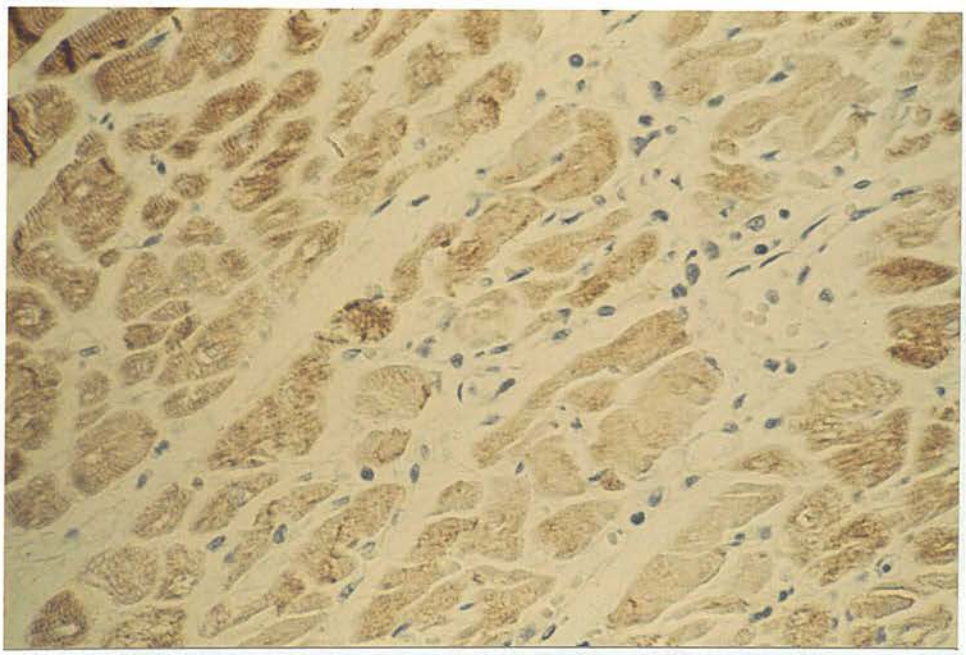


Figure 7.38X25 Desmin-Myocardial Infarction and Ischaemia.

A gradation in the intensity of staining can be seen from the normal (upper left corner) to ischaemic fibres to necrotic myocytes (lower left).

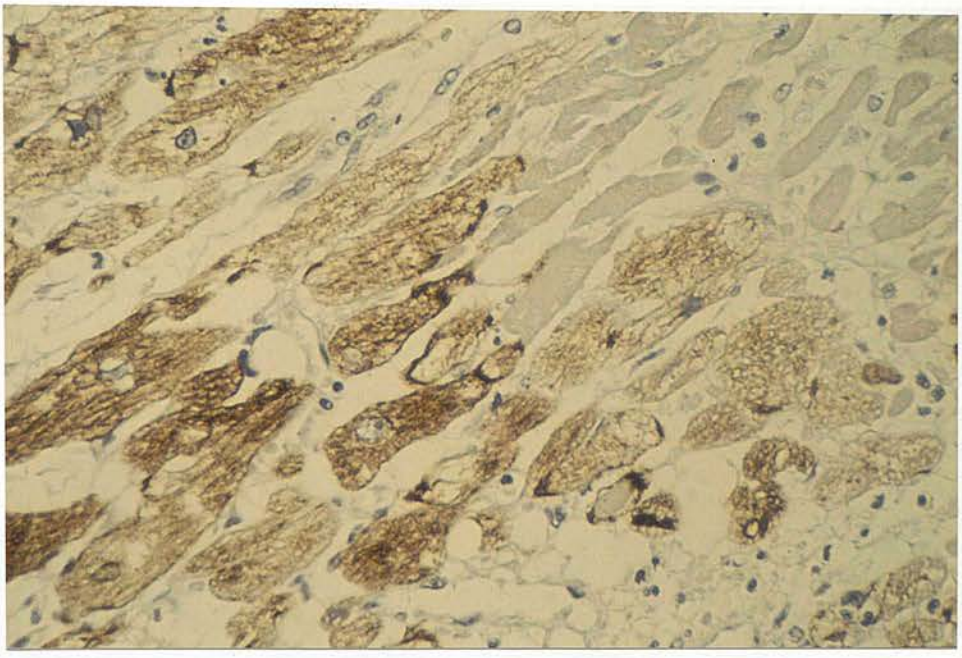


Figure 7.39 X25 Desmin-Myocardial Infarction

The infarcted myocytes show complete loss of both cytoplasmic, ICDs and cross-striation staining. Some of the necrotic fibres show a pronounced vacuolated, floccular appearance.

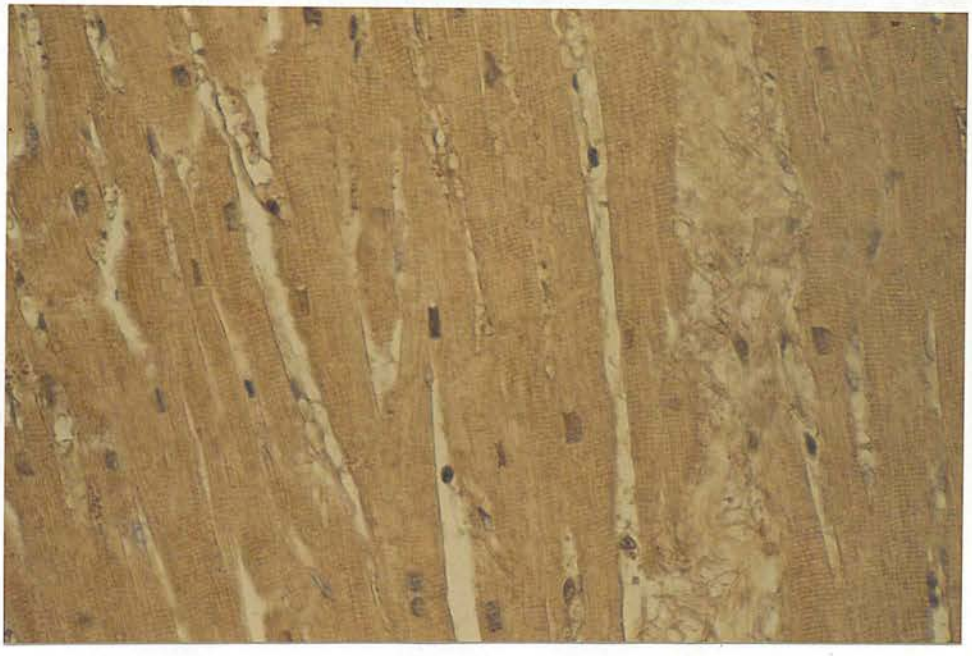


Figure 7.40 X25 Myoglobin-Normal Myocardium.

Diffuse intracytoplasmic staining with faint cross-striation.

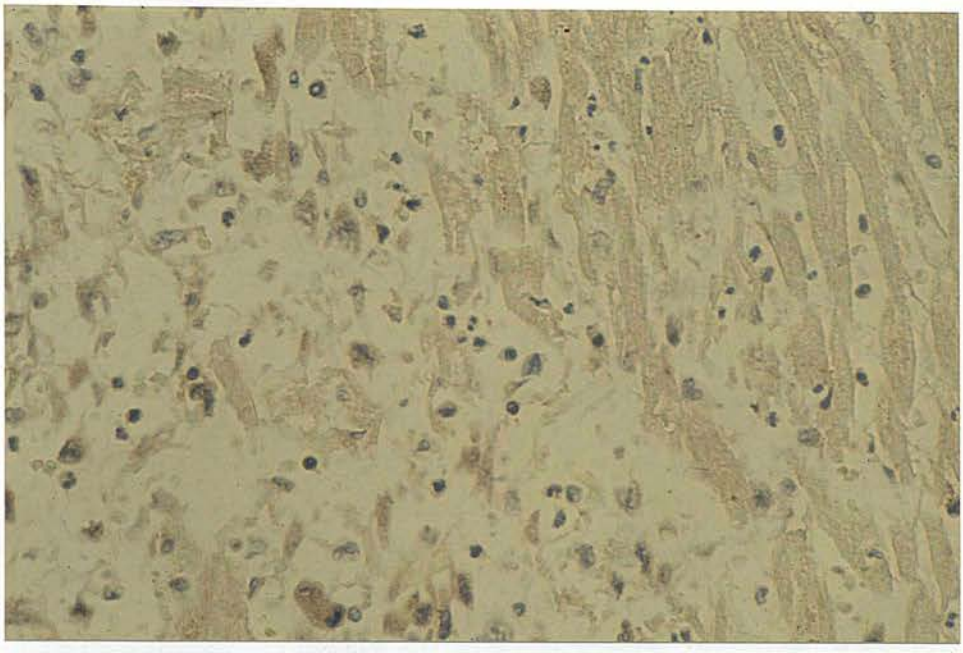


Figure 7.41 X25 Myoglobin-Myocardial Infarction.

Loss of staining in infarcted area with minimal residual positive striated staining in the damaged area (right upper corner).

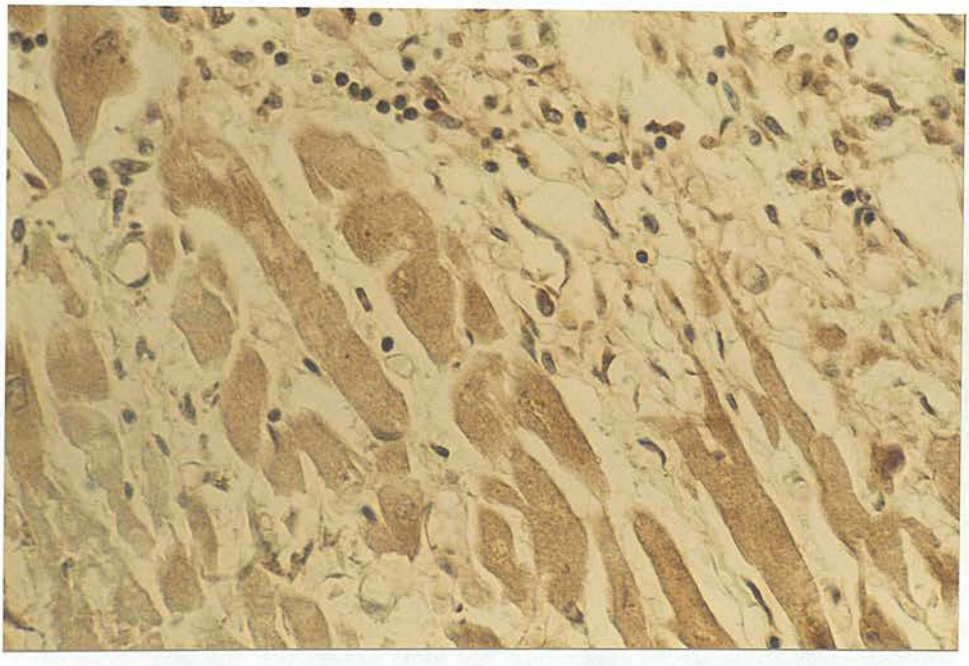


Figure 7.42 X25 Myoglobin-Ischaemic Myocardium.

Residual patchy positive intracytoplasmic staining in ischaemic fibres.

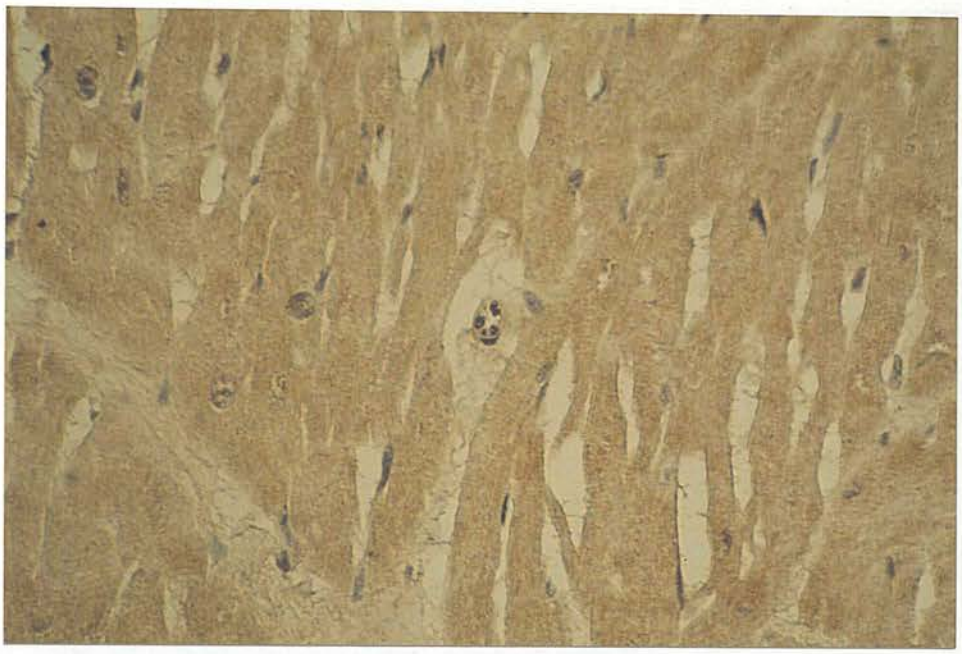


Figure 7.43 X25 Myosin-Normal Myocardium.

Diffuse but patchy cytoplasmic staining with focal demonstration of faint striation staining. The cell membrane stains focally as a thin linear band in some fibres.

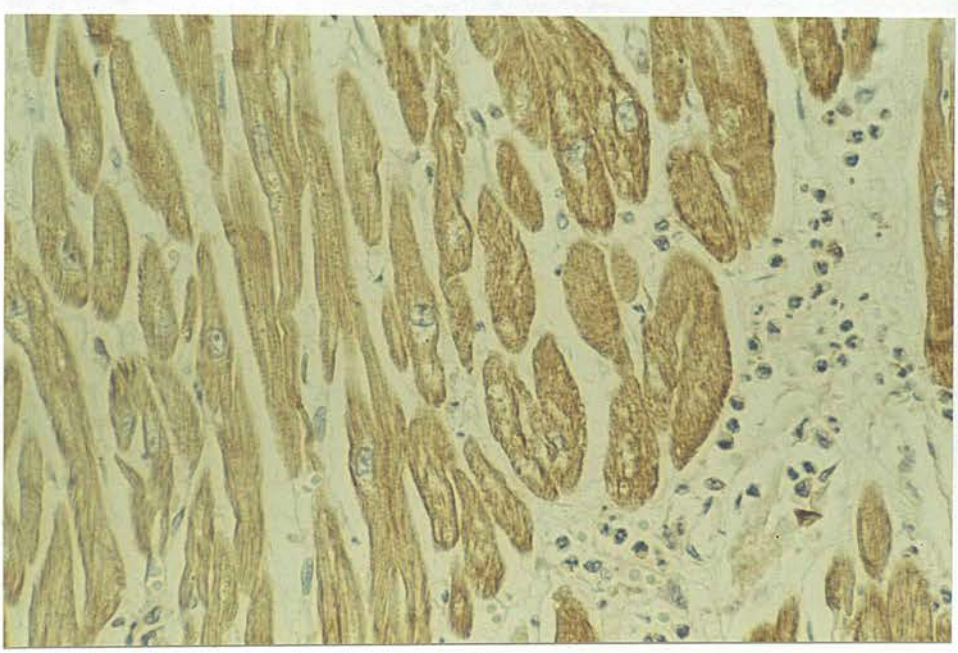


Figure 7.44 X25 Myosin-Myocardial Infarction & Ischaemia.

The necrotic myocytes surrounded by neutrophils show almost complete loss of staining. The non-necrotic cells show residual vaculated cytoplasmic staining.

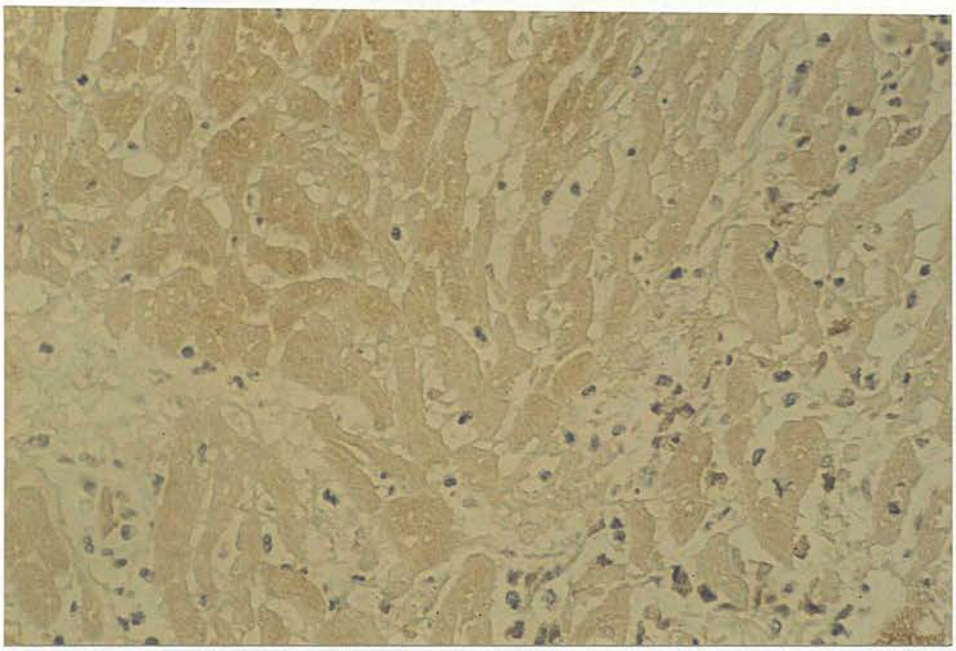


Figure 7.45 X25 Myosin–Myocardial Infarction & Ischaemia.

As figure 44.

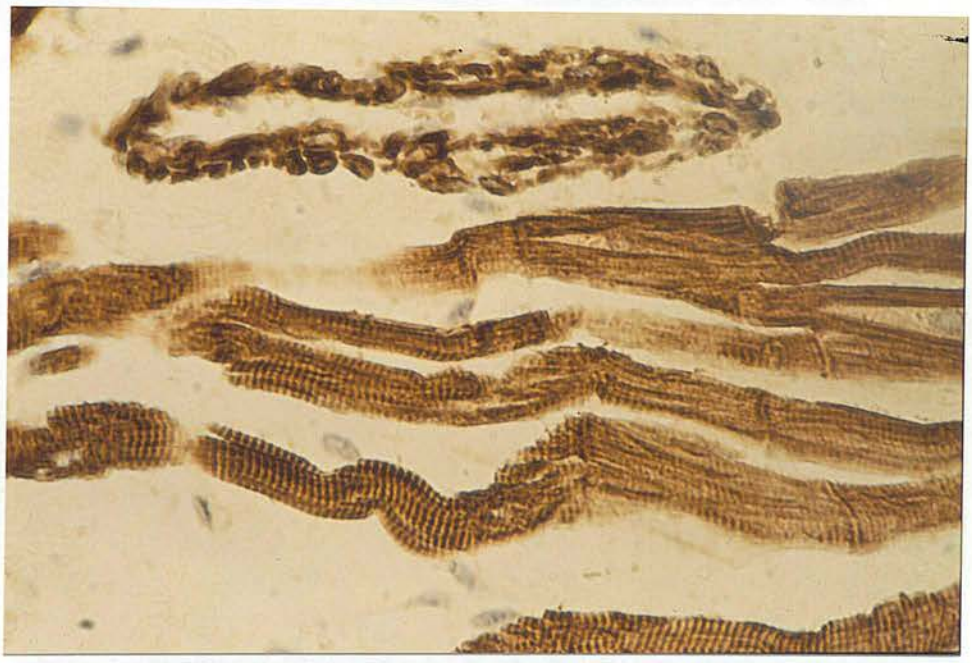


Figure 7.46 X40 Tropomyosin–Normal Myocardium.

The myocytes show a prominent, patterned striated cytoplasmic staining. The blood vessels show staining of their endothelial and medial layers.

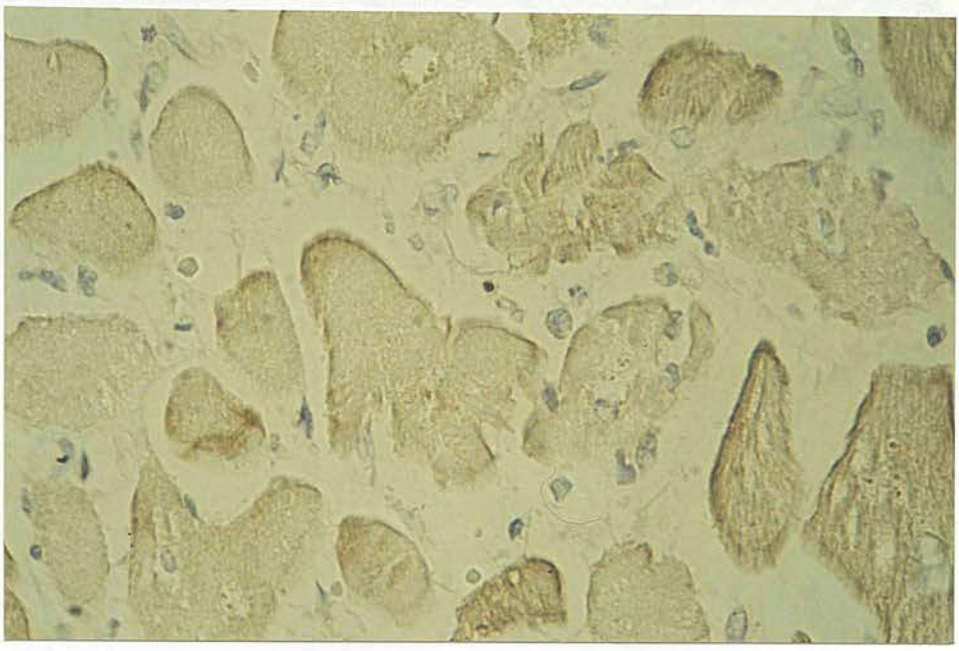


Figure 7.47 X40 Tropomysin - Myocardial Infarction and Ischaemia.

The ischaemic myocytes in contrast to the necrotic fibres show marked depletion of cytoplasmic staining and only focal membranous staining can be seen.

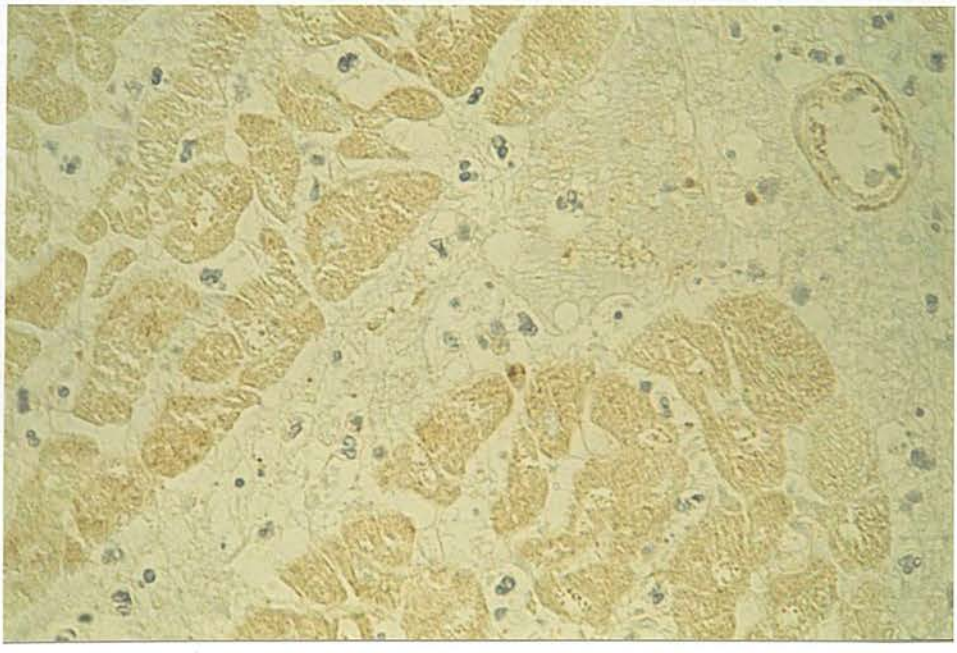


Figure 7.48 X40 Tropomyosin - Myocardial Infarction and Ischaemia.

In addition to near-total loss of cytoplasmic and membranous staining, there is also marked depletion of endothelial and muscle wall staining intensity in blood vessels.

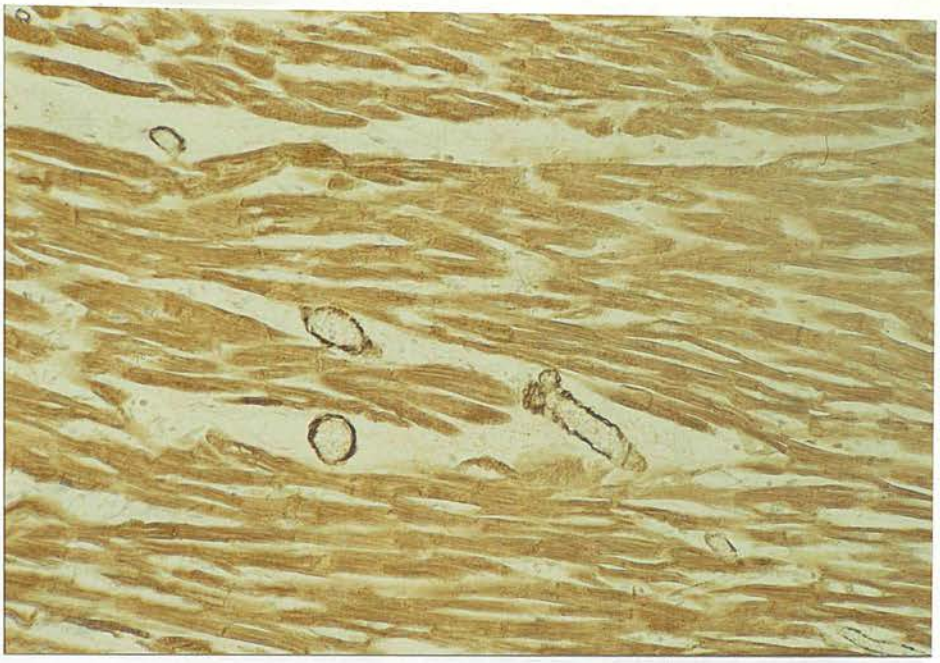


Figure 7.49 X10 Troponin–Normal Myocardium.

Diffuse cytoplasmic staining. Vague staining of the cross striation. The vascular walls show more pronounced staining. The capillaries are not stained.

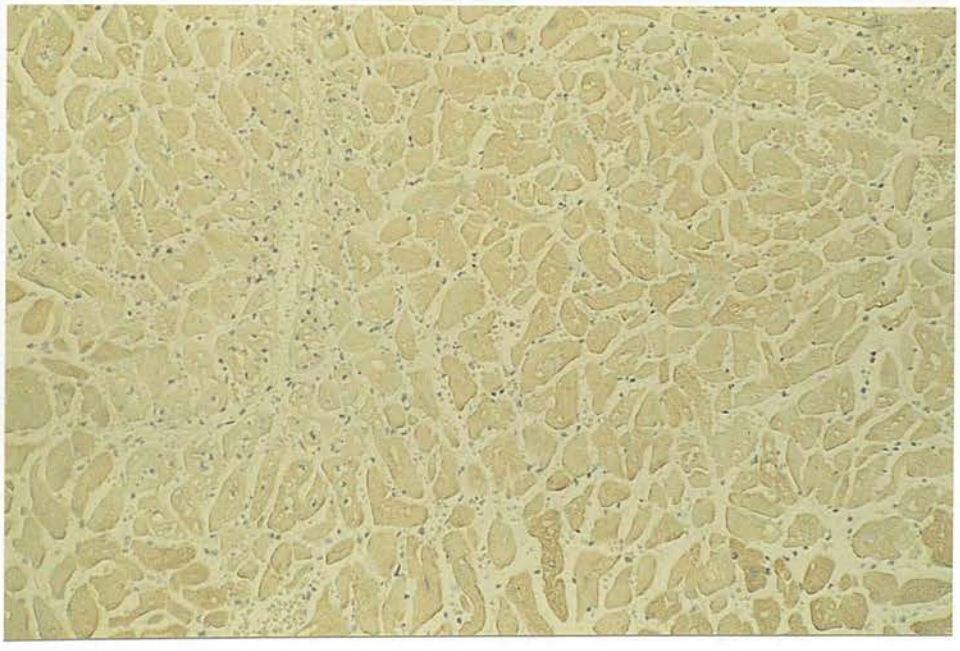


Figure 7.50 X25 Troponin - Myocardial infarction.

The anucleate infarcted myocytes show loss of intensity of cytoplasmic staining with a stippled appearance of the residual positive staining.

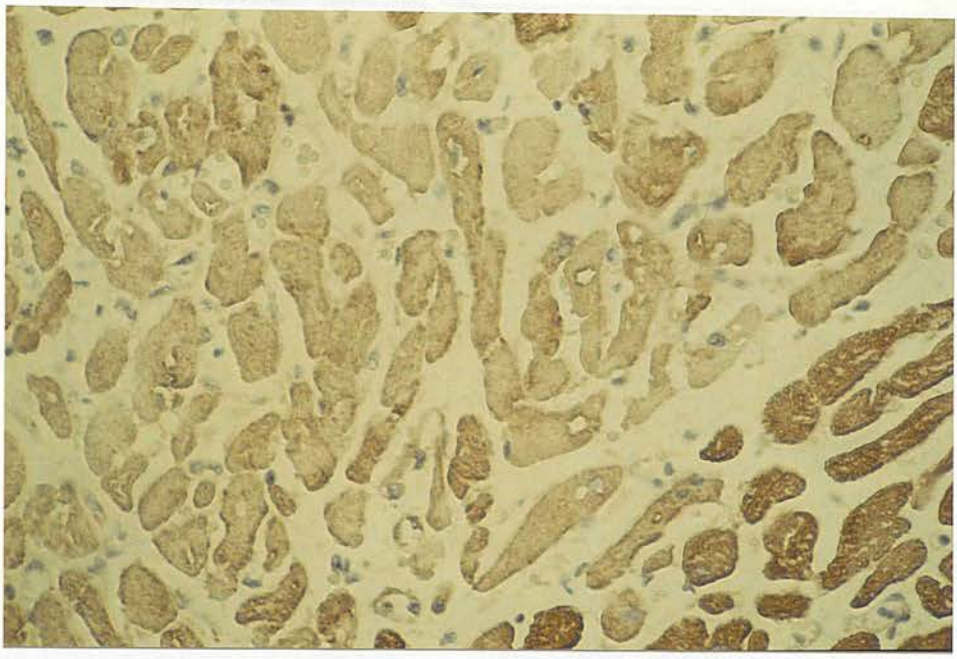


Figure 7.51 X25 Tropomyosin - Ischaemic zone at the edge of Myocardial Infarction.

Intensity and diffusness of staining is altered with a variegated staining pattern of the myocyte.

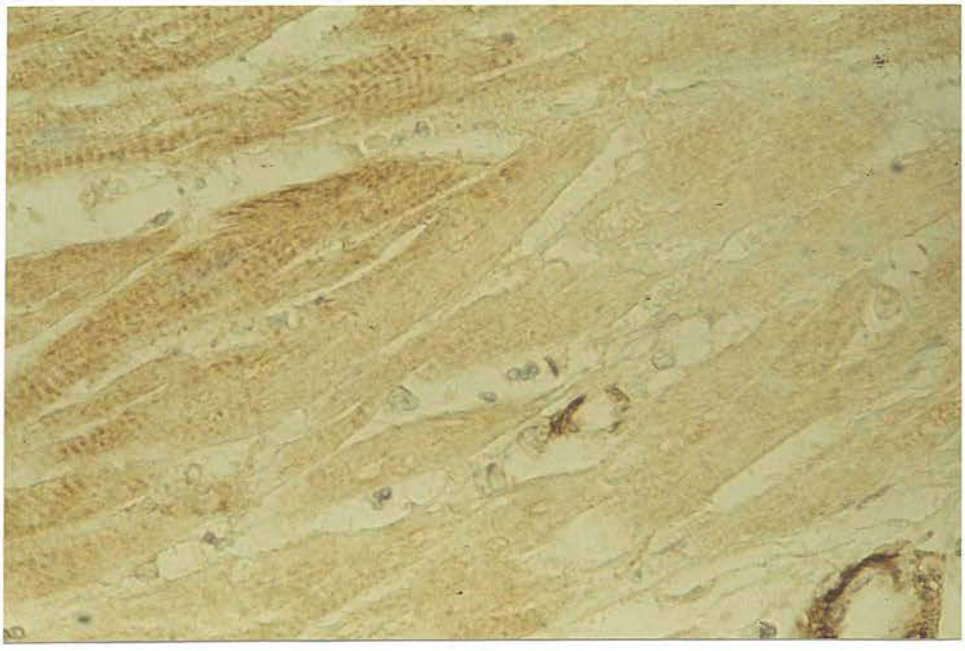


Figure 7.52 X40 Tropomyosin - Edge of Myocardial Infarction.

In these longitudunally cut myocytes there is a gradation of loss of staining intensity and striated pattern of staining. The vascular wall staining (lower left corner) is retained.

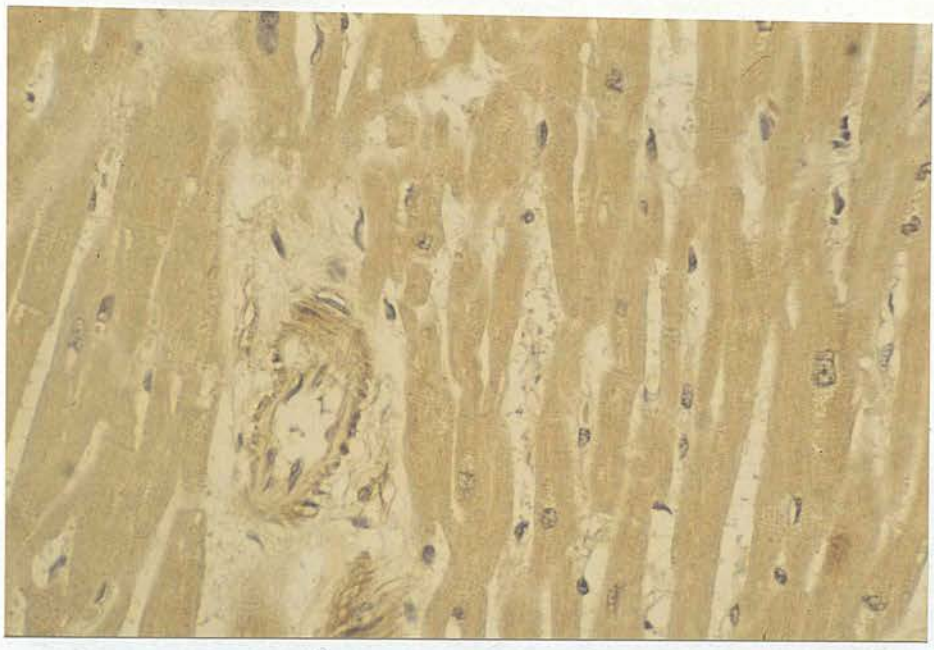


Figure 7.53 X25 Tubulin–Normal Myocardium.

Bland and non-patterned positive staining of myocytic cytoplasm and positive staining of vascular wall endothelium.

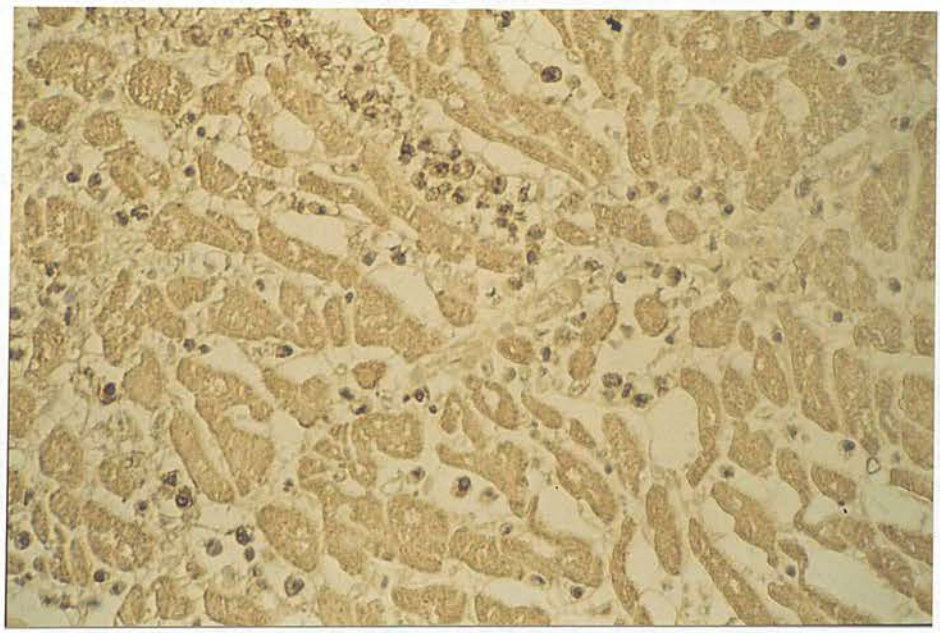


Figure 7.54 X25 Tubulin–Myocardial Infarction.

The necrotic fibres infiltrated with PMNs show a loss of intensity of staining. There is a vacuolated pattern in some of the fibres but no striations are seen.

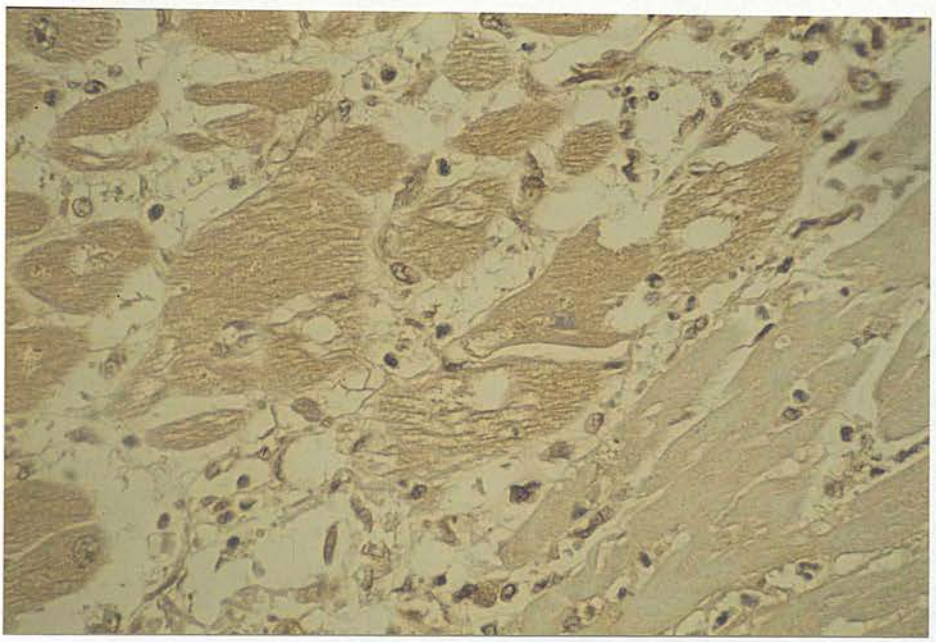


Figure 7.55X25 Tubulin - Myocardial Infarction & Ischaemia.

Variation from diffuse non-patterned staining of the cytoplasm of ischaemic fibres to hardly stained, necrotic fibres; some macrophages are also positively stained.

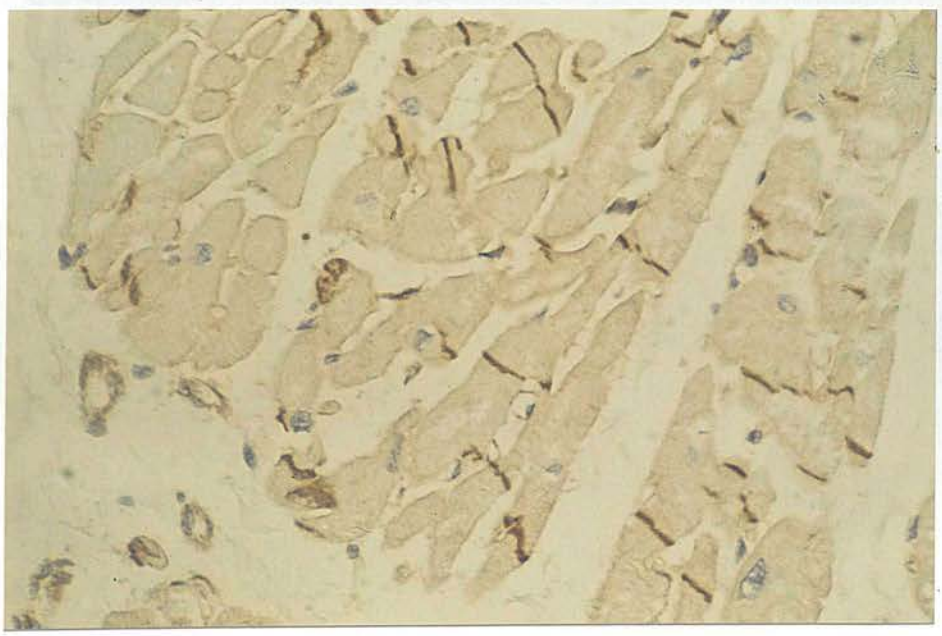


Figure 7.56 X25 Vinculin–Normal Myocardium.

ICDs show up as uniformly densely stained bands. No other part of the myocyte stain positively.

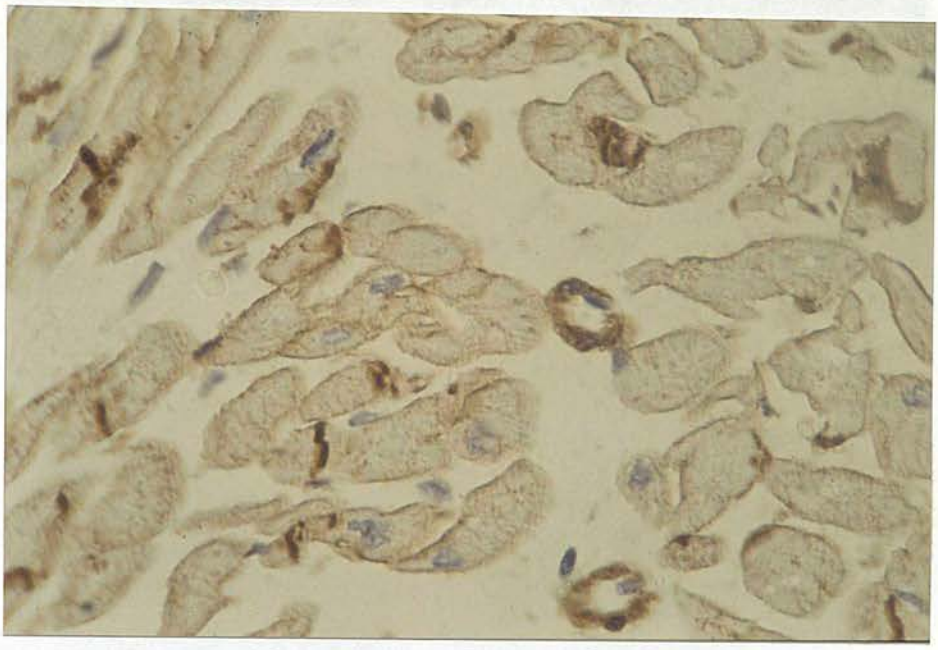


Figure 7.57 X40 Vinculin–Normal Myocardium.

Blood vessels show marked staining of their wall. The outer membrane staining of the myocytes is well seen focally as well as the densely stained ICDs .

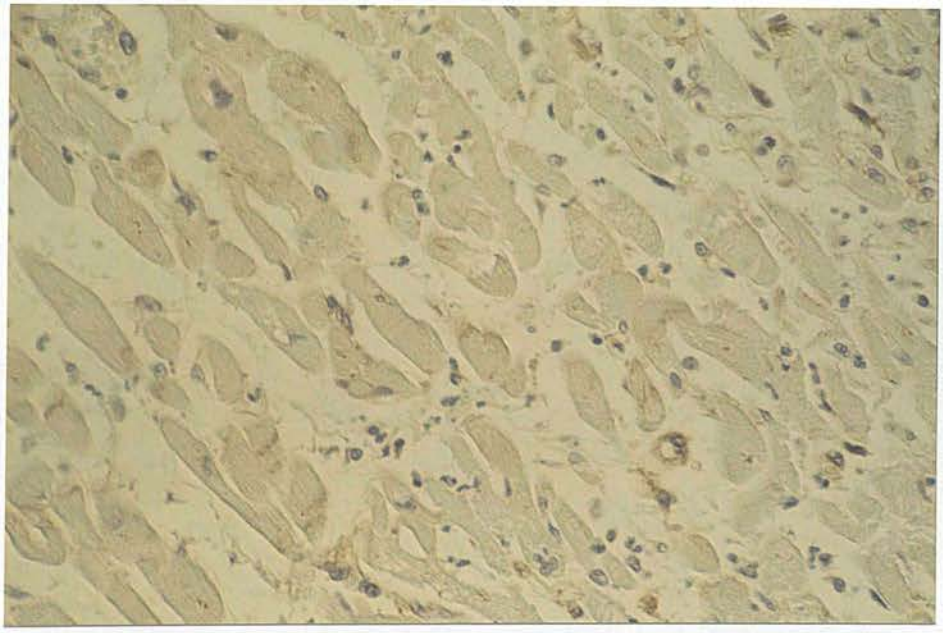


Figure 7.58 X25 Vinculin-Normal & Myocardial Infarction.

Loss of staining of the ICDs, vascular walls and other intracytoplasmic staining.

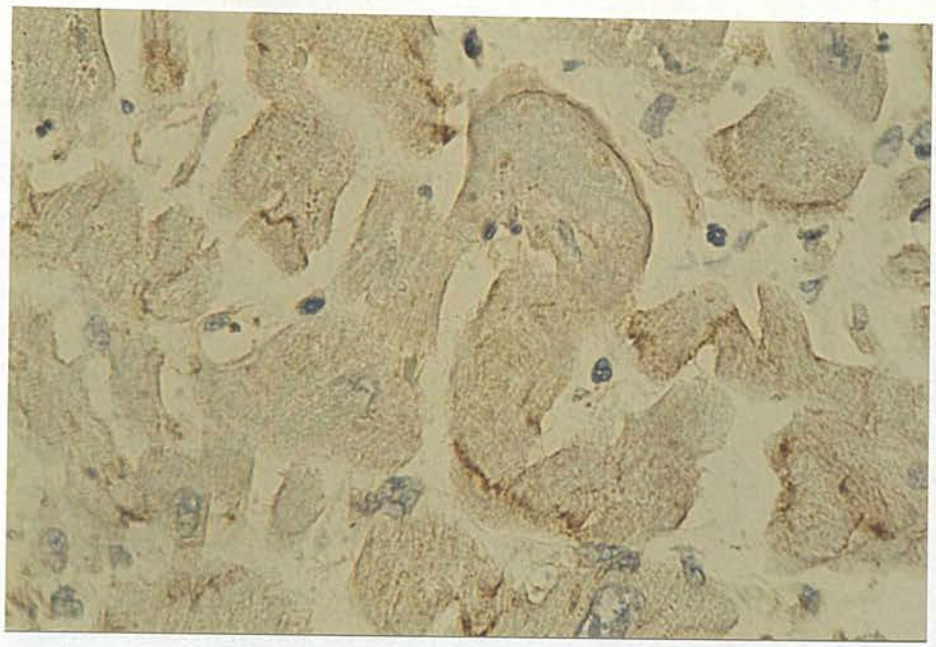


Figure 7.59X 40 Vinculin-Myocardial Infarction & Ischaemia.

Most of the necrotic myocytes show loss of intercalated discs staining. In some the ICDs staining show a fine irregular serrated pattern instead of a more regular dense pattern as found in normal cells. There is patchy discontinuous linear membranous staining.

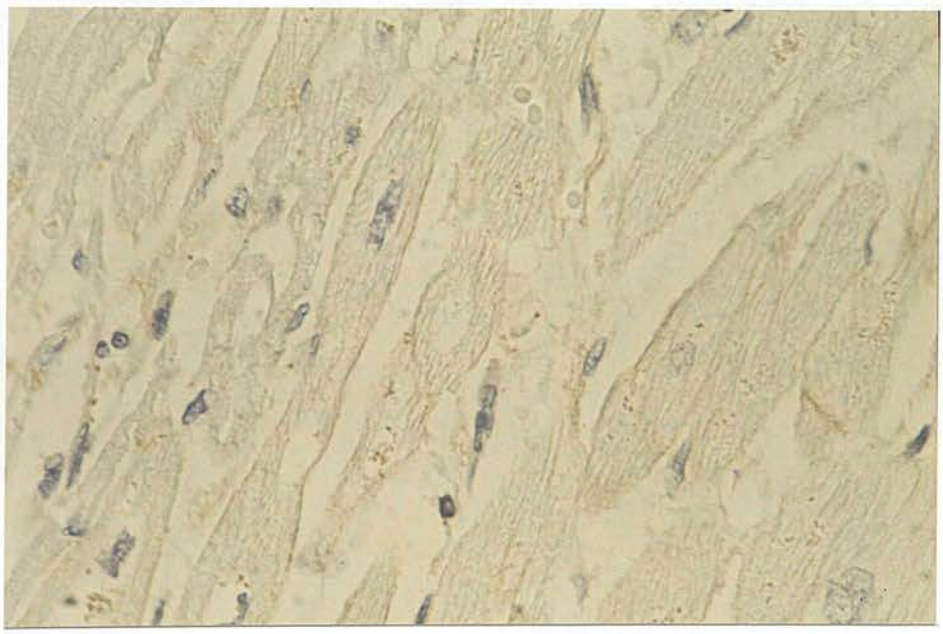


Figure 7.60X 40 Vinculin-Myocardial Infarction & Ischaemia.

The ischaemic myocytes show loss of ICDs staining. The myocytes on the right lower corner show that the ICDs staining has become more diffuse.

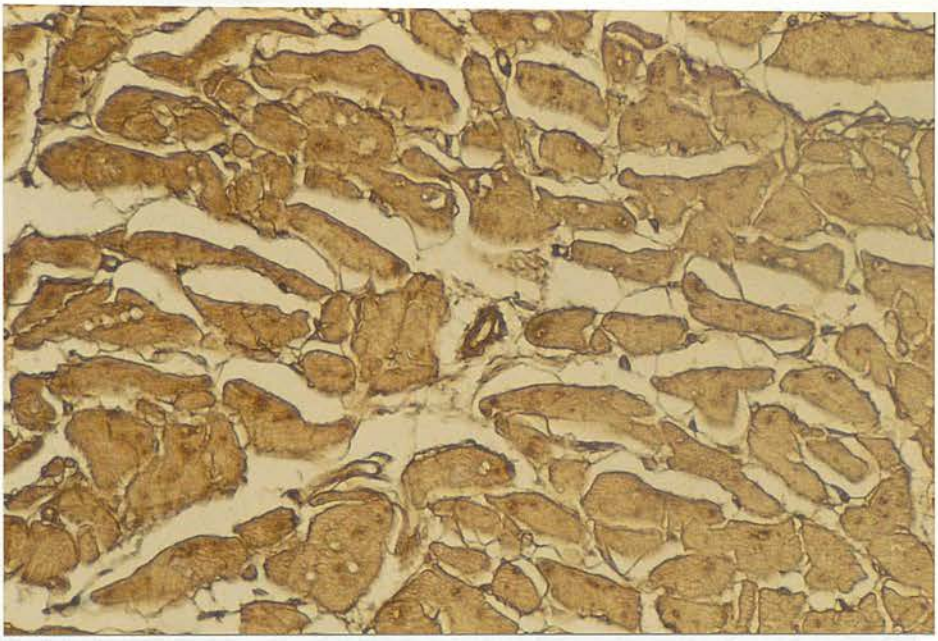


Figure 7.61 X25 Con A–Normal Myocardium.

The cardiac myocytes show diffuse cytoplasmic staining which in areas is vaguely striated. Staining of the outer cell membrane is also noted. Vascular wall staining is prominent.

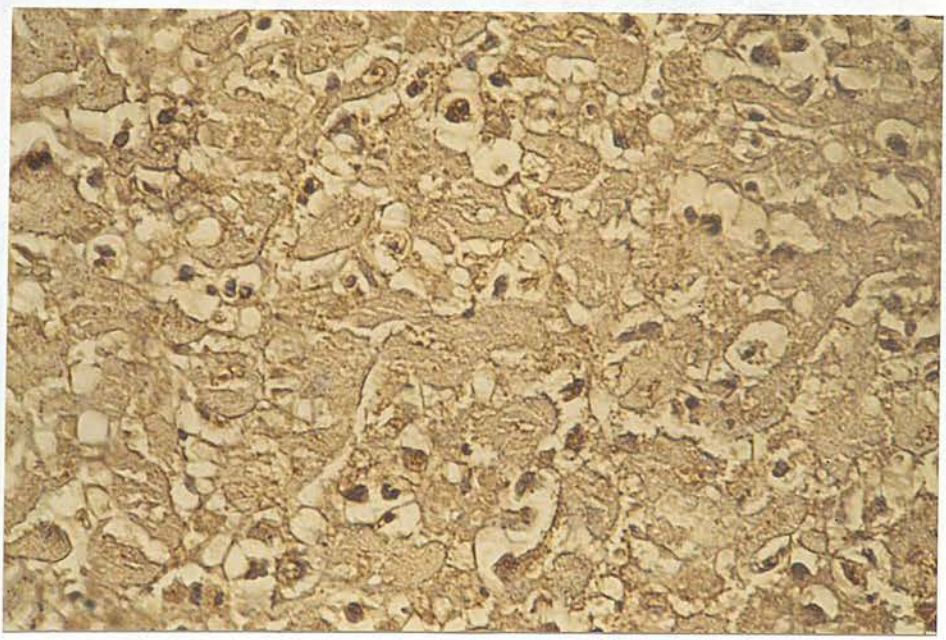


Figure 7.62 X40 Con A–Myocardial Infarction.

Most of the necrotic myocytes show an almost total loss of cytoplasmic staining. There is some focal membranous staining and residual particulate staining in parts of the cytoplasm.

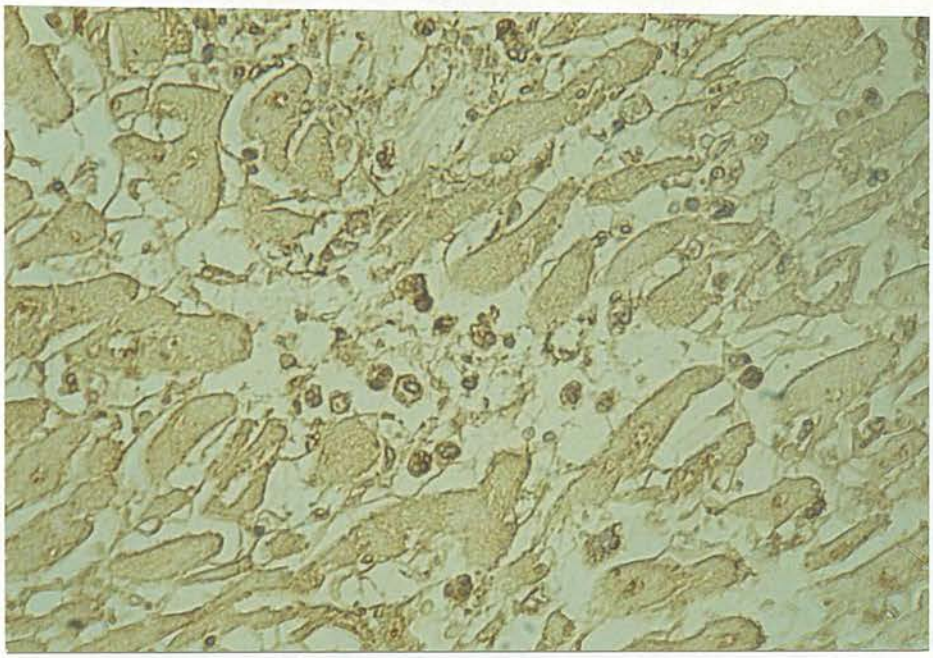


Figure 7.63 X40 Con A Myocardial Infarction.

There is complete loss of cytoplasmic staining of the infarcted myocytes. Their external membranes still retain focal staining.

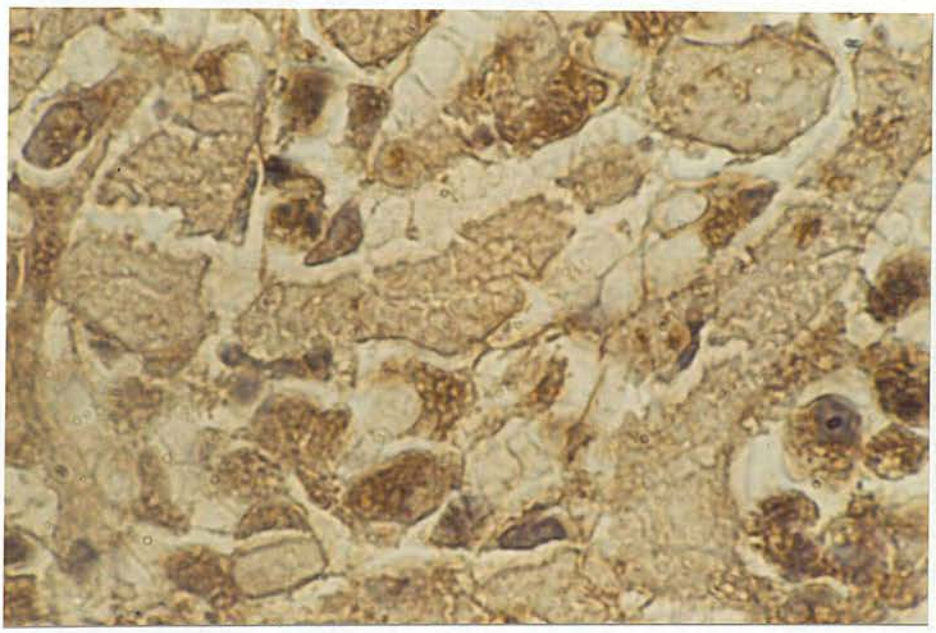


Figure 7.64 X100 Con A-Myocardial Infarction.

The necrotic myocytes show the marked disruption of the continuity of the cell membrane. The inflammatory cells in contrast show cytoplasmic staining.

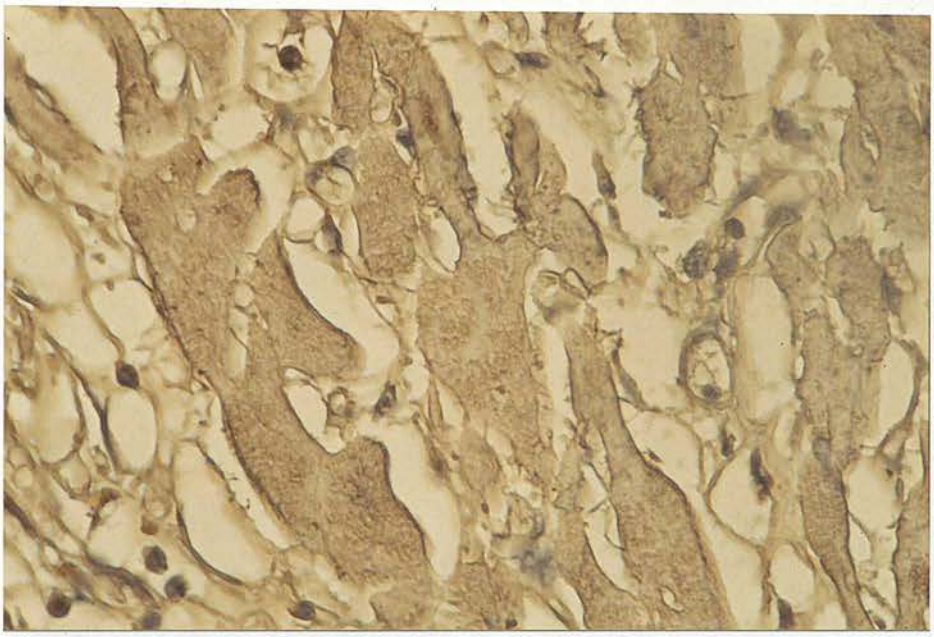


Figure 7.65 X40 Con A-Ischaemic myocardium.

The ischaemic cells show a decreased intensity of cytoplasmic staining and patchy outer membranous positivity.

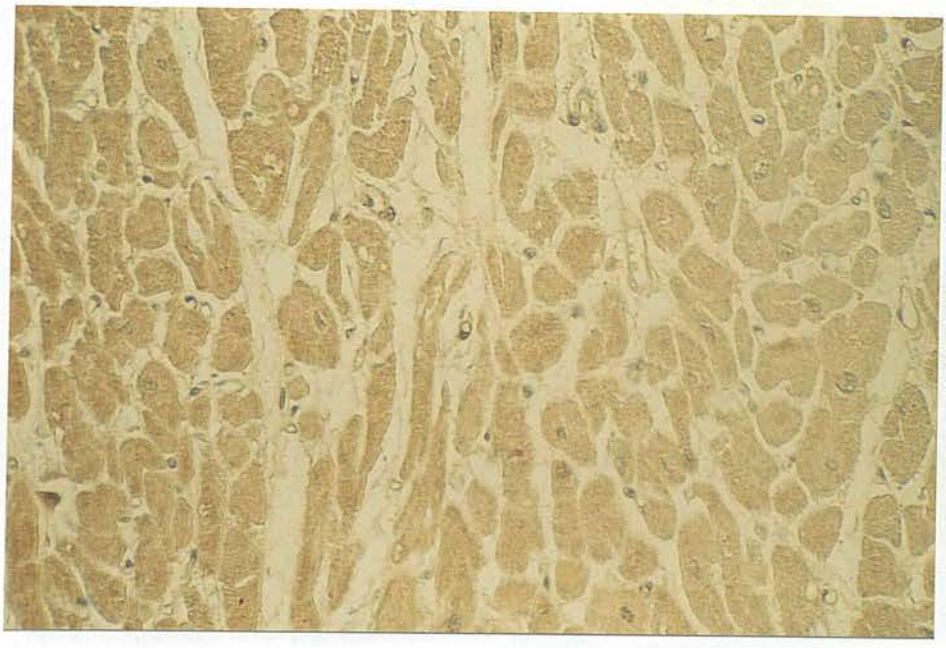


Figure 7.66 X25 DBA–Normal Myocardium

Diffuse cytoplasmic staining is present and is accentuated focally.

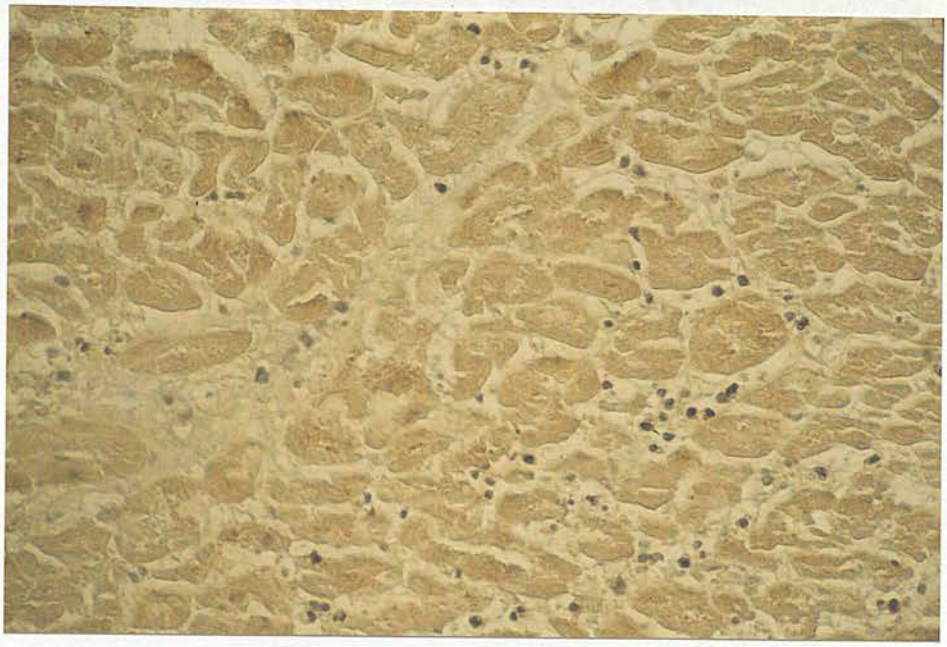


Figure 7.67 X25 DBA-Myocardial Infarction.

Necrotic myocytes with marked loss of cytoplasmic stain. The capillaries also show absence of staining.

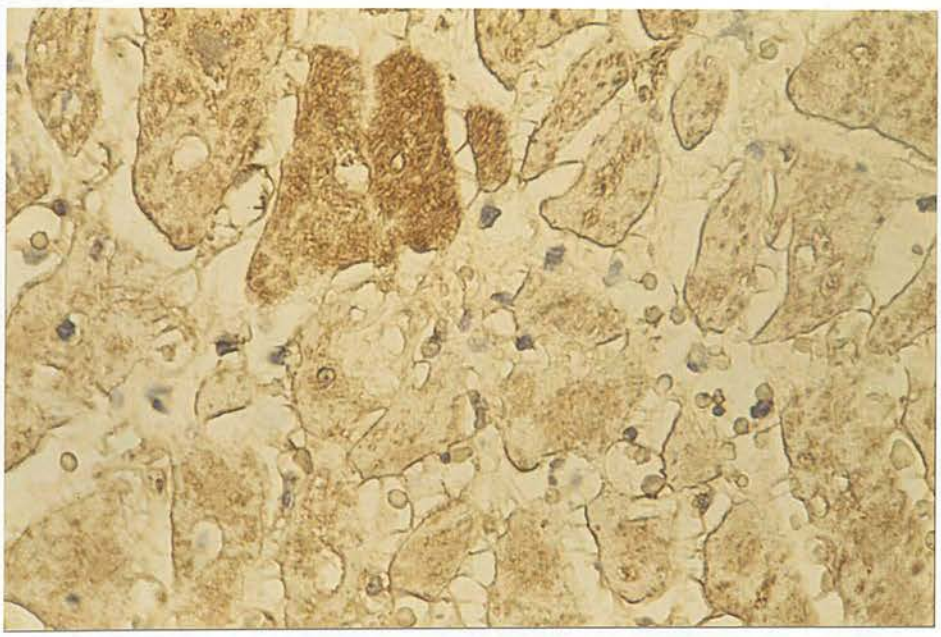


Figure 7.68 X40 DBA-Myocardial Infarction.

Focal discontinuous membranous staining of myocytes. Isolated within infarcted zones cells show diffuse granular cytoplasmic staining.

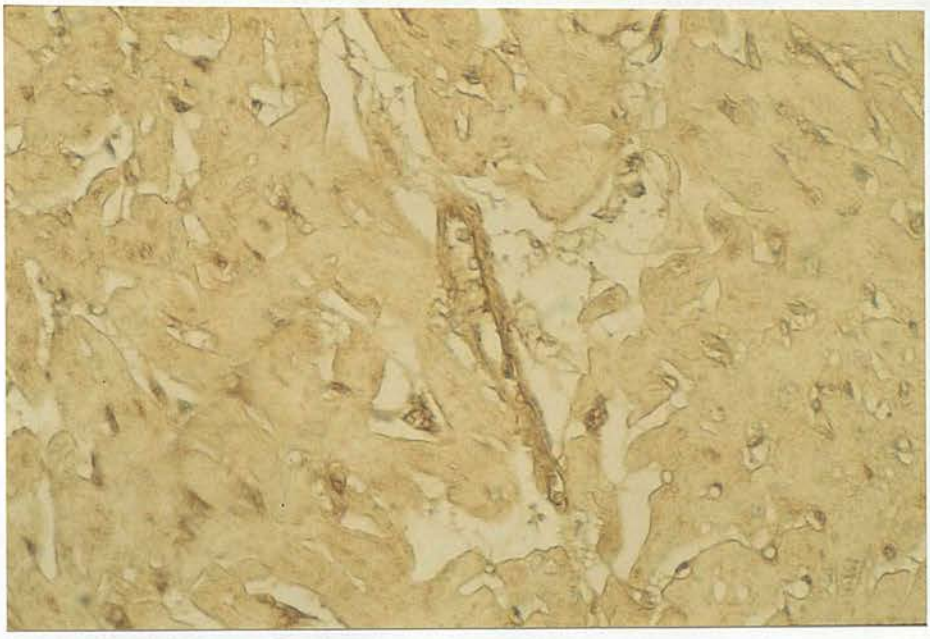


Figure 7.69 X25 PNA–Normal Myocardium.

Bland, almost hyalinised, cytoplasmic staining as well as focal membranous staining of the myocytes.

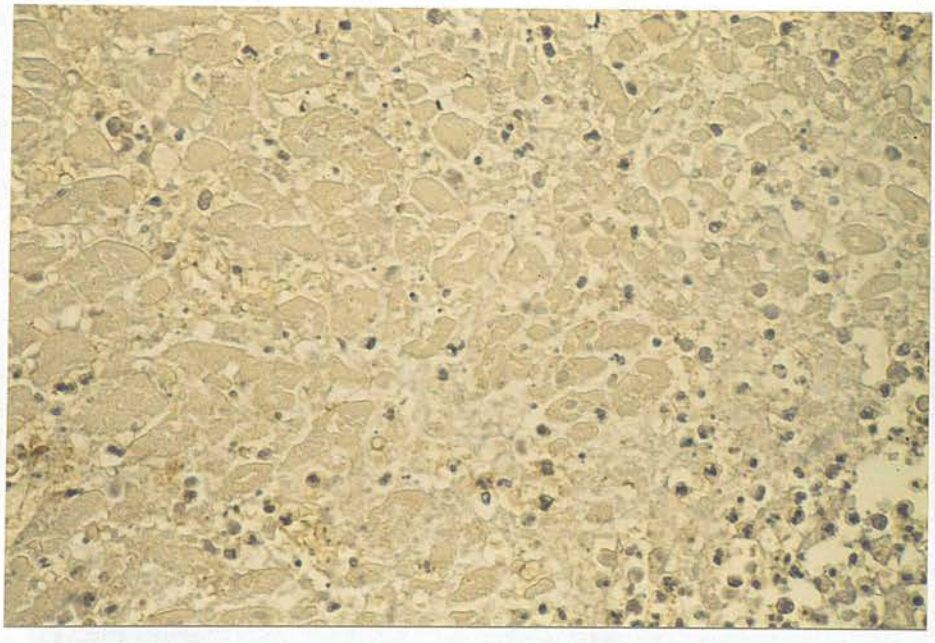


Figure 7.70 X25 PNA–Myocardial Infarction.

The necrotic myocytes show almost complete loss of cytoplasmic staining. The inflammatory cells show granular cytoplasmic staining.

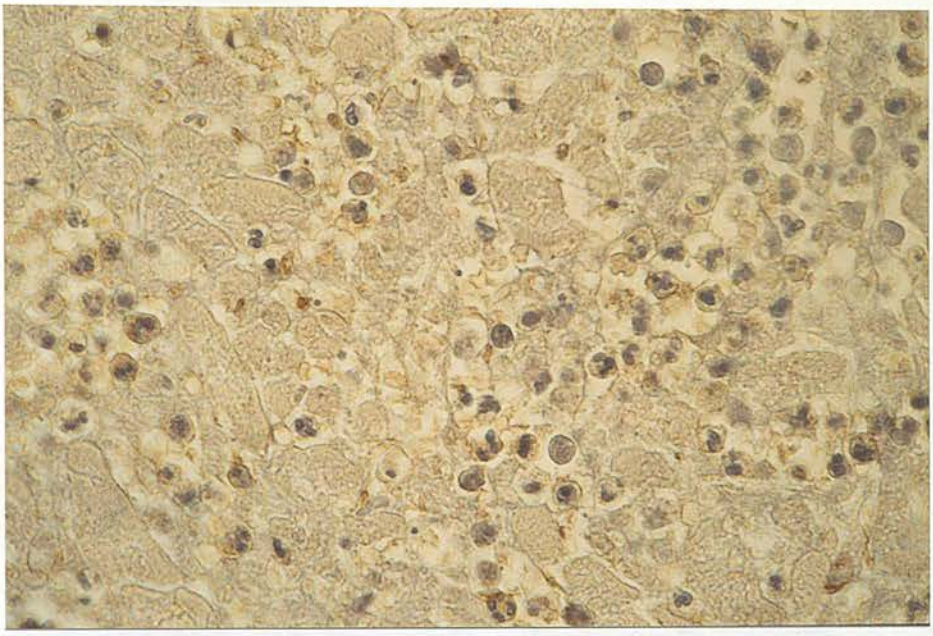


Figure 7.71 X40 PNA–Myocardial Infarction.

The necrotic myocytes show a similar picture to that seen in the previous figure but at high power - almost complete loss of cytoplasmic staining. The acute inflammatory cells show granular cytoplasmic staining.

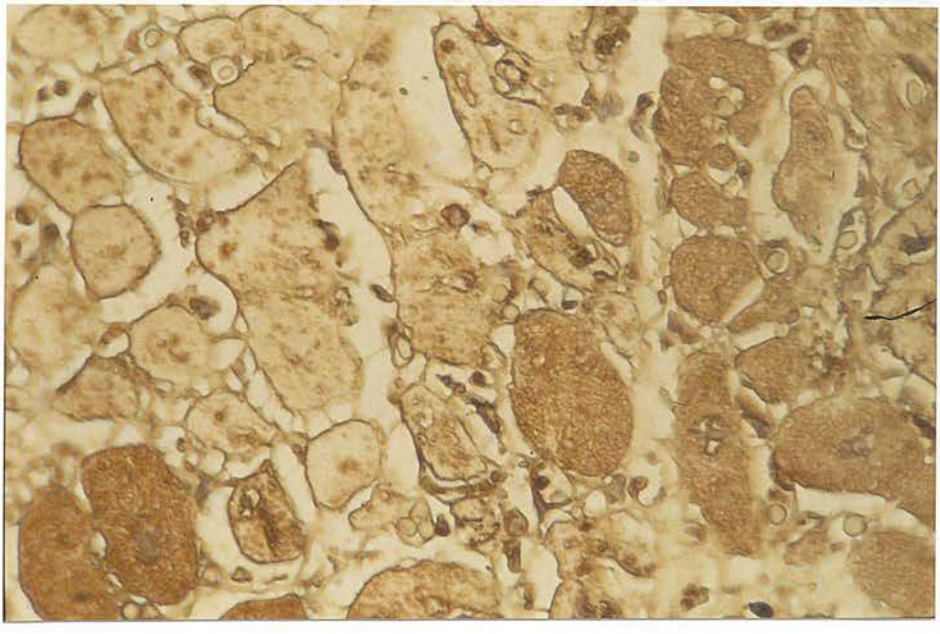


Figure 7.72 X40 PNA–Myocardial Infarction & Ischaemia

The necrotic myocytes (left upper side) show only very minimal residual staining contrasting with diffuse punctate non-patterned cytoplasmic staining of the non-necrotic myocytes.

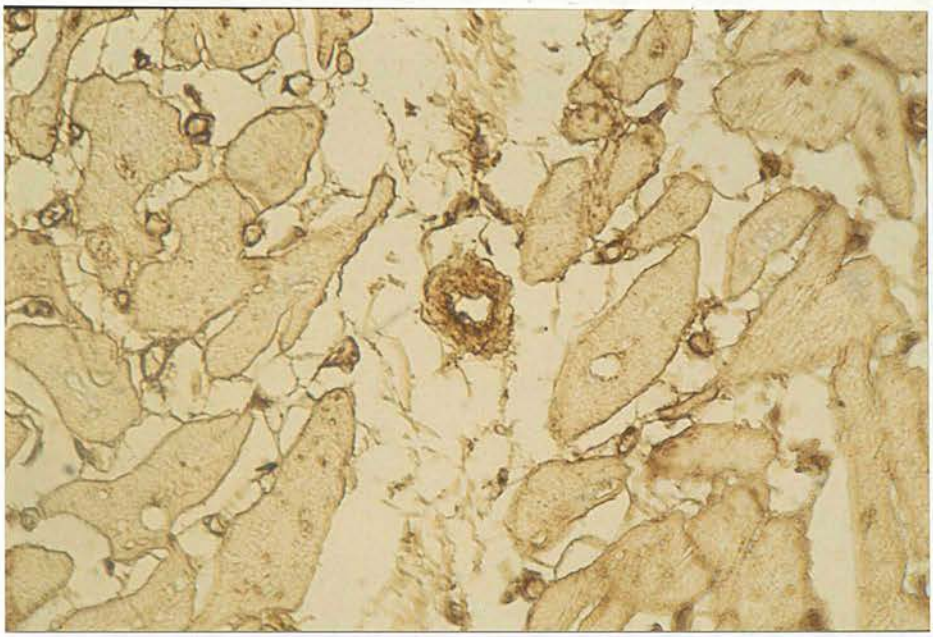


Figure 7.73 X25 RCA–Normal Myocardium.

Linear, thin external membranous staining of the myocytes. The muscular arterioles and capillaries show positive endothelial staining.

Figure 7.74 X40 RCA–Normal Myocardium.

The myocytes show linear membranous and striated cytoplasmic staining. The capillaries all show positive endothelial staining.

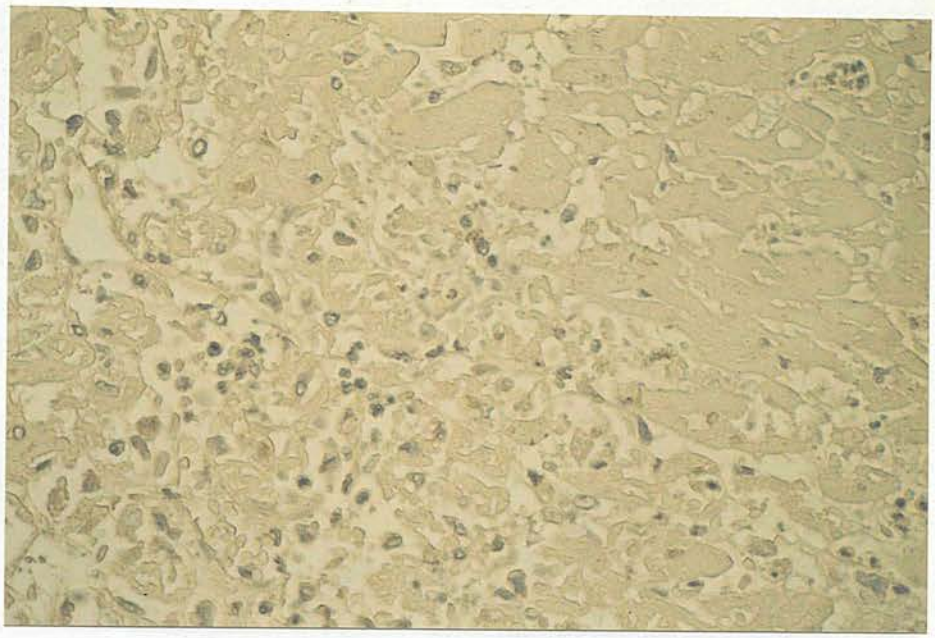


Figure 7.75 X25 RCA–Myocardial Infarction.

The myocytes in the infarcted zone show total depletion of positive stain.

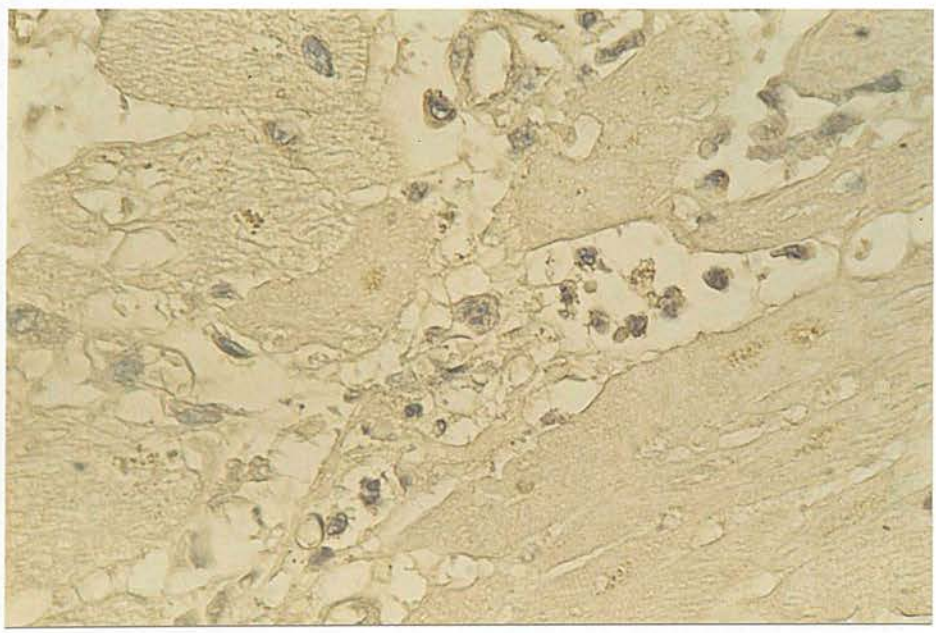


Figure 7.76 X40 RCA–Myocardial Infarction.

Focal membranous staining is seen in some myocytes. The polymorpho-nuclear neutrophils show cytoplasmic stain.

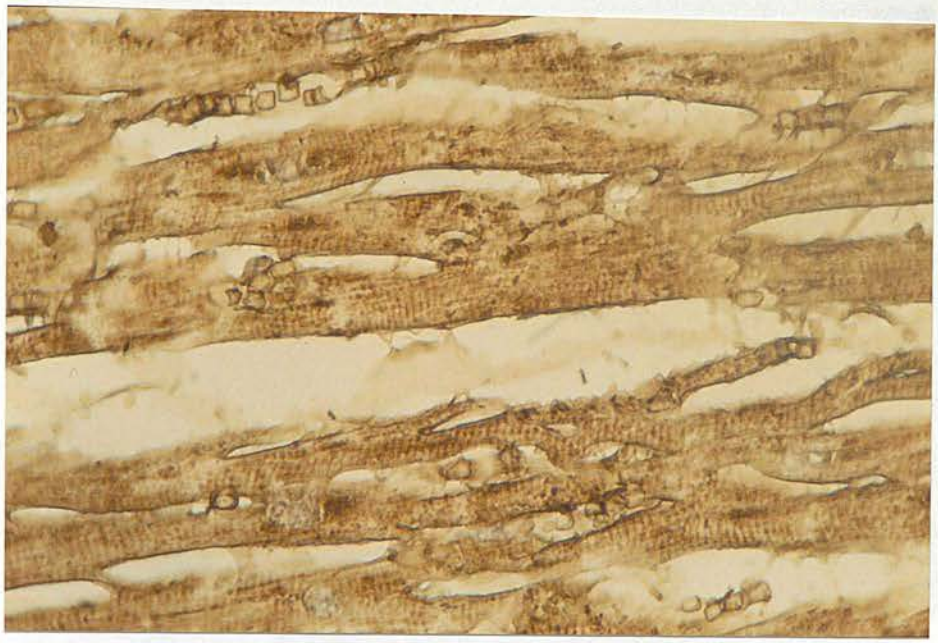


Figure 7.77 X40 SBA–Normal Myocardium.

The myocytes show prominent membranous and striated cytoplasmic staining.

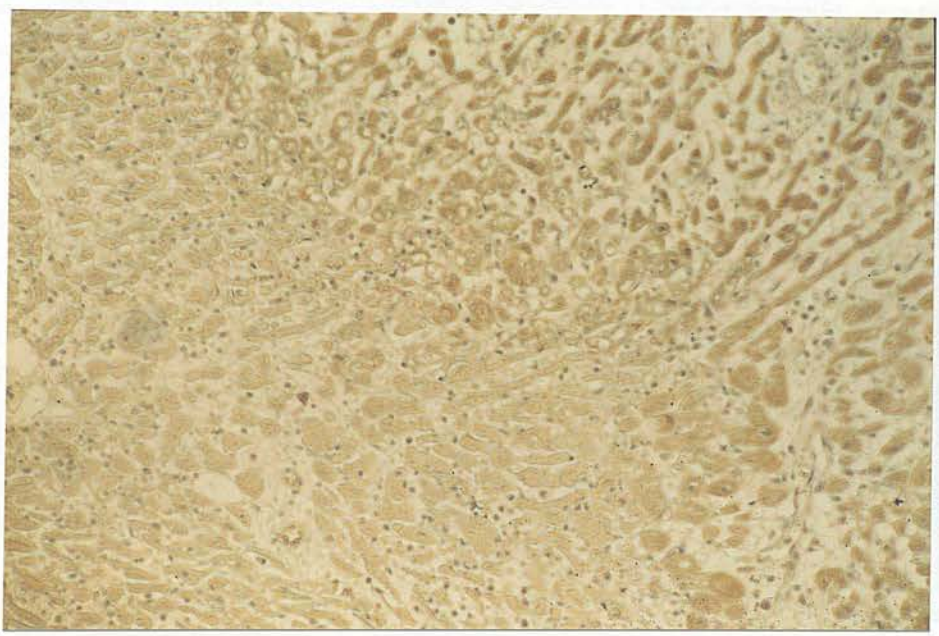


Figure 7.78 X10 SBA-Myocardial Infarction.

Edge of myocardial infarction with gradation between necrotic myocytes (on the left lower half) with no staining, and the ischaemic myocytes (on the right upper half) with residual cytoplasmic staining.

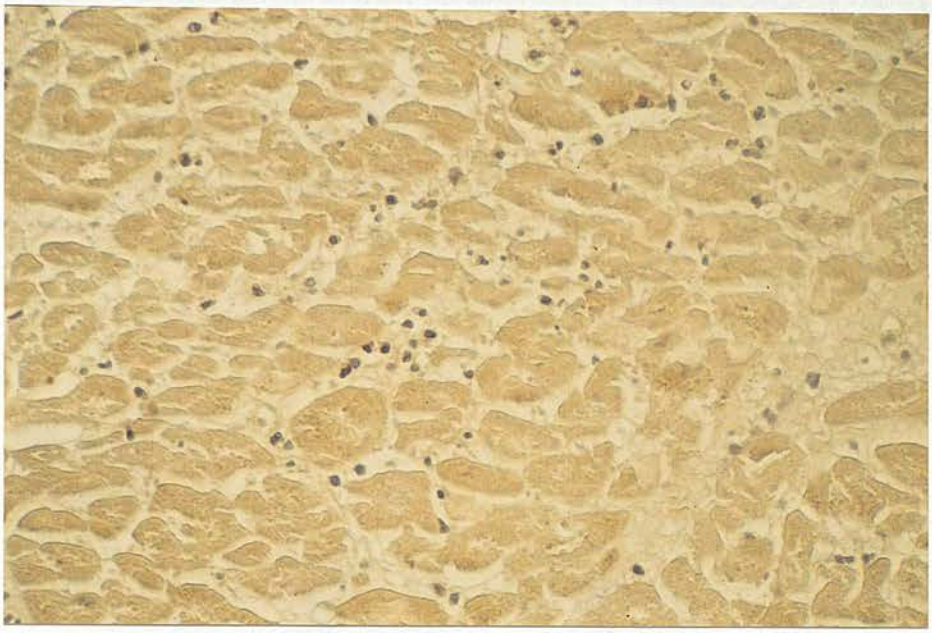


Figure 7.79 X25 SBA-Myocardial Infarction.

The necrotic myocytes show marked depletion of their cytoplasmic staining and loss of striated pattern of staining.

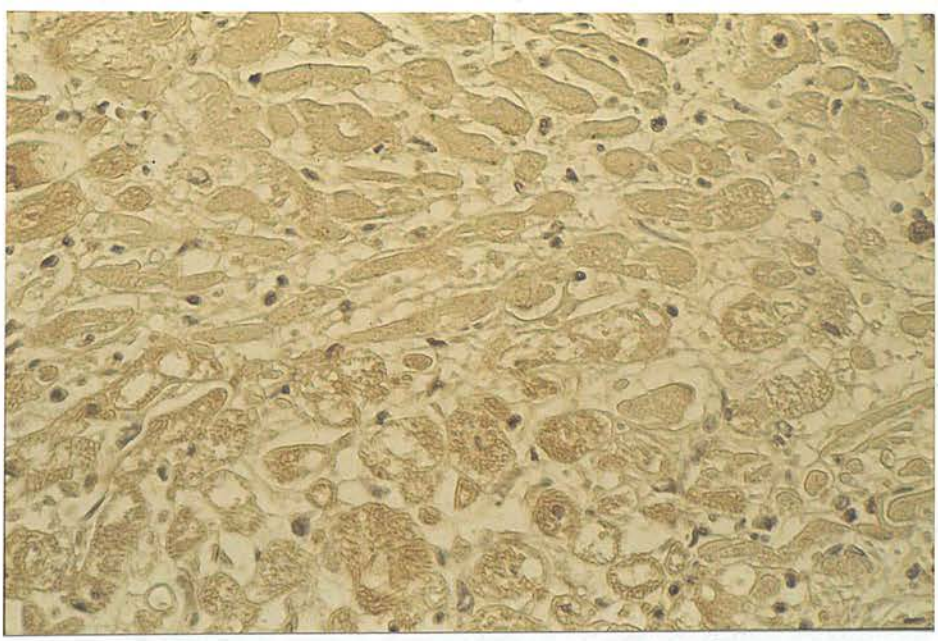


Figure 7.80 X25 SBA-Myocardial Ischaemia.

Graduation from myocytes (ischaemic) with granular cytoplasmic staining to necrotic fibres with a depleted stain.

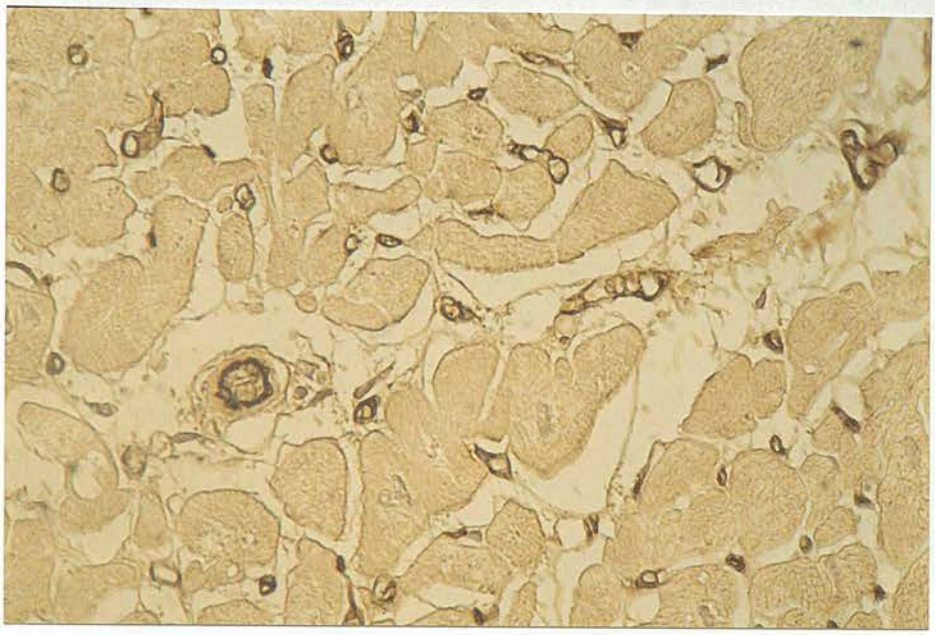


Figure 7.81 X25 UEA–Normal Myocardium.

The myocytes show faint granular cytoplasmic and external membranous staining. The blood vessels including the capillaries show positive endothelial staining. Red blood cells are also stained.

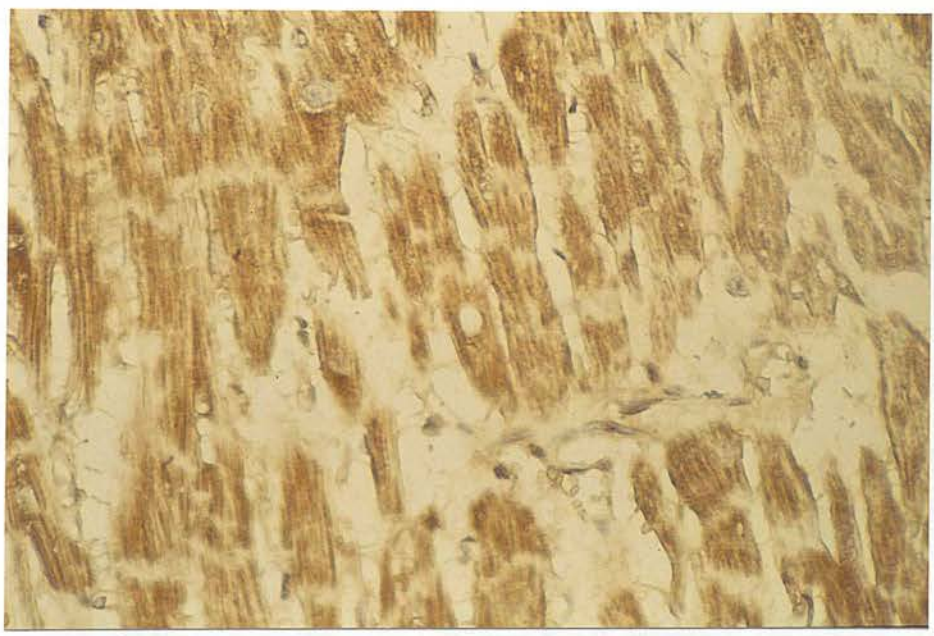


Figure 7.82 X25 UEA–Normal Myocardium.

The myocytes show longitudinally accenuated and aligned positive staining. A few myocytes show vacuoles within their cytoplasm.

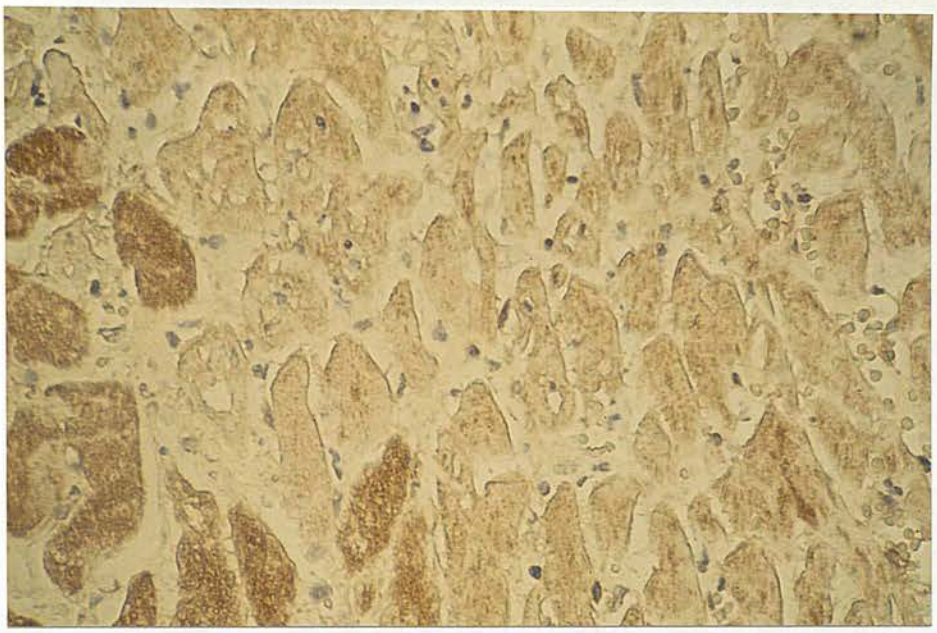


Figure 7.83 X25 UEA–Normal Myocardium & Myocardial Ischaemia.

Few normal myocytes (on the left mid and lower corners) show granular focally striated cytoplasmic and also membranous staining. The remaining ischaemic myocytes show marked depletion of the stain.

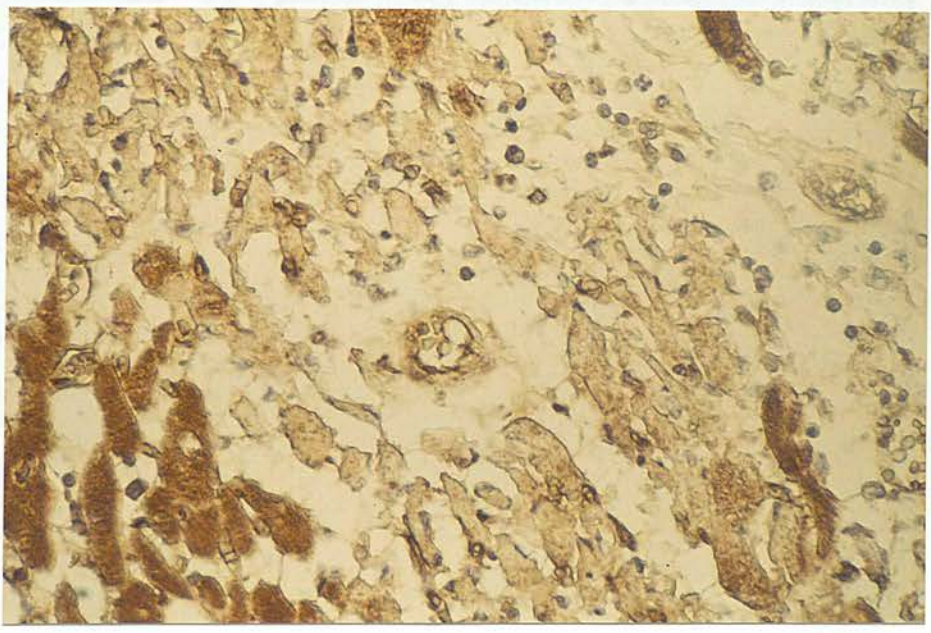


Figure 7.84 X25 UEA-Myocardial Infarction & Ischaemia.

The necrotic myocytes show almost total loss of cytoplasmic staining. Vascular staining is maintained but is focal and discontinuous. This contrasts with normally stained myocytes on left lower corner.

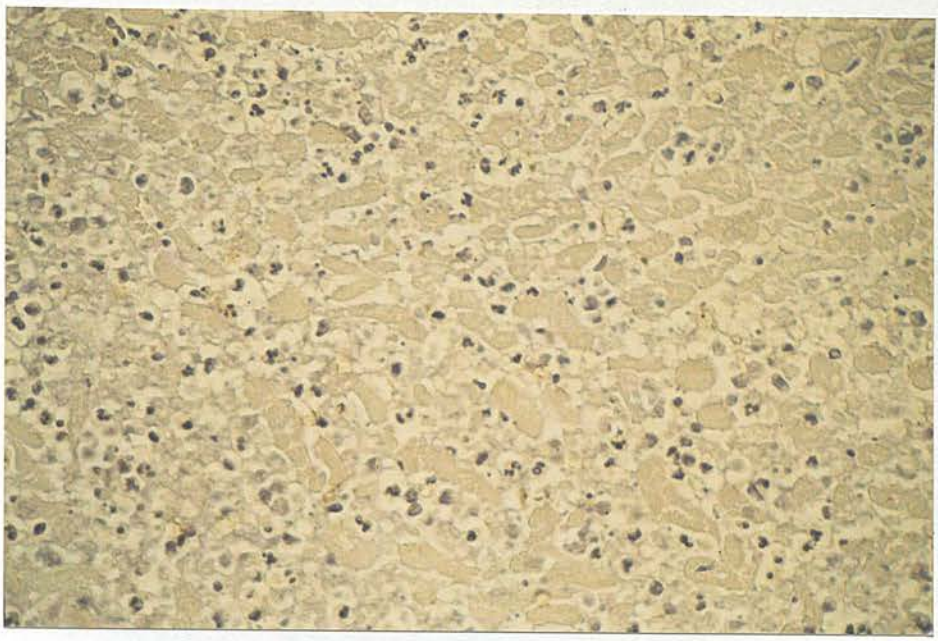


Figure 7.85 X25 UEA-Myocardial Infarction.

All the cytoplasmic components show loss of staining except for a focal linear membranous in some necrotic myocytes and capillaries.

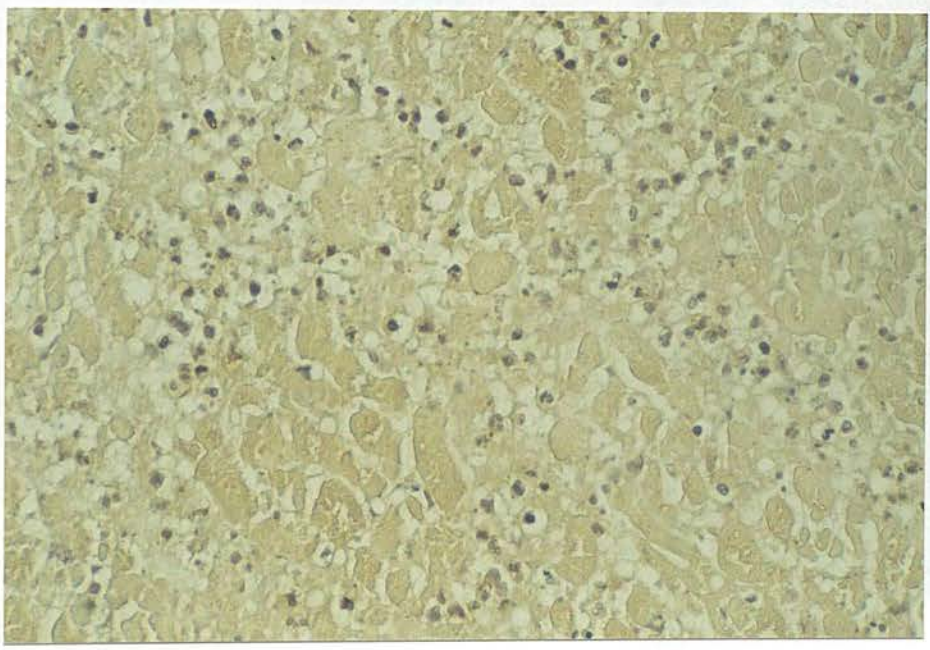


Figure 7.86 X40 UEA-Myocardial Infarction.

As figure 85.

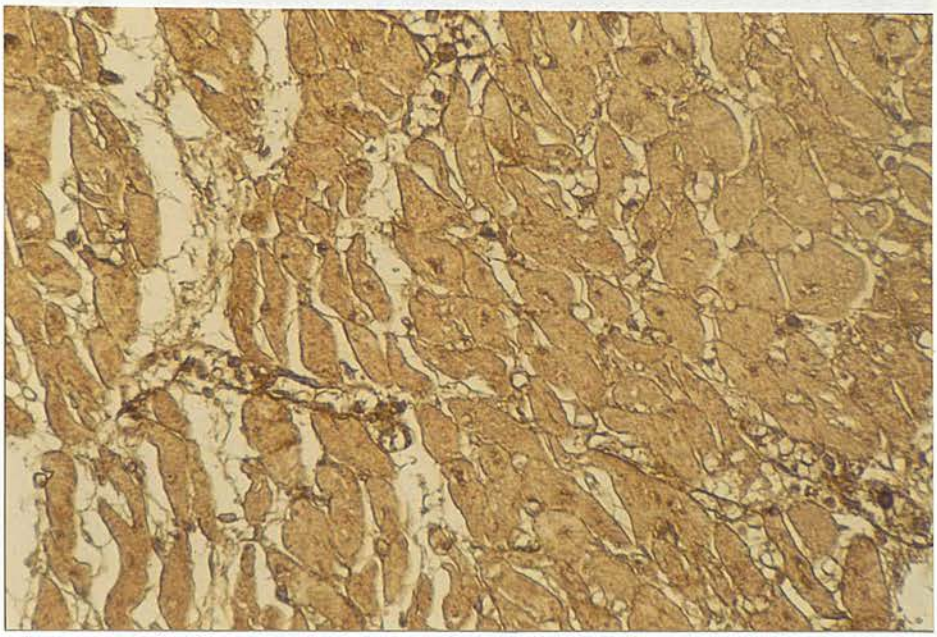


Figure 7.87 X25 WGA–Normal Myocardium.

The myocytes show bland granular cytoplasmic staining and thin membrous staining.

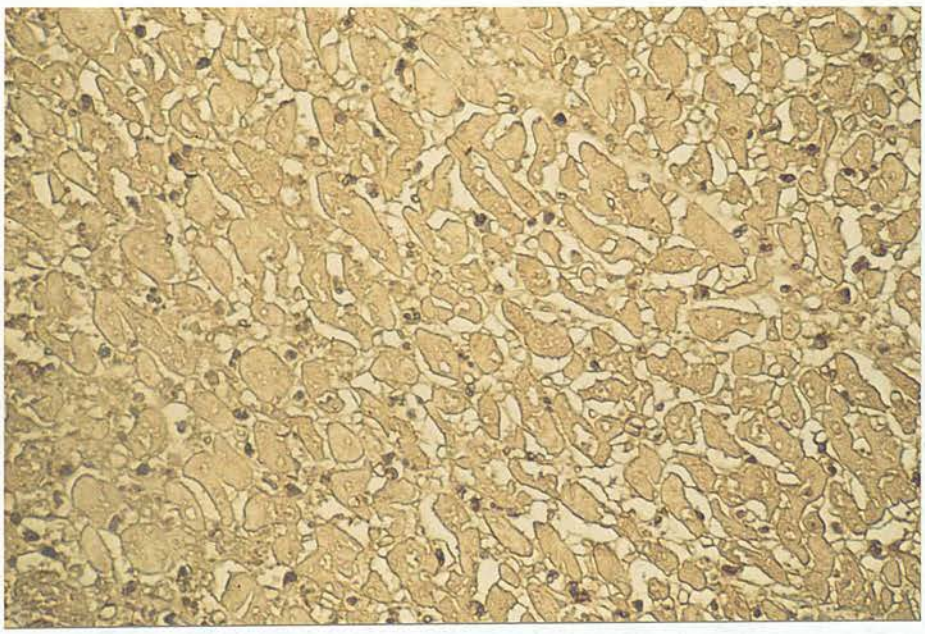


Figure 7.88 X25 WGA-Myocardial Infarction.

Myocytes damaged within an infarct show some decrease in the intensity of cytoplasmic staining. There is still prominent membranous stain.

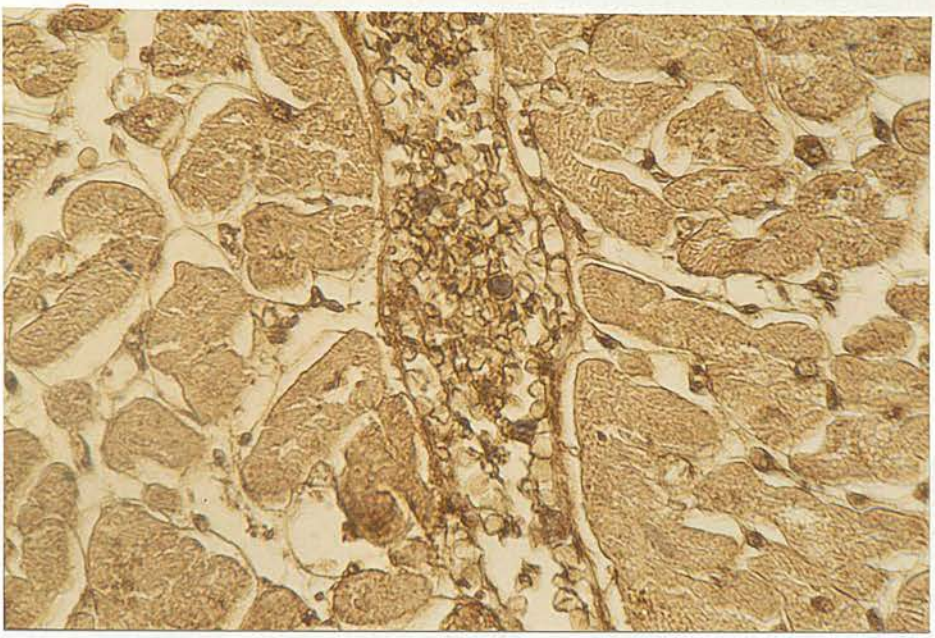


Figure 7.89 X40 WGA Myocardial Ischaemia.

The myocytes which are not frankly infarcted but ischaemic show some depletion of their staining but not to the degree seen within infarcts.

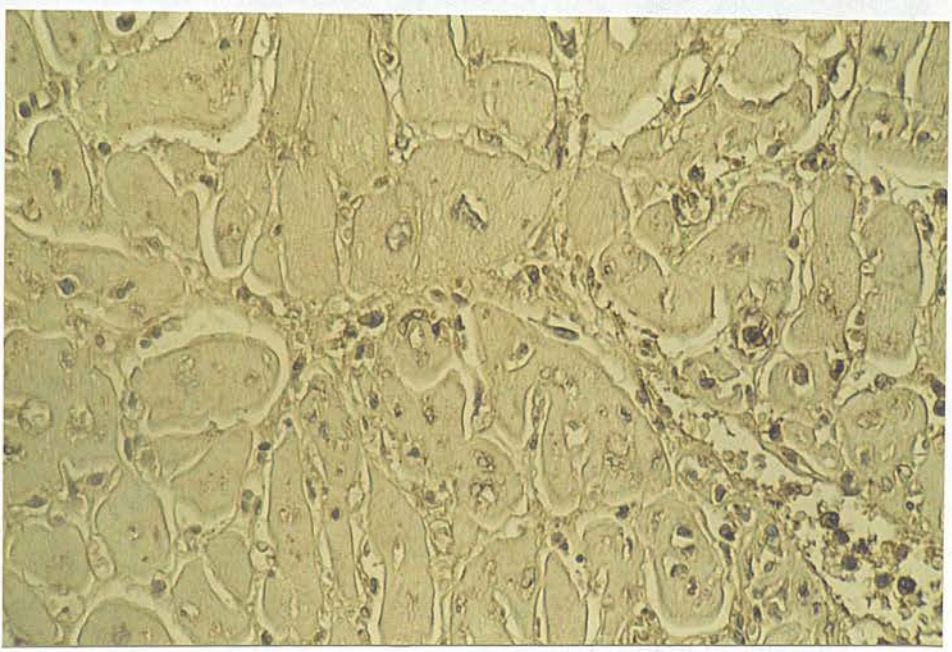


Figure 7.90 X25 WGA Myocardial Ischaemia.

The only positive staining present is on the outer membranes of these ischaemic myocytes.

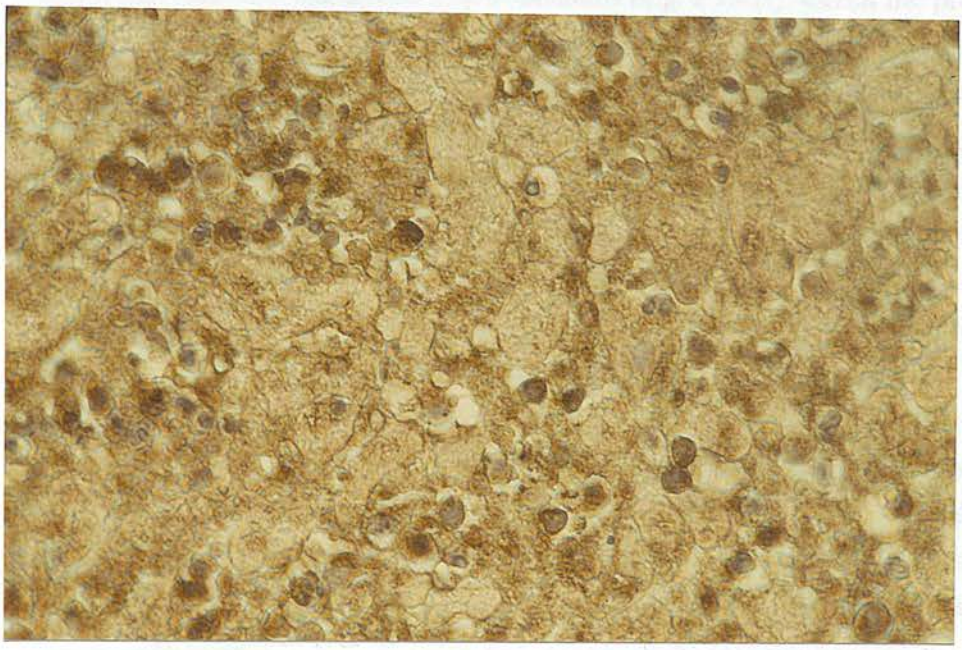


Figure 7.91 X40 WGA Myocardial Infarction.

The staining of necrotic myoctes in an infarct is diffuse and limited in its intensity causing a diffuse variegated pattern of intensity. Cell walls can be identified only in some areas but actual delineation of myocytes is not possible. Inflammatory cells retain their cytoplasmic intense staining as well as red blood cells.

CHAPTER 8.

DISCUSSION.

8.A. General Comments on Previous Studies.

A basic and recurring problem for the pathologist is the detection at post-mortem examination of early myocardial damage caused by ischaemia when such damage is not visible either to the naked eye or on histological slides which have been stained by ordinary H & E or by specialised tinctorial stains (e.g. PTAH). Given the predominance of ischaemic heart disease and atheroma worldwide, this problem is encountered wherever pathologists have to determine with accuracy the cause of death in instances of sudden unexpected, non suspicious deaths and in accidents where heart disease may be a complicating or a contributory cause. The position in which the pathologist often finds himself in is that coronary arterial occlusive disease is present and had obviously been present for some years, but no superimposed acute coronary arterial event (e.g. thrombosis, haemorrhage into a plaque) to account for the sudden demise is identified and no other cause of death is found after an extensive autopsy.

Over the years attempts have been made to devise histological stains which by selectively staining acutely ischaemic areas would be able to identify such sites specifically and thus provide direct evidence of the acute event preceding and causing death. Among these can be classed the Lie's stain (HBFP) (Lie et al., 1971) and Arnold's stain (the modified LFB) (Arnold et al., 1985).

Evaluation of these staining methods by many workers in both human hearts and in models of myocardial ischaemia in a variety of experimental animals have come to the conclusion that these methods are neither reliable nor sensitive (Zugibe & Zugibe, 1973; Lichtig et al., 1975; Sakurai, 1977; Knight, 1979; Oehmichen et al., 1990). The research groups of the National Heart Hospital in London Nayar & Olsen (1974) and Al-Rufaie et al. (1983) were the exception to this general trend in that the Lie stain was shown by them to be useful and reliable in human autopsy cases.

Identification of early ischaemia by demonstration of ultra-structural changes, even in the animal experimental model, are plagued by inconsistencies and the ever-present problem of autolytic changes being mistaken for ischaemic damage. Originally, electron microscopy (EM) was said to be capabable of detecting foci of necrosis as early as 20 minutes after coronary arterial occlusion (Caufield & Klionsky, 1959; Sommers & Jennings, 1964; Jennings et al., 1965; Sulkin & Sulkin, 1965; Herdson et al., 1965; Wolfe et al., 1968; Jennings, 1969; Jennings et al., 1969; Poche, 1969; Heggtveit, 1969; Kloner et al., 1974; Jennings & Ganote, 1974). The lengthy and complicated technical such procedures involved make the routine use of EM in the detection of MIs in autopsy material remote and largely inaccessible (Caufield & Klionsky, 1959; Knight, 1969).

8.B. Immunohistochemistry.

8.B.1. Structural Components of Myocytes.

These problems have led to a move away from conventional histology to immunohistochemistry particularly as the antibodies that could be used for this purpose which became available commercially are specific, relatively cheap and reliable. The assumption that forms the basis of these histochemical studies is that ischaemia leads to damage to the cytoskeleton, to organelles and to membranes of the ischaemically injured myocytes with loss of anatomical normal patterns, and displacement and diffusion of structural proteins from the myocytes into immediately adjacent connective tissue and vascular channels. The loss of the structural integrity of the myocytes induced during the acute ischaemic episode or in the immediate re-perfusion phase. Vascular changes are also to be expected in relation to the damage caused by the acute loss of blood supply to an area of heart muscle; to some extent also this may involve activation of the complement cascade locally in the early phase of anoxia-induced inflammatory response with leakiness of the vascular endothelial pores and the deposition of activated complement in the vicinity of the infarcted zone.

Attempts have also been made at identifying changes of the enzymatic patterns within myocytes which should in turn reflect the damage known to occur to organelles such as mitochondria with ischaemia and consequent hypoxia. These have also been evaluated in several morbid anatomical human studies and in experimental situations (Shnitka &

Nachlas, 1963; Fine et al., 1966). These procedures often require that blocks of myocardium are snap frozen, enabling the histochemical reactions to be carried on frozen sections. The method is laborious and well outwith the remit of most departments dedicated to forensic pathology. Furthermore a significant number of patients die well before three hours from sustaining the infarct. Other enzymatic based tests using CK, AspAT and LDH-1 have also failed to provide reliable tissue markers of ischaemia (Herscher et al., 1984; Siegel et al., 1984).

Attempts have also been made for spontaneous and eosin-induced fluorescence on tissue sections to be correlated with early ischaemic damage (Carle, 1981). These methods only require simple tinctorial stains and an UV fluorescence microscope and therefore, if discriminatory, would have been ideal. Unfortunately they were also shown to be lacking in specificity and are indeed over-sensitive with consequent major difficulties in the interpretation of the findings (Siegel & Fishbein, 1982; Al-Rufaie et al., 1983; Saukko & Knight, 1989).

Leadbeatter et al. (1989; 1990) used protein markers, specifically myosin, myoglobin, caeruloplasmin, C-reactive protein (CRP), pre-albumin, and α-1 anti-trypsin and $C3_b$ of the complement cascade. The results obtained were conflicting in terms of the these acute phase reactants (namely α-1 anti-trypsin, CRP, and pre-albumin): only α-1 AT was a strong positive marker of ischaemia and the third component of complement ($C3_b$) was found to be unreliable. Myoglobin and myosin which are internal components of normal myocytes and are known to be released into the blood stream within a few hours of infarction (Kagen et al., 1975; Léger et al., 1990). The depletion of these markers from damaged cells was found to be useful but not with full reproducibility for every single case (Leadbeatter et al., 1989).

This led to the suggestion that these antibodies are identifying non-specific changes which were found in the heart muscle as a consequence of circulatory arrest and cardiac asystole i.e. these changes reflect death as a phenomenon but not specifically ischaemia-induced changes, they may possibly represent the effects of resuscitation. Such potential non-specificity could therefore cause problems with interpretation (Leadbeatter et al., 1990).

Brinkmann et al. (1993) in order to circumvent these problems applied and contrasted tinctorial methods (Lie's and Arnold's stains) and a battery of immunohistochemical markers namely: myoglobin, desmin, fibrinogen and $C5_b$-9. The results of these studies showed that the selected application of one single staining method in isolation whatever that was, did not improve the diagnosis of early myocardial ischaemic damage: a whole series of tinctorial stains and immunohistochemical markers are more useful and allow a more specific delineation of infarcted zones in comparison to the use of isolated markers.

The scope of the study reported in this thesis was to work on the same principles outlined above hoping that by immunohistochemical staining with a battery of antibodies, areas of acute ischaemia could be demonstrated reliably and reproducibly. Myocyte components originating mainly from the cytoskeleton, were specifically chosen for this study in an attempt at demonstrating the earliest ischaemic changes.

This study involved a bank of 13 specific antibodies and this is the largest and more comprehensive bank of antibodies to be used in any study of this type.

The use of myocardial infarcts in the control series allowed an in-built control in that within the immediate peri-infarct zones, on the lateral borders of the actual infarct zone, it is likely that some non-necrotic ischaemic damage of myocardial fibres would be present. The suggestion for the use of such controls was that if the changes which could be demonstrated in these areas, could also be observed in areas of non-infarct ischaemia then such changes could be specifically looked for in those autopsies in which the likeliest cause of death was non-infarct acute ischaemia. This assumption is based on observations made in animal models where in infarcts a gradation of damage can be observed ranging from necrosis to normality; however this peri-infarct zone of ischaemia is very variable in its location and extent, and may also be entirely absent completely in humans, and as such although useful as a control it is not incontrovertible.

Actin was one of the myocytic components which was studied. Actin filaments are distributed throughout the cytoplasm and they are associated with several other proteins that enable these filaments to perform their specific functions and serve to anchor many of the proteins crucial to the cell's survival and integrity (Roberts, 1994). Indeed Hashimoto et al. (1986) had already explored the stability of this structural protein and showed that

there was little change in the reactivity and degree of intensity of staining for actin in relation to post-mortem interval even up to 28 hours, suggesting that this structural protein is resistant to autolysis, this being an added bonus in autopsy tissue. The latter also demonstrated that this protein is lost from myocardial fibres sometime within a two hour period of infarction supervening. Nishida et al. (1987) showed in the experimental rat model of myocardial infarction, sporadic losses of anti-actin reactivity within the 30 minute period from the time when ischaemia was induced; as further time elapsed and there was a survival period after the onset of ischaemia, the degree of negativity of fibres for actin increased progressively. In our study, the observation is that actin staining is progressively less prominent as ischaemia turns into an infarction. This is completely in keeping with the experimental observations and indeed, actin was one of the best antibodies to demonstrate damage in the heart due to decreased blood supply.

Tsukada et al. (1987) developed a monoclonal antibody against actin and designated it as HHF35, this antibody recognizes a common epitope of α-cardiac, α-skeletal, α & γ smooth muscle actin isotypes, even in fixed, paraffin-embedded tissue. Although the HHF35 antibody has been used in several studies in relation to differentiation, diagnosis and classification of soft tissue tumours (Tsukada et al., 1987; Miettinen & Rapola, 1989), no study has utilised HHF35 actin antibody in the study of ischaemic and/or infarcted myocardial tissue (Rangdaeng & Truong, 1991). The actin which was used in this study has been produced from a similar tissue culture source.

Desmin is another cytoskeletal protein of myocardial cells which has been looked at in ischaemic hearts. Desmin can be equally well demonstrated by antibodies in tissues which had been fixed in formalin, Zenker's, or Bouin's fluid, particularly with prior pronase digestion. Desmin staining was evaluated by the antibody-DAKO-Clone DER11 which is an antibody chosen specifically because it reacts with an epitope highly resistant to adverse treatments such as prolonged fixation or decalcification (Truong et al., 1990; Rangdaeng & Truong 1991).

The desmin filament is responsible for the transmission of the stress and strain of the contractual force between cardiac myocytes (Roberts, 1994). It has a periodic staining pattern similar to α-actinin, but the bands of intense staining were broader, and no intensification of staining at intercalated discs (Ganote & Vander-Heide, 1987). In cardiac

myocytes, desmin filaments connect desmosomes from one muscle cell to another and form the scaffold for both the Z-disc and the myofibrils.

Brinkmann et al. (1993) have used antibodies against desmin among his immunohistochemical markers. Desmin and myoglobin, in general, reacted very much in parallel, and a significant loss of positive desmin staining was demonstrated with the onset of infarction. In this study reactivity with desmin gave very much the same pattern of staining. Loss of desmin staining was also shown to be as intense as that for actin. This was the case both in the peri-infarct zones and also in the areas where acutely fatal non-infarct ischaemia was likely to be present.

Myosin has been another cardiac myocyte component which has been investigated not only by simple immunohistochemistry, but also by 'in situ' studies using labelled cardiac-specific antibody to myosin bearing a radio-active label (Haber et al., 1982). Different iso-myosins have also been studied in an attempt at rendering the method even more specific (Yazaki et al., 1984; Fulghum et al., 1986; Hoffmann et al., 1987). In the two studies by Leadbeatter et al. (1989; 1990) myosin was one of the antibodies which was employed in the study of myocardial ischaemia. Loss of staining was identified in infarcted zones and the suggestion was that this immunohistochemical marker was, along with myoglobin, a useful 'in situ' negative marker of myocardial ischaemic/hypoxic damage (Leadbeatter et al., 1989; 1990).

The present study shows a capricious reaction with this antibody. The variability in the staining with it may to a large extent be related to the lack of commercial availability of cardiac myocyte-specific anti myosin. Indeed in the study being reported, anti-skeletal muscle myosin antibody had to be used. This has made the interpretation of the results with this antibody even more fraught. This specific antibody may be available within the next few months, and when this is available commercially, it could then be important to study its specificity and usefullness.

Myoglobin is another essential intracytoplasmic cardiac muscle component. Kent (1982) and Block et al. (1983) showed immunohistochemically that an early diffusion of myoglobin from dog myocardium occurred following coronary artery occlusion. Nomoto et al. (1987) confirmed this observation in other studies in a rat model. The rationale

behind the use of myoglobin as a marker is that given the low molecular weight of myoglobin (17,000 daltons, Kagen, 1978) it diffuses through the cell membrane that is not already deteriorated. Accordingly, the loss of myoglobin from the myocardium could be considered to indicate myocardial membrane wall i.e. intrinsic cellular injury. Fujiwara et al. (1988) showed that the pig myocardial ischaemic cells exhibited a definite myoglobin defect 4 hours after occlusion of the coronary artery, therefore, it was concluded by them that the myoglobin antibody immunohistochemical method using formalin-fixed, paraffin-embedded tissue, is a valuable tool for the detection of early infarcts (Fujiwara et al., 1988).

Both Leadbeatter et al. (1989; 1990) and Brinkmann et al. (1993) used antibodies against myoglobin; the latter researchers showed that reactivity with this antibody was in general very much in parallel with the changes in desmin reactivity. Indeed Brinkmann et al. (1993) showed that myoglobin produced a higher degree of sensitivity and they recommended that myoglobin was a good way of demonstrating myocardial ischaemic damage. In our study this antibody did not perform well and certainly not in a manner similar to that in the above publications. (Leadbeatter et al., 1989; 1990 and Brinkmann et al., 1993). The possible reasons for this are manifold; it is likely however, that the laboratory conditions, and the specific bench manner used in the laboratory in which these studies were carried out, resulted in the diametrically opposite findings in this study. The specific antibodies used in previous papers were not available in Edinburgh, and perhaps the antibodies from other commercial companies (Dako & Sigma) may have been manufactured in different ways and consequently, did not react with cardiac muscle. Indeed, the 'Dako' antibody failed to react totally, while only a very minimal reaction was obtained at high concentration with the sigma antibody.

Katagiri (1977), in a study in which electrophoretic analysis of crude extracts of structural proteins of the heart muscle was made found that, in addition to a decrease of myosin, α-actinin is also depleted in the frankly infarcted human myocardial tissue (Katagiri, 1977). Sashida et al. (1984) have shown in electrophoretic and electron microscopical studies of myofibrillar proteins of ischaemic dog myocardial tissue, that a decrease of the content of α-actinin and actin takes place in myocytes which are ischaemically damaged (Sashida et al., 1984). Using electron microscopical and fluorescent techniques, it was shown that α-

actinin is localised at intercalated discs, though its localisation is less intense than the localisation of vinculin to intercalated discs (Ganote & Vander-Heide, 1987).

This myofibrillar protein has not previously been investigated as an immunohistochemical ischaemia-marker in fixed cardiac tissue. This study shows that its pattern of staining, or rather the loss of this pattern, may be a useful further ischaemia marker.

Experimental work with vinculin suggested the use of this cellular component to identify hypoxic damage to cardiac myocyte. Steenbergen et al. (1987) found a loss of staining for vinculin in the ischaemic myocardium of the dog. Ganote & Vander-Heide (1987) using electron microscopical and immunofluorescence techniques showed a diffuse decrease of staining intensity for vinculin using specific immunohistochemical methods at intercalated discs, at sarcolemmal membranes and along the Z-bands in rat hearts which have been rendered ischaemic. This decrease in staining was shown to occur before the actual formation of membrane blebs and the subsequent rupture of the sarcolemmal membranes, and it was suggested that this earlier loss of vinculin caused a weakening of cytoskeletal membrane attachments which in turn produced changes in the actin binding protein, α-actinin which cross-links with actin and forms much of the dense membrane component of both the Z-band and the fascia adherens plaque.

Antibodies to vinculin have only become available comercially recently and these can be applied to paraffin-embedded, formalin-fixed tissues. Utilisation of this antibody in this study confirmed its usefulness by demonstrating that in ischaemic zones, intercalated discs staining is significantly depleted.

Troponin-T is a contractile protein which is unique to cardiac muscle and can be differentiated by immunological methods from its skeletal muscle isoform; indeed a commercially available kit utilising an enzyme immunoassay specific for cardiac troponin is now available (Mair et al., 1992). An experimental study in the dog heart analyzing troponin/tropomyosin complex by gel electrophoresis has demonstrated that alteration in troponin sub-units may be a marker for early infarction in this context (Katgiri, 1977; Katgiri et al., 1981).

Given the marked specificity of this structural protein, an attempt was made to obtain from commercial sources an antibody that would demonstrate this cardiac myocytic protein but unfortunately no such commercially available antibody source could be identified. Given the known cross-reactivity with skeletal troponin, two different commercially produced anti-skeletal muscle antibodies were tried in this study, with diametrically different results between these antibodies. The antibody purchased from 'Sigma' showed no positivity in normal myocardium and the antibody bought from 'Serotec' did show cross-reactivity and a positive staining of the myocardium.

Given these difficulties with this particular antibody, its results have to be considered as suspect, and in order to secure a better interpretation and a further assessment of their specificity, it will be necessary to repeat these particular studies on human tissue once better and more specific anti-cardiac troponins becomes commercially available. Biochemical measurements of troponins (Anderson et al., 1992; Gerhardt & Ljungdahl, 1993; Adams et al., 1993a; Zabel et al., 1993; Adams et al., 1994) in human clinical cases an aid to the early diagnosis of myocardial infarction, have raised the profile of troponins further and it appears to hold a potential for very specific demonstration of ischaemia.

It has also been suggested that as part of irreversible ischaemic damage, the microtubular network of the cell's cytoskeleton is damaged as a result of the disruption produced by the ischaemia (Iwai et al., 1990; Armstrong & Ganote, 1992). Tubulin is the structural sub-unit protein of microtubules and monoclonal antibodies that recognize discrete forms of tubulin have become commercially available. As tubulin is unfortunately a very complex set of proteins and it is difficult to ensure the specificity of such antibodies. Studies by Armstrong & Ganote (1992) using fluorescence microscopy and quantitative flow cytometry showed that ischaemic myocytes exhibited a decrease in tubulin reactivity. The commercially available anti-Tubulin was produced against both its α and the β sub-groups, which were originally extracted from the brains of chicken embryos; it is therefore not surprising that when this antibody was reacted with human tissue the results were disappointing. If these structural changes are to be demonstrated adequately it may indeed be necessary to produce specific anti-human myocardial tubulins, but this work was outwith the ambit of this study.

Other structural proteins which have been investigated particularly by Katagiri (1977) and Katigiri et al. (1981) from Showa University School of Medicine, Tokyo was tropomyosin. In these above studies this protein was investigated utilising SDC polyacrylamide gel electrophoresis; reduction in tropomyosin content was observed as a delayed phenomenon following infarction. Commercially available monoclonal anti-tropomyosin has recently become available and this antibody was therefore tried in this case series.

8.B.2. Reaction to Ischaemia.

Another site where disruption by ischaemia may also occur is at sites of cell-cell contact. Cadherin molecules (A-CAM) are proteins which are associated with these sites and antibodies specific to N-Cadherin suggest that this protein is specifically associated with intercellular junctions (Takeichi, 1988; Geiger & Ayalon, 1992; Goncharova et al., 1992). The 'in vivo' changes in calcium transport for which the functional cadherin-mediated inter-cellular contacts are responsible, further raised the potential for such proteins in their use as cytochemical markers of ischaemia. The commercial antibodies which have become available has enabled a study of this protein in infarcted and ischaemic zones. This has not been previously attempted.

This study showed that the specific staining of ICD becomes less pronounced and sharp as ischaemia develops.

Products of complement activation have been identified on cardiac myocytes after a period of ischaemia (McManus et al., 1983): immunoreactive C3 was detectable on swollen myocytes of the baboon heart as early as four hours after coronary arterial ligation (Pinkard et al. 1980). Radio-actively labelled complement fragments were used for investigation in the dog and the rat experimental myocardial infarct model (Rossen et al., 1985). Schäfer et al. (1986) carried out complement studies on formalin fixed hearts and showed that complement in addition to be being deposited on myocytes, was also found to be identifiable subendocardially in arteries within the infarcted tissue (Schäfer et al., 1986). Leadbeatter et al., (1989) had made use of $C3_b$ as an immunohistochemical marker but found that the pattern of staining with this antibody was very variable and unreliable. This antibody was therefore abandoned in the study by the same workers in their later

study in 1990. Brinkmann et al. (1993) showed mostly parallel reactivity when $C5_b$-9 was compared with fibrinogen staining.

In this study the antibody to the membrane attack complex (MAC) i.e. $C5_b$-9 did not identify with any degree of certainty areas of ischaemia; in these areas only occasional fibres showed some deposition of $C5_b$-9 on their membranes and in their cytoplasm, but the fibres that showed such were elegantly demonstrated. The infrequency and inconsistency of this finding detracts from the usefulness of this antibody to demonstrate non-infarcted ischaemic zones.

The glycoprotein CD59 (protectin) which is anchored to the cell membrane has been identified as a major complement-mediated 'lysis restricting factor' (Meri et al., 1991). This antigen is known to be expressed on erythrocytes, vascular endothelium, and the parenchyma of such organs as the liver, kidney, lung and pancreas; it has also been demonstrated in human myocardium. Väkevä et al. (1992) have shown a loss of expression of CD59 in association with the deposition of complement membrane attack complex ($C5_b$-9), which is deposited on the human myocardium in myocardial infarction.

The immunoperoxidase technique which has been utilised in this study demonstrated positivity to protectin throughout the cytoplasm (with absence of cytoplasmic membrane and nuclear membrane) of normal non-ischaemic myocytes. No staining of blood vessels was demonstrable. This is in contrast to the work of Meri et al. (1991) who with an immunofluorescence technique showed staining of the sarcolemma of the myocytes only, as well as of the endothelium of blood vessels. The cytoplasmic staining in the immunoperoxidase method appeared to have either a particulate or a patterned striated distribution mimicking the pattern of other cytoplasmic structural components of cardiac myocytes. The pattern and intensity of staining disappeared in infarcted areas and was substantially decreased in ischaemic zones to suggest that utilisation of this antibody may be another useful tool in diagnosing non-infarct ischaemia.

8.B.3. Capillary Changes.

Monoclonal antibodies to CD34 have been observed to bind to capillary endothelium in both normal and neoplastic tissue (Fina et al., 1990). This antibody has not been

previously used in the study of ischaemic tissue, though it is a well-established phenomenon that damage to capillaries is integrally associated with acute infarction (Jennings et al., 1982).

It was confirmed in this study that this monoclonal antibody reacts with the interfascicular intricate capillary network of the human myocardium and that in infarcted areas a disruption of the orderly capillary network is visualisable. In ischaemic zones it could be shown that the capillary network showed different staining properties than in the normal non-ischaemic areas of the myocardium. This antibody thus appears to have significant potential as an ischaemic marker by demonstrating damage to the framework of blood vessels where an acute depletion of blood supply to the myocardium has taken place.

8.C. Use of Lectin Staining.

Lectin histochemistry is particularly useful in the analysis of the components of plasma membranes because these compounds bind specifically to the carbohydrate residues which are present on cell surfaces. The latter include free polysaccharides which are attached loosely to the membrane and intrinsic membrane carbohydrates which through covalent bonds are strongly linked to membrane proteins (glycoproteins) and to the membrane lipids (glycolipids) (Ponder, 1983; Cooper, 1984; Goldstein & Portez, 1986; Damjanov, 1987; Hennigar & Hennigar, 1987; Spicer & Schulte, 1988; Goldstein et al., 1990). These complex carbohydrates play an essential role in permeability regulation of the membrane as well as cell recognition, adhesion and locaL immunological phenomena (Sharon, 1984; Barondes, 1988; Sharon & Lis, 1989; Bryant et al., 1991).

Current views of cell death emphasize the role of the surface membrane in the sequence of events: a trivial damage to the membrane results in the uncontrolled influx of calcium into the cell with diversion of intra-cellular energy production to calcium transport, a high intra-cellular (mitochondrial) concentration of calcium activation of calcium-sensitive proteins and phospholipase A_2 with further damage to the cell and in the case of muscle fibres, increased contractility (Ashraf & Halverson, 1977; Jennings & Reimer, 1981; Reimer et al., 1981; Reimer et al., 1983; Reimer & Ideker, 1987; Ganote, 1983; Jennings et al., 1986; Jennings et al., 1990; Opie, 1993).

These aberrations have stimulated interest in the changes of lectin binding on the cell surface of skeletal muscle tissue and cardiac muscle tissue and later on also in endothelial linings. Bonilla et al. (1980) studied patients with Duchenne muscular dystrophy (DMD) and showed alterations in binding to Con A at the cell surface of skeletal muscle fibres which suggested an alteration in either the distribution or the accessibility of α-D manose, α-D-glucose and β-D-fructose which are the carbohydrate residues which bind specifically with Con A. This helped to explain the ultrastructural and combined electrophysiological and cytochemical studies by previous workers (Mokri & Engel, 1975; Engel et al., 1977). Defects in the cell membrane of the skeletal muscle were also demonstrated in cases of polymyositis (Bonilla et al., 1980).

Refined staining for different carbohydrate-binding sites were carried out on skeletal muscle with the aid of lectins and alkaline phosphatase-conjugated avidin-staining procedures (Kirkeby et al., 1991) and these studies were carried out on both human and experimental animal tissues. Capaldi et al. (1985) examined skeletal muscle tissues with fifteen lectin-horseradish peroxidase (HRP) conjugates. These studies showed that lectin-binding is not determined solely by specific monosaccharide binding thus if lectins are being used for any comprehensive histochemical investigation of tissue glycoconjugates, a large number of lectins will have to be employed on the same tissue if the results are to be interpreted meaningfully (Capaldi et al., 1985).

The little previous work carried out with lectins in relation to cardiac myocytes appeared to concentrate on animal tissues: indeed it was shown with guinea pig and rat cardiac myocytes that the carbohydrates α-mannose, α-glucose and N-acetyl-glucosamine and N-acetyl-neuraminic acid are evenly distributed on the cell surfaces of the cardiac myocytes from these animals (Grahal et al., 1984; Stegemann et al., 1990). β-galactosyl residues which were also distributed over the cell surface were concentrated on the Z lines. Intra-cytoplasmic staining with demonstration of striation was also easily identifiable with fluorescent Con A, WGA, LFA and RCA-I. Species-dependent differences were however demonstrable indicating the need for base-line studies to be carried out in each type of species that is examined (Stegemann et al., 1990). Lectins have also been employed in more specific intra-cellular organelle localisations using cultured myocardial cells (Iida & Page, 1989).

The binding of lectins to vascular endothelium is also well established. The pattern of carbohydrate residues which are to be found on vascular endothelium has also been studied in several species and in all, terminal sialyl and β-galactosyl residues were identified. In human endothelium the α-non-reducing terminal α-fucosyl residue was identifiable (Alroy et al., 1987). The influence of the endothelial cell layer on vascular permeability also lends itself to investigation by lectin binding and indeed WGA binding has been used in studying cerebral blood vessel permeability and the blood brain barrier in cerebral ischaemia by Nishida et al. (1986) in which WGA was the lectin used in an immunoelectronmicroscopical study (Nishida et al., 1986).

The utilisation of lectins in the literature does not appear to have been applied to the situation of myocardial ischaemia except by Väkevä et al. (1992) who applied only two lectins (PNA and WGA) in an immunofluorescenct study to proven cases of myocardial infarction. This was the reason that it was decided to stain human myocardial tissues with a battery of lectins: Con A, DBA, PNA, RCA-I, SBA, UEA-1 and WGA all of which could identify sugars on formalin-fixed, paraffin-embedded human tissues both on myocytes and a vascular endothelium.

On the whole, although changes in staining patterns from normal, in the intensity, distribution and detail of the staining pattern could be shown by the lectin staining in the areas of complete infarction, the changes were not as pronounced in ischaemia zones. Although no distinctive pattern could be identified in areas which were ischaemic but without frank necrosis, damage associated with lectin staining changes could be identified in ischaemia zones suggesting that they could also be useful methods particularly if tested in a bank.

Normal cardiac myocytes showed staining of the surfaces with particularly with Con A could in areas demonstrate a bi-laminal pattern. Disruption of this surface staining was a pattern that recurred with all the lectins which were investigated. Intracytoplasmic striations that was demonstrable with the number of the lectins was patchy and although no striations could be identified in areas of infarction given that the normal staining pattern was patchy and inconsistent, this change could not qualify as the distinctive feature of ischaemic necrosis.

Vascular endothelial staining was also demonstrable, however, little distinctive change could be seen as a consequence of ischaemia in the pattern of staining.

Staining with lectins has to be taken into account the use of neuraminidase and pre-treatment with this 'un-masking enzyme' was required for a number of the lectins used in the study. The requirement for this pre-treatment appears to vary from one tissue to another and if other lectins are employed other than the ones used it would be necessary to recover this phenomenon in a separate pilot study.

8.D. Conclusion.

In conclusion the assumption that has formed the basic underlying rationale for this study was that as a direct consequence of a diminished blood supply to areas of the myocardium there will be damage to the myocytes. This damage does not demonstrate itself at autopsy on naked eye examination and cannot be demonstrated with any specificity and reliability by ordinary light microscope tinctorial techniques. Yet if the ischaemia is long enough it could have caused the death of the patient even if this interval may be less than one hour. The work of others such as Leadbeatter et al. (1989; 1990) and Brinkmann et al. (1993) on heart muscle in humans, the work on experimental animals of (Khaw et al., 1984; Nishida et al., 1987; Nomoto et al., 1987; Iwai et al., 1990; Armstrong & Ganote, 1992) and the work on skeletal muscle (Fechner et al., 1991; Fechner et al., 1993) suggest strongly that ischaemia leads to acute structural damage with disruption of the structural components of the myocyte, and their eventual liberation into blood stream (Katus et al., 1984; Osuna et al., 1990; Larue et al., 1991; Silva et al., 1991; Arénegé et al., 1993) and into the pericardium (Burns et al., 1992) of these structural components. These have supplemented the well established enzymatic assays that are routinely carried out in the clinical context to diagnose and follow-up patients with myocardial infarcts (Adams et al., 1993b; Gunn et al., 1993)

These disruptions, losses and displacements were studied in hearts in which the cause of death was myocardial ischaemia in the presence of acute coronary arterial occlusion but without microscopical evidence of damage; as a positive control actual recent well shown myocardial infarcts with the typical histological changes of acute inflammation were assumed to have immediately adjacent to the infarcted zone an area of severe ischaemia

which show the changes that would be expected with the passage of time after the onset of ischaemia, in gradual transformation from an acutely ischaemic site into a site of coagulative necrosis and inflammation.

Two distinct groups of studies were carried out, both based on the demonstration of cardiac myocytic components by an immunoperoxidase technique. In the first study commercially available, mostly monoclonal antibodies were utilised, and in the second study lectins which seek out carbohydrates based radicals, and also available commercially, were employed in combination with a peroxidase label.

8.E. Concluding Remarks.

1. The demonstration of acute myocardial ischaemic damage at autopsy in the absence of macroscopical and light microscopical tinctorial changes, still remains a problem seeking a solution; this matter may have a very particular significance in the medico-legal field when it may be essential for the reporting pathologist to demonstrate the presence of cardiac ischaemic damage occurring in the peri-mortem period in a manner that can stand up to the scrutiny of the witness box.

2. Tinctorial stains including such methods as the Lie stain and Arnold's stain which have been billed as being of specific assistance in resolving this problem are not of any major assistance in the resolution of this problem in that their reproducibility and their specificity leave much to be desired.

3. Immunoperoxidase staining can be used to demonstrate loss of proteinacious myocytic components and cell wall damage; by and large, the antibodies which are available commercially for this purpose are shown to be specific, sensitive and reproducibly reliable.

4. The use of single antibodies in isolation for the demonstration of myocardial ischaemia is of little value; however the data obtained from the use of banks of such antibodies may together collaborate one another, particularly if serial sections from the same block of heart muscle are reacting identically.

5. This study has suggested that the larger the antibody bank that is used, the more comfortable one may be with the conclusion that a particular area of myocardium does indeed show ischaemic damage.

6. The antibodies which have been found to be most useful for this purpose are: actin, desmin and troponin-T and these three antibodies should form a useful initial screen for ischaemia to be corroborated by others as required.

7. Antibodies to more intrinsic and more recently isolated myocardial components such as vinculin and tropomyosin have been tried for the first time in this study and shown to add further discriminatory power to such an antibody panel.

8. Changes to the surface carbohydrate moieties in areas of ischaemia can be demonstrated by using stainng with lectins; these peroxidase-labelled lectins are also commercially available and this study has shown that they can be utilised in a manner similar to that of the antibodies. The demonstration of such changes had not previously been carried out in the human situation.

9. Demonstration of changes in lectin binding may corroborate even further the presence of ischaemic damage and should, therefore, be used together with antibodies on those cases where the demonstration of ischaemia may be of crucial evidential importance.

10. The manner in which the pathologist chooses the area of the heart which is to be subjected to these types of staining in any specific case remains a difficult matter which cannot be resolved. Preliminary screening tests by a small panel of antibodies may have to be carried out on a large number of carefully labelled blocks from a heart which is being looked at. This investigation can then be widened out to a larger panel of antibodies as required utilising serial sections from the more promising blocks. The choice of the area in the left ventricle where one is most likely to find ischaemia has to be worked out from the distribution, dominance and occlusive pattern of an individual's coronary arterial circulation. Fixation in neutral buffered formalin (NBF) of the heart blocks provides entirely satisfactory results.

11. From the studies which have been carried out it is difficult to be absolutely certain that the staining changes which were demonstrated as to have been caused by ischaemia and nothing else, and furthermore that the changes demonstrated have caused the death of that particular patient. By primary exclusion from the series of cases studied, hearts on whom peri-mortem resuscitation had taken place, artefactual changes resulting from this have been excluded. It is also a well established phenomenon that non-specific changes may appear in the myocardial cells in the agonal period in a variety of clinical conditions particularly those associated with the release of high levels of catecholamines; these changes are more likely to be patchy as compared with the compact, well-delineated and geographical distribution of the ischaemic zones.

12. It may also be the case that some ischaemic hearts will not manifest abnormalities in their component cells either because of the small time interval between ischaemia developing and death or because of other non-specific reasons. The absence of morphological changes, therefore, does not incontrovertibly exclude ischaemia as the cause of death.

13. These studies may require to be repeated on animal experiments where such conditions can be carefully delineated and controlled. If animal experiments can demonstrate changes similar to those identified in the human situation, then these tests would have that much more significance.

14. Changes in the capillary network at the site of the ischaemic damage as demonstrable by CD34 and in the cell-to-cell adhesion sites by N-cadherin have been shown in a similar immunoperoxidase-based technique in the course of this study. These antibodies, and others like them, need to be explored further and utilised for this purpose.

15. The immunoperoxidase methods which form the basis of this study, and all other similar stains, are capricious and to obtain reasonable and meaningful results the laboratory which carries out such stains should be carrying out such methods routinely to ensure their reproducibility and a potential reliance on the findings.

16. The reliability of the histological demonstration of ischaemia should as far as possible be further confirmed by biochemical studies particularly in samples of the pericardial fluid

collected at autopsy. The liberation from the myocardium into the pericardium of similar organelle and cell-wall components would further bolster any conclusions that could be made from the immunoperoxidase studies. A study of such biochemical tests in parallel with the histological testing may assist further in proving the usefulness of the combination of these two methods.

17. Antibody and lectin staining is expensive and laborious and as such is not a method that is likely to be offered routinely as part of a medico-legal service; it should be reserved for those cases in which the proof of myocardial ischaemic damage would assist in criminal proceedings, an inquest or civil litigation when its demonstration is absolutely crucial to the case under review. However if these stains are to have such major evidential significance and scrutiny, they would need to be carried out in a laboratory where such testing is carried out on a regular well-controlled basis.

CHAPTER 9.

REFERENCES.

Adams J.E., Abendschein D.R. & Jaffe A.S., (1993b) Biochemical markers of myocardial injury. Is MB creatine kinase the choice for the 1990s?. *Circulation*; **88(2)**: 750-763.

Adams J.E., Bodor G.S., Davila-Roman V.G., Delmez J.A., Apple F.S., Ladenson J.H. & Jaffe A.S., (1993a) Cardiac troponin I. A marker with high specificity for cardiac injury. *Circulation*; **88(1)**: 101-106.

Adams J.E., Sicard G.A., Allen B.T., Bridwell K.H., Lenke L.G., Davila-Roman V.G., Bodor G.S., Ladenson J.H. & Jaffe A.S., (1994) Diagnosis of perioperative myocardial infarction with measurement of cardiac troponin I. *N Engl J Med*; **330**: 670-674.

Aebi U., Cohn J., Buhle L. & Gerace L., (1986) The nuclear lamina is a meshwork of intermediate-type filaments. *Nature*; **323**: 560-564.

Al-Alousi L.M., (1990) Medico-legal problems of ischaemic heart disease and myocardial infarction. *Med Sci Law*; **30(1)**: 67-81.

Alroy J., Goyal V. & Skutelsky E., (1987) Lectin histochemistry of mammalian endothelium. *Histochemis;* **86**: 603-607.

Alroy J., Ucci A.A. & Pereira M.E.A., (1984) Lectins: Histochemical probes for specific carbohydrate residues. In: DeLellis R.A. (ed.). *Advances in Immunohistochemistry*. Raven Press. New York, pp 67-88.

Alroy J., Ucci A.A., & Periera M.E.A., (1988) Lectin histochemistry: An update. In: DeLellis R.A. (ed.). *Advances in Immunohistochemistry*. Raven Press. New York, pp 93-131.

Al-Rufaie H.K., Florio R.A. & Olsen E.G.J., (1983) Comparison of the haematoxylin basic fuchsin picric acid method and the fluorescence of haematoxylin and eosin stained sections for the identification of early myocardial infarction. *J Clin Pathol;* **36**: 646-649.

Altschule M.D., (1985) The coronary occlusion story. Prolonged neglect of early clinicopathologic findings and of the experimental animal physiology they stimulated. *Chest*; **87(1)**: 81-84.

Anderson P.A.W., Malouf N.N., Oakeley A.E., Pagani E.D. & Allen P.D., (1992) Troponin T isoform expression in the normal and failing human left ventricle: A correlation with myofibrillar ATPase activity. *Basic Res Cardiol*; **87** (suppl): 117-127.

Apple F.S., (1992) Acute myocardial infarction and coronary reperfusion. Serum cardiac markers for the 1990's. *Clin Chem*; **97(2)**: 217-226.

Aránega A.E., Reina., Muros M.A., Alvarez L., Prados J. & Aranega A., (1993) Circulating α-Actin Protein in acute myocardial infarction. *Int J Cardiol*; **38**: 49-55.

Armiger L.C., Wheeler E.E., Geraghty D.E. & Herdson P.B., (1977) An experimental evaluation of staining techniques for the detection of early ischaemic injury to the myocardium. *Pathology*; **9**: 161-171.

Armstrong S.C. & Ganote C.E., (1992) Flow cytometric analysis of isolated adult cardiomyocytes: Vinculin and tubulin fluorescence during metabolic inhibition and ischaemia. *J Mol Cell Cardiol*; **24**: 149-162.

Arnold G., Kaiser C. & Fischer R., (1985) Myofibrillar degeneration. A common type of myocardial lesion and its selective identification by a modified Luxol Fast Blue stain. *Pathol Res Pract;* **180**: 405-415.

Ashraf M. & Halverson C., (1977) Structural changes in the freeze-fractured sarcolemma of ischaemic myocardium. *Am J Pathol;* **88**: 583-594.

Aub J.C., Tieslau C. & Lankester A., (1963) Reactions of normal and tumour cell surfaces to enzymes, I. Wheat-germ lipase and associated mucopolysaccharides. *Proc Natl Acad Sci USA*; **50**: 613-619.

Auerbach R., Alby L. & Marrisey L., (1985) Expression of organ-specific antigens on capillary endothelial cells. *Microvasc Res*; **29**: 401-411.

Baldry P.E., (1971) In: Baldry P.E. (ed.). *The Battle Against Heart Disease*. Cambridge University Press. pp 13-14.

Baroldi G., (1975) Different types of myocardial necrosis in coronary heart disease: A pathophysiologic review of their functional significance. *Am Heart J*; **89**: 742-752.

Barondes S.H., (1988) Bifunctional properties of lectins: lectins redefined. *Trends Biochem Sci*; **13**: 480-482.

Baumgarten W., (1899) Infarction in the heart. III. *Am J Physiol*; **2:** 243-265.

Belloni P.N. & Nicolson G.L., (1988) Differential expression of cell surface glycoproteins on various organ-derived microvascular endothelia and endothelial cell cultures. *J Cell Physiol*; **136**: 398-410.

Bernardes W. & Avrameas S., (1971) Ultrastructual visualization of cellular carbohydrate components by means of concanavalin A. *Exp Cell Res*; **64**: 232-236.

Bershadsky A.D., Tint I.S. & Svitkina T.M., (1987) Association of intermediate filaments with vinculin-containing adhesion plaques of fibroblasts. *Cell Motil Cytoskel;* **8**: 274-283.

Bershadsky A.D. & Vasiliev J.M., (1988) Cytoskeleton. In: Siekevitz P. (ed.). *Cellular Organelles*. Plenum Press. London.

Bevilacqua M.P., Stengelin S., Gimbron Jr. M. & Seed B., (1989) Endothelial leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. *Science*; **243**: 1160-1165.

Biesecker G., (1983) Biology of disease. Membrane attack complex of complement as a pathologic mediator. *Lab Invest*; **49(3)**: 237-249.

Biesecker G., Katz S. & Koffler D., (1981) Renal localization of the membrane attack complex in systemic lupus erythematosus nephritis. *J Exp Med*; **154**: 1779-1794.

Biesecker G., Lavin L., Ziskind M. & Koffler D., (1982) Cutaneous localization of the membrane attack complex in discoid and systemic lupus erythematosus. *N Engl J Med*; **306**: 264-270.

Bing R.J., Castellanos A., Gradel E., Lupton C. & Siegel A., (1956) Experimental myocardial infarction: Circulatory, biochemical and pathologic changes. *Am J Med Sci*; **232**: 533-554.

Block M.I., Said J.W., Siegel R.J. & Fishbein M.C., (1983) Myocardial myoglobin following coronary artery occlusion: An immunohistochemical study. *Am J pathol*; **111**: 374-379.

Boenish T., (1989) Antibodies and controls. In: Naish S.J. (ed.). *Immunochemical Staining Methods*. DAKO Corporation, Carpinteria. California, pp 1-21.

Bonilla E. & Moggio M., (1987) Electron cytochemical study of the muscle cell surface. *Histochemis;* **86**: 503-507.

Bonilla E., Schotland D.L. & Wakayama Y., (1978) Duchenne dystrophy. Focal alterations in the distribution of Concanavalin A binding sites at the muscle cell surface. *Ann Neurol*; **4**: 117-123.

Bonilla E., Schotland D.L. & Wakayama Y., (1980) Application of lectin cytochemistry to the study of human neuromuscular disease. *Muscle and Nerve*; **3**: 28-35.

Bouchardy B. & Majno G., (1971/2). A new approach to the histologic diagnosis of early myocardial infarcts. *Cardiology*; **56**: 327-332.

Boyd W.C. & Reguera R.M., (1949) Haemagglutinating substances for human cells in various plants. *J Immunol*; **62**: 333-339.

Boyd W.C. & Shapleigh E., (1954) Specific precipitating activity of plant agglutinins (Lectins). *Science*; **119**: 419.

Braunwald E., (1992) *Heart Disease. A Textbook of Cardiovascular Medicine*. 4th. ed. W.B. Saunders Company. London, pp 1188.

Brinkmann B., Sepulchre M.A. & Fechner G., (1993) The application of selected histochemical and immunohistochemical markers and procedures to the diagnosis of early myocardial damage. *Int J Legal Med;* **106**: 135-141.

Bryant R.W., Granzow C.A., Siegel M.I., Egan R.W. & Motasim-Billah M., (1991) Wheat germ agglutinin and other selected lectins increase synthesis of decay-accelerating factor in human endothelial cells. *J Immunol*; **147(6)** (suppl-15): 1856-1862.

Bryant R.E., Thomas W.A. & O'Neal R.M., (1958) An electron microscopic study of myocardial ischaemia in the rat. *Circ Res*; **6**: 699-709.

Burger M.M. & Goldberg A.R., (1967) Identification of tumour-specific determinant on neoplastic cell surfaces. *Proc Natl Acad Sci USA*; **57**: 359-366.

Burkitt H.G., Young B. & Heath J.W., (1993) *Wheater's Functional Histology. A Text and Colour Atlas*. 3rd. ed. Churchill Livingstone. London, pp 107-112.

Burns J., Milroy C.M., Hulewicz B., West C.R., Walkley S.M. & Roberts N.B., (1992) Necropsy study of association between sudden death and cardiac enzymes. *J Clin Pathol*; **45**: 217-220.

Burridge K. & Connell L., (1983) A new protein of adhesion plaques and ruffling membranes. *J Cell Biol*; **97**: 359-367.

Burridge K., Fath K., Kelly T., Nuckolls G. & Turner C., (1988) Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annu Rev Cell Biol*; **4**: 487-525.

Burridge K., Nuckolls G., Otey C., Pavalko F., Simon K. & Turner C., (1990) Actin-membrane interaction in focal adhesions. *Cell Differ Dev*; **32**: 337-342.

Capaldi M.J., Dunn M.J., Sewry C.A. & Dubwitz V., (1985) Lectin binding in human skeletal muscle: a comparison of 15 different lectins. *Histochem J*; **17**: 81-92.

Carle B.N., (1981) Autofluorescence in the identification of myocardial infarcts. *Hum Pathol*; **12(7)**: 643-646.

Caulfield J. & Klionsky B., (1959) Myocardial ischaemia and early infarction: An electron microscopic study. *Am J Pathol;* **35**: 489-523.

Chiffelle T.L. & Putt F.A., (1951) Propylene and ethylene glycol as solvents for sudan IV and sudan black B. *Stain Technol*; **26**:51-56.

Chio K.S., Reiss U., Fletchor B. & Tappel A.L., (1969) Peroxidation of subcellular organelles: Formation of Lipofuscin like fluorescent pigments. *Science*; **166**: 1535-1536.

Cooke P.H. & Chase R.H., (1971) Potassium chloride-insoluble myofilaments in vertebrate smooth muscle cells. *Exp Cell Res*; **66**: 417-425.

Cooper H.S., (1984) Lectins as probes in histochemistry and immunohistochemistry. *Hum Pathol;* **15(10)**: 904-906.

Cormack D.H., (1993) Muscle tissue. *Essential Histology*. J. B. Lippincott Company. Philadelphia, pp 215-227.

Cotran R.S., Kumar V. & Robbins S.L., (1989) *Robbins Pathologic Basis of Disease* 4th. ed. W.B. Saunders Company. London, pp 23-25.

Crout J.R. & Jennings R.B., (1957) An improved histochemical method for the demonstration of potassium. *J Histochem*; **57**: 170-177.

Curran R.C. & Gregory J., (1977) The unmasking of antigens in paraffin sections of tissue by trypsin. *Experientia*; **33**: 1400-1401.

Damjanov I., (1987) Biology of disease. Lectin cytochemistry and histochemistry. *Lab Invest;* **57(1)**: 5-20.

Danguy A., Kiss R. & Pasteels J., (1988) Lectins in histochemistry. *Biol Struct Morph*; **1(3)**: 93-106.

Darr D., McCormack K.M., Manning T., Dunnston S., Winson D.C., Schulte B.A., Buller T. & Pinnell S.R., (1990) Comparision of *Dolichos bilorus* lectin and other lectin-horseradish peroxidase conjugates in staining of cutaneous blood vessels in the hairless mini-pig. *J Cutan Pathol*; **17**: 9-15.

Dauber I.M., VanBenthuysen K.M., McMurtry I.F., Wheeler G.S., Lesnefsky E.J., Horwitz L.D. & Weil J.V., (1990) Functional coronary microvascular injury evident as increased permeability due to brief ischemia and reperfusion. *Circ Res;* **66**: 986-998.

Debray H., Decout D., Strecker G., Spik G. & Montreuil J., (1981) Specificity of twelve lectins towards oligosaccharides and glycopeptides related to N-glycosylproteins. *Eur J Biochem*; **117**: 41-55.

Debus E., Weber K. & Osborn M., (1983) Monoclonal antibodies to desmin, the muscle-specific intermediate filament protein. *EMBO J;* **2**: 2305-2312.

Derias N.W. & Adams C.W.M., (1979) The non-specific nature of the myocardial wavy fibre. *Histopathology*; **3**: 241-245.

Dixon H.B.F., (1981) Defining a lectin. *Nature*; **292**: 192.

Dock G., (1939) Historical notes on coronary occlusion: From Heberden to Osler. Frank Billings Lecture. *JAMA;* **113(7)**: 563-568.

Dolber P.C., Beyer E.C., Junker J.L. & Spach M.S., (1992) Distribution of gap junctions in dog and rat ventricle studied with a double-label technique. *J Mol Cell Cardiol*; **24**: 1443-1457.

Dunn M.J., Sewry C.A. & Dubowitz V., (1982) Cytochemical studies of lectin binding by diseased human muscle. *J Neurol Sci;* **55**: 147-159.

Ehrlich P. & Morgenroth J., (1910) Contributions to the theory of lysin action. In: Ehrlich P. (ed.). *Studies in Immunity*. Collected and translated by Bolduan C. 2nd. ed. Chapman & Hall Ltd. London, pp 1-10.

Engel A.G. & Biesecker G., (1982) Complement activation in muscle fiber necrosis: demonstration of the membrane attack complex of complement in necrotic fibers. *Ann Neurol*; **12**: 289-296.

Engel A.G., Lambert E.H. & Howard F.M., (1977) Immune complexes (IgG & C_3) at the motor end plate in myasthenia gravis. Ultrastructural and light microscopic localization and electrophysiologic correlations. *Mayo Clin Proc*; **52**: 267-280.

Engler R.L., Schmid-Schonbein G.W. & Pavelec R.S., (1983) Leukocyte capillary plugging in the myocardial ischemia and reperfusion in the dog. *Am J Pathol;* **111(1)**: 98-111.

Fallon J.T., (1981) Postmortem: histochemical techniquues. In: Wagner G.S. (ed). *Myocardial Infarction and Intervention.* Martinus Nijhoff Publishers. The Hague, pp 373-384.

Fazel A.R., Thomson R.P., Sumida H. & Schilte B.A., (1989a) Lectin histochemistry of the embryonic heart: Fucose-specific lectin binding sites in developing rats and chicks. *Am J Anat*; **184**: 76-84.

Fazel A.R., Thomson R.P., Sumida H. & Schilte B.A., (1989b) Lectin histochemistry of the embryonic heart: expression of terminal and penultimate galactose residues in developing rats and chicks. *Am J Anat*; **184**: 85-94.

Fechner G., Bajanowski T., Brinkmann B., (1993) Immunohistochemical alterations after muscle trauma. *Int J Legal Med*; **105**: 203-207.

Fechner G., Hauser R., Sepulchre M.A. & Brinkmann B., (1991) Immunohistochemical investigations to demonstrate vital direct traumatic damage of skeletal muscle. *Int J Legal Med*; **104**: 215-219.

Fina L., Molgaard H.V., Robertson D., Bradley N.J., Monoghan D.D., Delia D., Sutherland D.R., Baker M.A. & Greaves M.F., (1990) Expression of CD34 gene in vascular endothelial cells. *Blood*; **75**: 2417-2426.

Fine G., Morales A. & Scerpella J.R., (1966) Experimental myocardial infarction. *Arch Pathol*; **82**: 4-8.

Fishbein M.C., Maclean M.B. & Maroko P.R., (1978) The histopathologic evolution of myocardial infarction. *Chest*; **73(6)**: 843-849.

Fonkalsrud E.W., Sanchez M., Zerubaul R., Lassatetta L. & Smeesters C., (1976) Arterial endothelial changes after ischaemia and perfusion. *Surg Gynecol Obstet*; **142**: 715-721.

Fujimato T. & Singer S.J., (1988) Immunocytochemical studies of endothelial cells in vivo. II. Chicken aortic and capillary endothelial cells exhibit different cell surface distributions of the integrin complex. *J Histochem Cytochem;* **36(10)**: 1309-1317.

Fujiwara H., Onodera T., Tanaka M., Miyazaki S., Wu D-J., Matsuda M., Kawamura A., Ishida M., Takemura G., Fujiwara Y., Fujiwara T. & Kawai C., (1989) Acceleration of cell necrosis following reperfusion after ischaemia in the pig heart without collateral circulation. *Am J Cardiol*; **63**: 14E-18E.

Fujiwara H., Tanaka M., Miyazaki S., Matsuda M., Kawai C., Fujiwara T., Onodera T., Wu D-J. & Sasayama S., (1988) Detection of early myocardial infarction in formalin-fixed, paraffin-embedded tissue. *Am J Cardiovasc Pathol*; **2**: 57-61.

Fulghum T.G., Mahon M.M.C., Aretz T., Khaw B.A., Fallon J.T., Haber E. & Powel Jr. W.M.J., (1986) The time course of canine ischaemic myocardial necrosis evaluated by immunocytochemistry using cardiac myosin specific monoclonal antibodies in vivo. *J Appl Cardiol*; **1**: 385-401.

Fye W.B., (1991) Acute myocardial infarction. A Historical summary. In: Gersh B.J. & Rahimtoola S. H. (eds.). *Acute Myocardial Infarction.* Elsevier. New York, pp 3-13.

Galen R.S., (1975) The enzyme diagnosis of myocardial infarction. *Hum Pathol;* **6**: 141-155.

Ganote C., (1983) Contraction band nerosis and irreversible myocardial injury. *J Mol Cell Cardiol*; **15**: 67-73.

Ganote C.E. & Armstrong S.C., (1993) Ischaemia and the myocyte cytoskeleton: review and speculation. *Cardiovasc Res*; **27**: 1387-1403.

Ganote C.E. & Vander-Heide R.S., (1987) Cytoskeletal lesions in anoxic myocardial injury. A conventional and high-voltage electron-microscopic and immunofluorescence study. *Am J Pathol*; **129**: 327-344.

Geiger B., (1979) A 130 K protein from chicken gizzard: Its localization at the termini of microfilament bundles in cultured chicken cells. *Cell*; **18**: 193-205.

Geiger B. & Ayalon O., (1992) Cadherins. *Annu Rev Cell Biol*; **8**: 307-332.

Geiger B., Tokuyasu K.T., Dutton A.H. & Singer S.J., (1980) Vinculin, an intracellular protein localized at specialized sites where microfilament bundles terminate at cell membranes. *Proc Natl Acad Sci USA*; **77**: 4127-4131.

Gerhardt W., Katus H., Ravkilde J., Hamm C., Jørgensen P.J., Peheim E., Ljungdahl L. & Löfdahl P., (1991) S-Troponin T in suspected ischemic myocardial injury compared with mass catalytic concentrations of S-Creatine kinase isoenzyme MB. *Clin Chem;* **37**: 1405-1411.

Gerhardt W. & Ljungdahl L., (1993) Rational diagnostic strategy in diagnosis of ischaemic myocardial injury. S-troponin T and S-CK MB (mass) time series using individual baseline values. *Scand J Clin Lab Invest;* **53** (suppl-215): 47-59.

Goldstein M.A. & Entman M.l., (1979) Microtubules in mammalian heart muscle. *J Cell Biol*; **80**: 183-195.

Goldstein I.J., Hughes R.C., Monsigny M., Osawa T. & Sharon N., (1980) What should be called a lectin?. *Nature*; **285**: 66.

Goldstein I.J., Kaku H. Knibbs R.N. & Rinderle S.J., (1990) Carbohydrate binding specificity of some unique new lectins. In: Elder H.Y. (ed.). *Transactions of the Royal Microscopical Society.* New Series Vol. 1. MICRO 90. Adam Hilger. I.O.P. Publishing Ltd. Bristol, pp 631-636.

Goldstein I.J. & Poretz R.D., (1986) Isolation, physicochemical characterisation and carbohydrate-binding specificity of lectins. In: Liener I.E., Sharon N. & Goldstein I.J. (eds.). *The Lectins. Properties, functions and applications in biology and medicine.* Academic Press Inc., Harcourt Brace Jovanovich. London, pp 35-49.

Gonatas N.K. & Avrameas S., (1973) Detection of plasma membrane carbohydrates with lectin peroxidase conjugates. *J Cell Biol;* **59**: 436-443.

Goncharova E.J., Kam Z., Geiger B. (1992). The involvement of adherens junction components in myofibrillogenesis in cultured cardiac myocytes. *Development*; **114**: 173-183.

Goodman S.R., (1994) Cytoskeleton. *Medical Cell Biology.* J. B. Lippincott Company. Philadelphia, pp 61-75.

Gozes I. & Barnstable C.J., (1982) Monoclonal antibodies that recognize discrete forms of tubulin. *Proc Natl Acad Sci USA*; **79**: 2579-2583.

Gräfe M., Graf W., Auch-Schwelk W., Terbeek D., Hertel H. & Eleck E., (1993) Cultivation and characterization of micro-and macrovascular endothelial cells from the human heart. *Euro Heart J*; **14** (suppl-1): 74-81.

Grahl B., Meyberg M., Konig C. & Gercken G., (1984) Carbohydrate structures at the plasma membrane of isolated adult rat cardiac myocytes. *Pflügers Archiv;* **402** (suppl): R26.

Gros D., Bruce B., Challice C.E. & Schrevel J., (1982) Ultrastructral location of concanavlin A and wheat germ agglutinin binding sites in adult and embryonic mouse myocardium. *J Histochem Cytochem;* **30(3)**: 193-200.

Gulati A.K., Reddi A.H. & Zalewski A.A., (1982) Distribution of fibronectin in normal and regenerating skeletal muscle. *Anat Rec*; **204**: 175-183.

Gulati A.K., Reddi A.H. & Zalewski A.A., (1983) Changes in the basement zone components during skeletal muscle fibre degeneration and regeneration. *J Cell Biol*; **97**: 957-962.

Gulati A.K. & Zalewski A.A., (1985) An immunofluorescent analysis of lectin binding to normal and regenerating skeletal muscle of rat. *Anat Rec;* **212**: 113-117.

Gunn I.R., Mir N.S., Parnham A.J., Caslake C.E., Matthews D.M. & O'Brien I.A.D., (1993) Emergency serum creatine kinase MB isoenzyme concentration in parients with suspected myocardial infarction. *Health Bull*; **51**: 166-176.

Haber E., Katus H.A., Hurrel J.G., Matsueda G.R., Ehrlich P., Zurawski V.R. & Khaw B.A., (1982) Detection and quantification of myocardial cell death: application of monoclonal antibodies specific for cardiac myosin. *J Moll Cell Cardiol*; **14**: 139-146.

Hangartner J.R.W., Marley N.J., Whitehead A., Thomas A.C. & Davies M.J., (1985) The assessment of cardiac hypertrophy at autopsy. *Histopathology*; **9**: 1295-1306.

Hashimoto S., Nishida S., Hiruma S., Takahashi M., Enomoto Y. & Sakatani K., (1986) Immunohistochemical reaction of myocardial fibers with actin antiserum in autopsy cases of myocardial infarction. *Histol Histopathol;* **1**: 241-249.

Heggtveit H.A., (1969) Contributions of electron microscopy to the study of myocardial ischaemia. *Bull WHO;* **41**: 865-872.

Helliwell T.R., (1988) Lectin binding and desmin staining during Bupivicaine-induced necrosis and regeneration in rat skeletal muscle. *J Pathol;* **155**: 317-326.

Helliwell T.R., Gunhan O. & Edwards R.H.T., (1989) Lectin binding and desmin expression during necrosis, regeneration, and neurogenic atrophy of human skeletal muscle. *J Pathol;* **159**: 43-51.

Hendley D.D., Mildvan A.S., Reporter M.C. & Strehler B.L., (1963) The properties of isolated human cardiac age pigment. II. Chemical and enzymatic properties. *J Gerontol*; **18**: 250-259.

Hennigar L.M. & Hennigar R.A., (1987) Histochemical specificity of β-galactose binding lectins from arachis hypogaea (Peanut) and Ricinus communis (castor bean). *Stain Technol*; **62(5)**: 317-325.

Henquell L., Odoroff C.L. & Honig C.R., (1976) Coronary intercapillary distance during growth: relation to Pt_{O_2} and aerobic capacity. *Am J Physiol*; **231(6)**: 1852-1859.

Herdson P.B., Kaltenbach J.P. & Jennings R.B., (1969) Fine structures and biochemical changes in dog myocardium during autolysis. *Am J Pathol;* **57**: 539-557.

Herdson P.B., Sommers H.M. & Jennings R.B., (1965) A comparative study of the fine structure of normal and ischaemic dog myocardium with special reference to early changes following temporary occlusion of a coronary artery. *Am J Pathol;* **46**: 367-386.

Herscher L.L., Siegel R.J., Said J.W., Edwalds G.M., Moran M.M. & Fishbein M.C., (1984) Distribution of LDH-1 in normal ischaemic and necrotic myocardium. An immunoperoxidase study. *Am J Clin Pathol;* **81**: 198-203.

Hibbs R.G. & Black W.C., (1963) Electron microscopy of post-mortem changes in the rat myocardium. *Anat Rec*; **147**: 261-272.

Hoffmann U., Axmann C. & Palm N., (1987) Atrial and ventricular myosins from human hearts. II. Isoenzyme distribution after myocadial infarction. *Basic Res Cardiol*; **83**: 359-369.

Holt S., Wilkinson A., Suresh M.R., Mate G., Reid W.B., Longenecker B.M., McPherson A. & Noujaim A.A., (1984) Radiolabeled peanut lectin for the scintigraphic detection of cancer. *Cancer Lett*; **25**: 55-60.

Holthöfer H., (1983). Lectin binding sites in kidney. A comparative study of 14 animal species. *J Histochem Cytochem*; **31**: 531-537.

Holthöfer H., Virtanen I., Kairiniemi A.L., Hormia M., Linder E. & Miettinen A., (1982) Ulex europaeus 1 lectin as a marker for vascular endothelium in human tissues. *Lab Invest*; **47**: 60-66.

Holthöfer H., Vitranen I., Pettersson E., Tornroth T., Alfthan O., Linder E. & Miettinen A., (1981) Lectins as fluoresence microscopic markers for saccharides in the human kidney. *Lab Invest*; **45**: 391-399.

Hougen H.P., Valenzuela A., Lachica E. & Villanueva E., (1992) Sudden cardiac death: A comparative study of morphological, histochemical and biochemical methods. *Forensic Sci Int;* **52**: 161-169.

Hsu S.M. & Raine L., (1982) Versatility of biotin-labeled lectins and avidin-biotin-peroxidase complex for localization of carbohydrate in tissue sections. *J Histochem Cytochem*; **30(2)**; 157-161.

Hsu S.M., Raine L. & Fanger H., (1981) The use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase technique. A comparison between ABC and unlabeled antibody (PAP) procedures. *J Histochem Cytochem*; **29**: 577-580.

Huang S.N., (1975) Immunohistochemical demonstration of hepatitis B core and surface antigens in paraffin sections. *Lab Invest*; **33**: 88-95.

Huang S.N., Minassian H. & More J.D., (1976) Application of immunofluorescent staining on paraffin sections improved by trypsin digestion. *Lab. Invest*; **35**: 383-390.

Iida H. & Page R. (1989) Localisation of wheat-germ agglutinin-binding sites in the Golgi complex of cultured rat atrial myocytes. *Cell Tissue Res*; **257**: 325-331.

Ishiyama I., Kamiya M., Rose M., Komuro E. & Takatsu A., (1982) Fulminant deletion of myoglobin from myocardial fibers in state of acute cardiac failure inducing sudden cardiac arrest. *Lancet*; **II**: 1468-1469.

Iwai K., Hori M., Kitabatake A., Kurihara H., Uchida K., Inoue M. & Kamada T., (1990) Disruption of microtubules as an early sign of irreversible ischaemic injury. *Circ Res;* **67(3)**: 694-706.

Jacobsen M., Clausen P.P. & Smidth S., (1980) The effect of fixation and trypsinization on the immunohistochemical demonstration of intracellular immunoglobulin in paraffin-embedded material. *Acta Pathol Microbiol Scand;* **88**: 369-376.

Jennings R.B., (1969) Symposium on the pre-hospital phase of acute myocardial infarction part II. Early phase of myocardial ischaemic injury and infarction. *Am J Cardiol*; **24(6)**: 753-765.

Jennings R.B., Baum J.H. & Herdson P.B., (1965) Fine structural changes in myocardial ischaemic injury. *Arch Pathol;* **79**: 135-143.

Jennnings R.B. & Ganote C.E., (1974) Structural changes in myocardium during acute ischaemia. *Circ Res*; **34** (suppl-III): III-156-III-172.

Jennings R.B., Kaltenbach J.P. & Smetters G.W., (1957) Enzymatic changes in acute myocardial ischemic injury. *Arch Pathol;* **64**: 10-16.

Jennings R.B., Kloner R.A., Ganote C.E., Hawkins H.K., Reimer K.A., (1982) Changes in capillary fine structure and function in acute myocardial ischaemic injury. In: Tillmanns H., Kubler W. & Zebe H. (eds.). *Microcirculation of the Heart.* Springer-Verlag. Berlin, pp 87-103.

Jennings R.B., Murry C.E., Steenbergen C. & Reimer K.A., (1990) Development of cell injury in sustained acute ischaemia. *Circulation*; **82** (suppl-II): II-2-II-12.

Jennings R.B. & Reimer K.A., (1981) Lethal myocardial ischemic injury. *Am J Pathol*; **102**: 241-245.

Jennings R.B., Reimer K.A. & Steenbergen C., (1986) Myocardial ischemia revisited. The osmolar load, membrane damage, and reperfusion. *J Mol Cell Cardiol*; **18**: 769-780.

Jennings R.B., Sommers H.M., Herdson P.B. & Kaltenbach J.P., (1969) Ischemic injury of myocardium. *Ann NY Acad Sci*; **156**: 61-78

Kadota E., Tanji K., Nishida S., Takahashi M., Maeda M., Hiruma S., Enomoto Y., Hashimoto S. & Akai F., (1986) Lectin (UEA-1) reaction of capillary endothelium with reference to permeability in autopsied cases of cerebral infarction. *Histol Histopathol;* **1**: 219-226.

Kagen L.J., (1978) Myoglobin: Methods and diagnostic uses. *Crit Rev Clin Lab Sci*; **9**: 273-302.

Kagen L.J., Scheidt S., Roberts L., Porter A. & Paul H., (1975) Myoglobinemia following acute myocardial infarction. *Am J Med;* **58**: 177-182.

Karpati G., (1979) The principles of skeletal muscle histochemistry in neuromuscular diseases. In: Vinken P.J. & Bruyn G.W. (eds.). *Handbook of Clinical Neurology. Diseases of Muscle.* Vol. 40. Part 1. North-Holland Publishing Company. Oxford, pp 1-61.

Karsner H.T. & Dwyer J.E., (1916) Studies in infarction. IV. Experimental bland infarction of the myocardium, myocardial regeneration and cicatrization. *J Med Res*; **34**: 21-38.

Katagiri T., (1977) Changes of cardiac structural proteins and myocardial infarction. *Jpn Heart J*; **18**: 711-721.

Katagiri T., Kobayashi Y., Sasai Y., Toba K. & Niitani H., (1981) Alterations in cardiac troponin subunits in myocardial infarction. *Jpn Heart J;* **22(4)**: 653-664.

Katus H.A., Remppis A., Looser S., Hallermeier K., Scheffold T. & Kübler W., (1989) Enzyme linked immunoassay for cardiac troponin-T for the detection of acute myocardial infarction in patients. *J Mol Cell Cardiol*; **21**: 1349-1353.

Katus H.A., Yasuda T., Gold H.K., Leinbach R.C., Strauss H.W., Waksmonsk C., Haber E. & Khaw B.A., (1984) Diagnosis of acute myocardial infarction by detection of circulating cardiac myosin light chains. *Am J Cardiol*; **54**: 964-970.

Kent S.P., (1982) Diffusion of myoglobin in the diagnosis of early myocardial ischaemia. *Lab Invest*; **46(3)**: 265-270.

Kent S.P. & Diseker M., (1955) Early myocardial ischaemia. *Lab Invest*; **4**: 398-405.

Khaw B.A., Beller G.A. & Haber E., (1978) Experimental myocardial infarct imaging following intravenous adminstration of iodine-131-labeled antibody F(ab')2 fragments specific for cardiac myosin. *Circulation*; **57**: 743-750.

Khaw B.A., Beller G.A., Haber E. & Smith T.W., (1976) Localization of cardiac myosin-specific antibody in myocardial infarction. *J Clin Invest*; **58**: 439-446.

Khaw B.A., Fallon J.T., Beller G.A. & Haber E., (1979) Specificity of localization of myosin-specific antibody fragments in experimental myocardial infarction. *Circulation*; **60**: 1527-1531.

Khaw B.A., Fallon J.T., Strauss H.W. & Haber E., (1980) Myocardial infarct imaging of antibodies to canine cardiac myosin with Indium-111-diethylenetriamine pentaacetic acid. *Science*; **209**: 295-297.

Khaw B.A., Gold H.K., Yasuda T., Leinbach R.C., Kanke M., Fallon J.T., Barlai-Kovach M., Strauss H.W., Sheehan F. & Haber E., (1986) Scintigraphic quantification of myocardial necrosis in patients after intravenous injection of myosin-specific antibody. *Circulation*; **74**: 501-508.

Khaw B.A., Mattis J.A., Melincoff G., Strauss H.W., Gold H.K. & Haber E., (1984) Monoclonal antibody to cardiac myosin: imaging of experimental myocardial infarction. *Hybridoma*; **3**: 11-22.

Khaw B.A., Strauss H.W., Pohost G.M., Fallon J.T., Katus H.A & Haber E., (1983) Relation of immediate and delayed thallium-201 distribution to localization of iodine-125 antimyosin antibody in acute experimental myocardial infarction. *Am J Cardiol*; **51**: 1428-1432.

Kirkeby S., Bog-Hansen T.C., Moe D. & Garbarsch C., (1991) Lectin binding in skeletal muscle. Evaluation of alkaline phosphatase conjugated avidin staining procedures. *Histochem J*; **23**: 345-354.

Klionsky B., (1960) Myocardial ischaemia and early infarction. A histochemical study. *Am J Pathol*; **36**: 575-591.

Kloner R.A., Ganote C.E., Whalen D.A., & Jennings R.B., (1974) Effect of a transient period of ischemia on myocardial cells. II. Fine structure during the first few minutes of reflow. *Am J Pathol*; **74**: 399-422.

Kloner R.A., Rude R.E., Carlson N., Naroko P.R., DeBoer L.W.V. & Braunwald E., (1980) Ultrastructural evidence of microvascular damage and myocardial cell injury after coronary artery occlusion: which comes first?. *Circulation*; **62**: 945-952.

Knight B.H. (1969) Myocardial ischaemia. In: Camps F.E. (ed.). *Recent Advances in Forensic Pathology*. J. & A. Churchill Ltd. London, pp 20-34.

Knight B.H., (1979) A further evaluation of the reliability of the HBFP stain in demonstrating myocardial damage. *Forensic Sci Int;* **13**: 179-181.

Kocourek J., (1986) Historical background. In: Liener I.E., Sharon N. & Goldstein I.J. (eds.). *The Lectins. Properties, functions, and applications in biology and medicine*. Academic Press Inc., Harcourt Brace Jovanovich. London, pp: 1-32.

Kocourek J. & Horejst V., (1981) Defining a lectin. *Nature;* **290**: 188.

Koffler D., Biesecker G., Noble B., Andres G.A. & Martinez-Hernandez A., (1983) Localization of the membrane attack complex (MAC) in experimental immune complex glomerulonephritis. *J Exp Med*; **157**: 1885.

Koobs D.H., Schultz R.L. & Jutzy R.V., (1978) The origin of lipofuscin and possible consequences to the myocardium. *Arch Path Lab Med*; **102**: 66-68.

Koteliansky V.E. & Gneushev G.N., (1983) Vinculin localization in cardiac muscle. *FEBS Lett;* **159**: 158-160.

Koteliansky V.E., Gneushev G.N., Shartava A.S., Shirinsky V.P., Glukhova M.A. & Goodman S.R. (1983) The regulation by vinculin of filamin, α-actinin, and spectrin tetramer-induced actin sol-gel transformation. *FEBS Lett*; **151(2)**: 206-210.

Laitinen L., Juusela H. & Virtanen I., (1990) Binding of the blood group-reactive lectins to human adult kidney specimens. *Anat Rec*; **226**: 10-17.

Larue C., Calzolari C., Leger J., Leger J. & Pau B., (1991) Immunoradiometric assay of myosin heavy chain fragments in plasma for investigation of myocardial infarction. *Clin Chem*; **37(1)**: 78-82.

Lazarides E., (1980) Intermediate filaments as mechanical integrators of cellular space. *Nature*; **283**: 249-256.

Lazarides E. & Hubbard B.D., (1976) Immunological characterization of the subunit of the 100 A filaments from muscle cells. *Proc Natl Acad Sci USA*; **73**: 4344-4348.

Leadbeatter S., Wawman H.M. & Jasan B., (1989) Immunocytochemical diagnosis of early myocardial ischaemic/hypoxic damage. *Forensic Sci Int;* **40**: 171-180.

Leadbeatter S., Wawman H.M. & Jasan B., (1990) Further evaluation of immunocytochemical staining in the diagnosis of early myocardial ischaemic/hypoxic damage. *Forensic Sci Int;* **45**: 135-141.

Leader M., Collins M., Patel J. & Henry K., (1986) Staining for factor VIII-related antigen and Ulex europaeus aglutinin 1(UEA-1) in 230 tumours. An assessment of their specificity for angiosarcoma and Kaposi's sarcoma. *Histopathology*; **10**: 1153-1162.

Léger J.C., Dehchesne C., Bouvagnet P., Pons F. & Léger J.J., (1984) Characterization and distribution of myosin variants in normal and pathological human hearts. *Eur Heart J*; **5** (suppl-F): 111-117.

Léger J.C., Ming C., Calzolari C., Gautier P., Mouton C., Grolleau R., Louisot P., Puech P., Peperstraete B., Staroukine M., Telerman M., Pau B. & Léger J.J., (1990) Assay of serum cardiac myosin heavy chain fragments in patients with acute myocardial infarction: determination of infarct size and long-term follow-up. *Am Heart J;* **120**: 781-790.

Leibowitz J.O., (1960) Amatus Lusitanus on sudden death. *J Hist Med & Allied Sci*; **15**: 364-371.

Levine S.A. & Brown C.L., (1929) Coronary thrombosis. VII. Pathological consideration. *Medicine*; **VIII (3)**: 345-418.

Lewis R.E., Johnson W.W. & Cruse J.M., (1983) Pitfalls and caveats in the methodology for immunoperoxidase staining in surgical pathologic diagnosis. *Surv Synth Path Res*; **1(1-2)**: 134-152.

Lichtig C., Brooks H., Chassagne G., Glasgov S. & Wissler R.W., (1975) Basic fuchsin picric acid method to detect acute myocardial ischaemia. *Arch Pathol;* **99**: 158-161.

Lie J.T., (1968) Detection of early myocardial infarction by the acid fuchsin staining technic. *Am J Clin Pathol*; **50(3)**: 317-319.

Lie J.T., (1978) Centenary of the first correct antemortem diagnosis of coronary thrombosis by Adam Hammer (1818-1878). English translation of the original report. *Am J Cardiol*; **42**: 849-852.

Lie J.T., Holley K.E., Kampa W.R. & Titus J.L., (1971) New histochemical method for morphological diagnosis of early stages of myocardial ischemia. *Mayo Clin Proc*; **46**: 319-327.

Lie J.T., Holley K.E. & Titus J.L., (1972) Fuchsinorrhagia-a new histochemical indication of inapparent early myocardial ischaemia. *Lab Med*; **3**: 37-40.

Lillie R.D., (1948) Histopathologic Technic. The Blakiston Co. Philadelphia, pp 249-250.

Linke R.P., (1983) Identification of AA-type amyloid in tissue sections using monoclonal antibodies. In: Peeters H. (ed.). *Protides Biol Fluids*. Vol. 31. Pergamon Press. Oxford, pp 835-838.

Linke R.P., (1984) Monoclonal antibodies against fibril protein AA. Production, specificity, and use for immunohistochemical localization and classification of AA-type amyloidosis. *J Histochem Cytochem*; **32**: 322-328.

Lis H. & Sharon N., (1977) Lectins: Their chemistry and application to immunology. In: Sela M. (ed.). *The Antigens*. Academic Press Inc., Harcourt Brace Jovanovich. London, pp 436-437.

Lis H. & Sharon N., (1986a) Lectins as molecules and as tools. *Annu Rev Biochem*; **55**: 35-67.

Lis H. & Sharon N., (1986b) Applications of lectins. In: Liener I.E., Sharon N. & Goldstein I.J. (eds.). *The Lectins. Properties, functions, and applications in biology and medicine*. Academic Press Inc., Harcourt Brace Jovanovich. London, pp 293.

Lloyd R.V., Foley J. & Judd W.J., (1984) Peanut lectin agglutinin and α-lactalbumin. *Arch Pathol Lab Med*; **108**: 392-395.

Lodge-Patch I., (1951) The ageing of cardiac infarcts and its influence on cardiac rupture. *Br Heart J*; **13**: 37-42.

Lott J.A. & Stang J.M., (1980) Serum enzymes and isoenzymes in the diagnosis and differntial diagnosis of myocardial ischaemia and necrosis. *Clim Chem,* **26**: 1241-1250.

Luna A., Carmona A. & Villanueva E., (1983) The postmortem determination of CK isoenzymes in pericardial fluid in various causes of death. *Forensic Sci Int;* **22**: 23-30.

Luna A., Villanueva E., Castellano M.A. & Jimenez G., (1982) The determination of CK, LDH and its isoenzymes in pericardial fluid and its application to the post-mortem diagnosis of myocardial infarction. *Forensic Sci Int;* **19**: 85-91.

Mahdavi V., Chambers A.P. & Nadal-Ginard B., (1984) Cardiac α– and β-myosin heavy chain genes are organised in tandem. *Proc Natl Acad Sci USA*; **81**: 2626-2630.

Mair J., Dienstl F. & Puschendorf B., (1992) Cardiac troponin T in the diagnosis of myocardial injury. *Crit Rev Clin Lab Sci*; **29(1)**: 31-57.

Malinin T.I., (1961) Feulgen-oxidized tanning-azo (FOTA) technique for concomitant staining of DNA and protein. *Stain Technol*; **36**: 198-200.

Malinin T.I., Stokes J.R., Hardy L.B., & Lumb G., (1968) Experimental coronary artery narrowing in swine. I. Morphologic and histochemical changes. *Johns Hopk Med J*; **122**: 102-111.

Malkin H.M., (1984) Julius Cohneim (1839-84). His life and contributions to pathology. *Ann Clin Lab Sci;* **14**: 335-342.

Mallory G.K., White P.D. & Salcedo-Salgar J., (1939) The speed of healing of myocardial infarction. *Am Heart J*; **18 (6)**: 647-671.

McGee-Russell S.M., (1958) Histochemical methods for calcium. *J Histochem;* **6**: 22-24.

McLean I.W. & Nakane P.K., (1974) Periodate-lysine-para-formaldehyde fixative. A new fixative for immunoelectron microscopy. *J Histochem Cytochem*; **22(12)**: 1077-1083.

McManus L.M., Kold W.P., Crawford M.H., O'Rourke R.A., Grover F.L. & Pinckard R.N., (1983) Complement localisation in ischaemic baboon myocardium. *Lab Invest*; **48**: 436-447.

Meri S., Waldmann H. & Lachmann P.J., (1991) Distribution of protectin (CD59), a complement membrane attack inhibitor, in normal human tissues. *Lab Invest;* **65**: 532-537

Miettinen M., Holthofer H., Lehto V-P., Miettinen A. & Virtanen I., (1983) Ulex europaeus 1 lectin as a marker for tumours derived from endothelial cells. *Am J Clin Pathol;* **79**: 32-36.

Miettinen M. & Rapola J., (1989) Immunohistochemical spectrum of rhabdomyosarcoma and rhabdomyosaroma-like tumours. *Am J Pathol*; **13(2)**: 120-132.

Milici A.J. & Porter G.A., (1991) Lectin and immunolabeling of microvascular endothelia. *J Electron Microsc Tech;* **19**: 305-315.

Millikan G.A., (1939) Muscle hemoglobin. *Physiol Rev*; **19:** 503-523.

Miyauchi T., Tomobe Y., Shiba R., Ishikawa T., Yanagisawa M., Kimura S., Sugishita Y., Ito I., Goto K. & Masaki T., (1990) Involvement of endothelium in the regeneration of human vascular tonus. Potent vasoconstrictor effect and existence in endothelial cells. *Circulation*; **81**: 1874-1880.

Mokri B. & Engel A.G., (1975) Duchenne dystrophy: Electron microscopic findings pointing to a basic or early abnormality in the plasma membrane of the muscle fibre. *Neurology*; **25**: 1111-1120.

Morales A.R. & Fine G., (1966) Early human myocardial infarction. A histochemical study. *Arch Pathol;* **82**: 9-14.

Morales A.R., Fine G. & Taber R.E., (1967) Cardiac surgery and myocardial necrosis. *Arch Pathol*; **83**: 71-79.

Mowry R.W. & Millican R.C., (1953) A histochemical study of the distribution and fate of dextran in tissues of the mouse. *Am J Pathol*; **29**: 523-545.

Mukai K., Rosai J. & Buggdorf W.H.C., (1980) Localization of factor VIII-related antigen in vascular endothelial cells using an immunoperoxidase method. *Am J Surg Pathol*; **4**: 273-276.

Mullane K.M. & Young M., (1992) The contribution of neutrophil activation and changes in endothelial function to myocardial ischemia-reperfusion injury. In: Yellon D.M. & Jennings R.B. (eds.). *Myocardial Protection: The Pathophysiology of Reperfusion and Reperfusion Injury*. Raven Press. New York, pp 59-83.

Nabel E.G., (1991) Biology of the impaired endothelium. *Am J Cardiol*; **68**: 6C-8C.

Nachlas M.M., Tsou K-C., DeSouza E., Cheng C-S. & Seligman A.M., (1957) Cytochemical demonstration of succinic dehydrogenase by the use of a new p-nitrophenyl substituted ditetrazole. *J Histochem*; **5**: 420-436.

Nag S., Robertson D.M. & Dinsdale H.B., (1981) Cerebrovascular permeability in mechanically induced hypertension. *Can J Neurol Sci;* **8**: 215-220.

Nayar A. & Olsen E.G.J., (1974) The use of the basic fuchsin stain in the recognition of early myocardial ischaemia. *Cardiovasc Res;* **8**: 391-394.

Nelson R.A., Jensen J., Gigli I. & Tamura N., (1966) Methods for the separation, purification and measurement of nine components of hemolytic complement in guinea-pig serum. *Immunochemistry*; **3**: 111-135.

Nicolson G.L. & Singer S.J., (1971) Ferritin-conjugated plant agglutinins as specific saccharide stains for electron microscopy: Application to saccharides bound to cell membranes. *Proc Natl Acad Sci USA*; **68(5)**: 942-945.

Nielsen K., Renaud S., Lemire Y. & Selye H., (1958) Fuchsinophilic degeneration of myocardial fibres. Meeting of the Canadian Federation of Biological Societies, Kingston, Ontario, June 9-11. pp 37-38.

Niggli V. & Burger M.M., (1987) Interaction of the cytoskeleton with the plasma membrane. *J Memb Biol*; **100**: 97-121.

Niles N.R. & Barnhouse D.L., (1967) The acid hematein stain and myocardial damage. *Arch Pathol;* **83**: 407-410.

Niles N.R., Bitensky L., Chayen J., Cunningham G.J. & Braimbridge M.V., (1964) The value of histochemistry in the analysis of myocardial dysfunction. *Lancet*; **1**: 963-965.

Nishida S., Akai Hiruma S., Maeda M., Tanji K. & Hashimoto S., (1986) Experimental study of WGA binding on the endothelial cell surface in cerebral ischaemia. *Histol Histopathol;* **1**: 69-74.

Nishida S., Hiruma S. & Hashimoto S., (1987) Immuno-histochemical change of actin in experimental myocardial ischaemia. Its usfulness to detect very early myocardial damages. *Histol Histopathol*; **2**: 417-428.

Nolan A.C., Clark W.A., Karwoski T. & Zak R., (1983) Patterns of cellular inury in myocardial ischaemia determined by monoclonal antimyosin. *Proc Natl Acad Sci USA*; **80**: 6046-6050.

Nomato K., Mori N., Shoji T. & Nakamura K., (1987) Early loss of myocardial myoglobin detected immunohistochemically following occlusion of the coronary artery in rats. *Exp Mol Pathol*; **47**: 390-402.

Novelli A., (1959) Rapid staining of mitochondria. *Experientia*; **15(7):** 274.

Nowell P.C., (1960) Phytohaemagglutinin: An inhibitor of mitosis in cultures of normal human leukocytes. *Cancer Res*; **20**: 462-466.

Oakley C.E. & Oakley B.R., (1989) Identification of γ-tubulin, a new member of the tubulin superfamily encoded by mipA gene of Aspergillus nidulans. *Nature*; **338**: 662-664.

Oehmichen M., Pedal I. & Hohmann P., (1990) Diagnostic significance of myofibrillar degeneration of cardiocytes in forensic pathology. *Forensic Sci Int;* **48**: 163-173.

Okun E.M., Factor S.M. & Kirk E.S., (1979) End-capillary loops in the heart: An explanation for discrete myocardial infarctions without border zones. *Science*; **206**: 565-569.

Opie L.H., (1991) *The Heart. Physiology and Metabolism.* 2nd. ed. Raven Press. New York.

Opie L.H., (1993) The mechanism of myocyte death in ischaemia. *Eur Heart J;* **14** (suppl-G): 31-33.

Ordonez N.G. & Batsakis J.G., (1984) Comparison of Ulex europaeus I lectin and Factor VIII-related antigen in vascular lesions. *Arch Pathol Lab Med*; **108**: 129-132.

Osler W., (1889) Rupture of the heart. *Med News*; **54**: 129-130.

Osler W., (1897) Lectures on angina pectoris and allied states. D. Appleton & Co. New York.

Osuna E., Carceles M.D.P., Jakobsson S.W. & Luna A., (1990) Biochemical and morphological markers in the postmortem diagnosis of ischaemic heart distress. *Acta Med Leg Soc* (Liege); **40**: 275-283.

Paljarvi L., Karjalainen K. & Kalimo H., (1982) Lectin histochemistry of normal and diseased human muscle. *Acta Neurol Scand;* **65** (suppl): 248-249.

Pardo J.V., Siliciano J.D. & Craig S.W., (1983) A vinculin containing cortical lattice in skeletal muscle: Transverse lattice elements ("costameres") mark sites of attachment between myofibrils and sarcolemma. *Proc Natn Acad Sci USA;* **80**: 1008-1012.

Pearse A.G.E., (1961) *Histochemistry. Theoretical and Applied.* 2nd. ed. J. & A. Churchill Ltd. London, pp 661-662.

Pena S.D.J., Gordon B.B., Karpati G. & Carpenter S., (1981) Lectin histochemistry of human skeletal muscle. *J Histochem Cytochem;* **29(4)**: 542-546.

Petrusz P., Ordonneau P. & Finley J.C.W., (1980) Criteria of reliability for light microscopic immunohistochemical staining. *Histochem J*; **12**: 333-348.

Petrusz P., Sar M., Ordronneau P. & Dimeo P., (1976) Specificity in immunocytochemical staining. *J Histochem Cytochem;* **24**: 1110-1112.

Pinckard R.N., O'Rourke R.A., Crawford M.H., Grover F.S., McManus L.M., Ghidoni J.J., Storrs S.B., Olson M.S., (1980) Complement localisation and mediation of ischaemic injury in baboon myocardium. *J Clin Invest;* **66**: 1050-1056.

Plendl J., Hartwell L. & Auerbach R., (1993) Organ-specific change in Dolichos Biflorus lectin binding by myocardial endothelial cells during in vitro cultivation. *In Vitro Cell Dev Biol;* **29A(1)**: 25-31.

Poche R., (1969) Ultrastructure of heart muscle under pathological conditions. *Ann N Y Acad Sci*; **156(1)**: 34-47.

Poley R.W., Fobes C.D. & Hall M.J., (1964) Fuchsinophilia in early myocardial infarction. *Acta Pathol*; **77**: 325-329.

Pollard T.D. & Cooper J.A., (1986) Actin and actin-binding proteins. A critical evaluation of mechanisms and functions. *Ann Rev Biochem*; **55**: 987-1035.

Ponder B.A.J., (1983) Lectin histochemistry. In: Polak M. & Noorden V. (eds.). *Immunocytochemistry. Practical Applications in Pathology and Biology*. P.S.G. Wright. London, pp 129-142.

Ponder B.A.J. & Wilkinson M.M., (1983) Organ-related differences in binding of Dolichos biflorus agglutinin to vascular endothelium. *Develop Biol*; **96**: 535-541.

Pratt R.M. & Gibson W.A., (1973) An immunofluorescent technique for observing the binding of Concanavalin A to frozen tissue sections. *J Histochem Cytochem*; **21**: 229-232.

Puchtler H., Sweat W.F., Terry M.S. & Conner H.M., (1969) A combined PAS-myofibril stain for demonstration of early lesions of striated muscle. *J Microscopy;* **89**: 329-338.

Puchtler H., Waldrop F.S., Meloan S.N., Terry M.S. & Conner H.M., (1970) Methacarn (methanol-carnoy) fixation. Practical and theoretical considerations. *Histochemis*; **21**: 97-116.

Rajs J. & Jakobsson S., (1976) Experiences with the haematoxylin basic fuchsin picric acid staining method for morphologic diagnosis of myocardial ischaemia. An experimental study in forensic pathology. *Forensic Sci*; **8**: 37-48.

Ramani P., Bradley N.J., Fletcher C.D.M., (1990) QBEND/10 a new monoclonal antibody to endothelium: assessment of its diagnostic utility in paraffin sections. *Histopathology*; **17**: 237-242.

Rangdaeng S., Fruong L.D., (1991) Comparative immunohistochemical staining for desmin and muscle-specific actin. A study of 576 cases. *Am J Clin Pathol;* **96**: 32-45.

Rapin A.M.C. & Burger M.M., (1974) Tumour cell surfaces: General alterations detected by agglutinins. *Adv Cancer Res*; **20**: 1-91.

Rapport L. & Samuel J.L., (1988) Microtubules in cardiac myocytes. *Int Rev Cytol*; **113**: 101-143.

Reichenbach D.D. & Beneditt E.P., (1968) Myofibrillar degeneration. A response of the myocardial cell to injury. *Arch Pathol*; **85**: 189-199.

Reid W.A., Brown D., Purvest D. & Barnest L., (1992) Diagnosis of the acute ischaemic heart by ion elution from myocardium. *J Pathol*; **167**: 205-209.

Reimer K.A. & Ideker R.E., (1987) Myocardial ischaemia and infarction: anatomic and biochemical substrates for ischaemic cell death and ventricular arrthythmias. *Hum Pathol;* **18(5)**: 462-475.

Reimer K.A., Jennings R.B. & Hill M.L., (1981) Total ischemia in dog hearts in vitro. 2. high energy phosphate depletion and associated defects in energy metabolism, cell volume regulation, and sarcolemmal integrity. *Circ Res*; **49**: 901-911.

Reimer K.A., Jennings R.B. & Tatum A.H., (1983) Pathobiology of acute myocardial ischaemia: metabolic, functional and ultrastructural studies. *Am J Cardiol*; **52**: 72A-81A.

Renkonen K.O., (1948) Studies on haemagglutinins present in seed of the same representatives of the family of leguminoseae. *Ann Med Exp Biol Fenn*; **26**: 66-72.

Rindfleisch G.E., (1872) *Manual of Pathological Histology to Serve as an Introduction to the Study of Morbid Anatomy.* Baxter E.B. (trans.). New Sydenham Society. London.

Roberts R., (1994) Introduction to the techniques of molecular biology and the molecular basis for cardiac growth. In: Schlant R.C. & Alexander R.W. (eds.). *The Heart, Arteries and Veins.* 8th. ed. McGraw-Hill, Inc. London, pp 21.

Robertson J.D., Bodenheimer T.S. & Stage D.E., (1963) The ultrastructure of Mauthner cell synapses and nodes in goldfish brains. *J Cell Biol*; **19**: 159-199.

Roitt I.M., Brostoff J. & Male D.K., (1993) Complement. In: *Immunology.* 3rd. ed. Mosby. London, pp 12.1-12.17.

Roseman S., (1975) Sugars of the cell membrane. In: Weismann G. & Cainborne R. (eds.). *Cell Membranes.* H. B. Publishing Co. New York, pp 55-64.

Rossen R.D., Swain J.L., Michael L.H., Weakley S., Giannini E. & Entman M.L., (1985) Selective accumulation of the first component of complement and leukocytes in ischaemic canine heart muscle. A possible initiator of an extra myocardial mechanism of ischaemic injury. *Circ Res*; **57**: 119-130.

Roth J., (1983a) Application of lectin-gold complexes for election microscopic localisation of glycoconjugates on thin sections. *J Histochem Cytochem;* **31**: 987-999.

Roth J., (1983b) Applications of immunocolloids in light microscopy. II. Demonstration of lectin-binding sites in paraffin sections by the use of lectin-gold or glycoprotein-gold complexes. *J Histochem Cytochem*; **31**: 547-552.

Roth J., Lucocq J.M. & Charest P.M., (1984) Light and electron microscopic demonstration of sialic acid residues with the lectin from limax flarus: A cytochemical affinity technique with the use of fetuin-gold complexes. *J Histochem Cytochem*; **32(11)**: 1167-1176.

Rus H.G., Niculescu F. & Vlaicu R., (1987) Presence of $C5_b$-9 complement complex and S-protein in human myocardial areas with necrosis and sclerosis. *Immunol Lett*; **16**: 15-20.

Ryan E.S., (1986) The endothelial surface and responses to injury. *Fed Proc*; **45**: 101-108.

Sakurai I., (1977) Pathology of ischaemic myocardium. Special references to (I) evaluation of morphological methods for detection of early myocardial infarcts and (II) lipid metabolism in infarcted myocardium. *Acta Pathol Jpn;* **27(5)**: 587-603.

Sarphie T.G., (1985) Anionic surface properties of aortic and mitral valve endothelium from New Zealand white rabbits. *Am J Anat*; **174**: 145-160.

Sashida H., Uchida K. & Abiko Y., (1984) Changes in cardiac ultrastructure and myofibrillar proteins during ischaemia in dogs, with special reference to changes in Z lines. *J Mol Cell Cardiol*; **16**: 1161-1172.

Saukko P. & Knight B., (1989) Evaluation of eosin-fluorescence in the diagnosis of sudden cardiac death. *Forensic Sci Int;* **40**: 285-290.

Schäfer H., Mathey D., Hugo F. & Bhakdi S., (1986) Deposition of the terminal $C5_b$-9 complement complex in infarcted areas of human myocardium. *J Immunol*; **137**: 1945-1949.

Schaper J., Froede R., Hein S., Buck A., Hashizume H., Speiser B., Friedl A. & Bleese N., (1991) Impairment of the myocardial ultrastructure and changes of the cytoskeleton in dilated cardiomyopathy. *Circulation*; **83**: 504-514.

Schlesinger M.J. & Reiner L., (1955) Focal myocytolysis of the heart. *Am J Pathol*; **31**: 443-459.

Schulte B.A. & Spicer S.S., (1983) Light microscopic histochemical detection of sugar residues in secretory glycoproteins of rodent and human tracheal glands with lectin-horseradish peroxidase conjugates and the galactose oxidase-schiff sequence. *J Histochem Cytochem*; **31(3)**: 391-403.

Schulte B.A., Spicer S.S., Miller R.L., (1984) Histochemical localization of sialoglycoconjugates with a sialic acid-specifc lectin from the slug Limax flauus. *Histochem J*; **16**: 1125-1132.

Sethna D.H., (1973) The story of coronary artery disease clinical features. *J Postgrad Med*; **19**: 105-122.

Severs N.J., (1985) Intercellular junctions and the cardiac intercalated disc. *Adv Myocardiol*; **5**: 223-242.

Sharon N., (1984) Surface carbohydrates and surface lectins are recognition determinants in phagocytosis. *Immunol Today*; **5(5)**: 143-147.

Sharon N., (1987) Bacterial lectins, cell-cell recognition and infectious disease. *FEBS Lett*; **217**: 145-157.

Sharon N. & Lis H., (1972) Lectin-agglutinating and sugar-specific proteins. *Science*; **177**: 949-959.

Sharon N. & Lis H., (1987) A century of lectin research (1888-1988). *Trends Biochem Sci*; **12**: 488-491.

Sharon N. & Lis H., (1989) Lectins as cell recognition molecules. *Science*; **246**: 227-234.

Shnitka T.K. & Nachlas M.M., (1963) Histochemical alterations in ischemic heart muscle and early myocardial infarction. *Am J Pathol;* **42(5)**: 507-521.

Siegel R.J., Edwalds G., Rej R. & Fishbein M.C., (1984) Distribution of cytosolic and mitochondrial aspartate aminotransferase in normal, ischaemic, and necrotic myocardium. An immunohistochemical study. *Lab Invest*; **51**: 648-54.

Siegel R.J. & Fishbein M.C., (1982) Evaluation of fluorescence microscopy for the detection of necrotic myocardium. *Hum Pathol*; **13(12)**: 1091-1094.

Silva D.P., Landt T., Porter S.E., Ladenson J.H., (1991) Development and application of monoclonal antibodies to human cardiac myoglobin in a rapid fluorescence immunoassay. *Clin Chem*; **37(8)**: 1356-1364.

Simionescu M., Simionescu N. & Palade G.E., (1981) Differential microdomains on the luminal surface of capillary endothelium. I. preferential distribution of anionic sites. *J Cell Biol*; **90**: 605-613.

Simionescu M., Simionescu N. & Palade G.E., (1982) Differential microdomains on the luminal surface of capillary endothelium: Distribution of lectin receptors. *J Cell Biol*; **94**: 406-413.

Simionescu M., Simionescu N. & Santoro F., (1985) Differentiated microdomains of the luminal plasmalemma of marine capillaries: Segmental variations in young and old animals. *J Cell Biol;* **100**: 1396-1407.

Simpson K.L., Houlihan J.M. & Holmes C.H., (1993) Complement regulatory proteins in early human fetal life: CD59, membrane co-factor protein (MCP) and decay-accelerating factor (DAF) are differentially expressed in the developing liver. *Immunology;* **80**: 183-140.

Sommers H.M. & Jennings R.B., (1964) Experimental acute myocardial infarction. Histologic and histochemical studies of early myocardial infarcts induced by temporary or permanent occlusion of a coronary artery. *Lab Invest*; **13**: 1491-1503.

Spicer S.S., Erlandsen S.L., Wilson A.C., Hammer M.F., Hennigar R.A. & Schulte B.A., (1987) Genetic differences in the histochemically defined structure of oligosaccharides in mice. *J Histochem Cytochem*; **35(II)**: 1231-1244.

Spicer S.S., Schulte B.A., (1988) Detection and differentiation of glycoconjugates in various cell types by lectin histochemistry. *Basic Appl Histochem*; **32**: 307-320.

Stobo J.D. & Rosenthal A.S., (1972) Biologically active concanavalin A complexes suitable for light and electron microscopy. *Exp Cell Res*; **70**: 443-447.

Stahl E., (1990) Screening of myocardial contraction bands: A comparison between two histological staining methods. *Forensic Sci Int;* **45**: 151-157.

Steenbergen C., Hill M.L. & Jennings R.B., (1987) Cytoskeletal damage during myocardial ischaemia: changes in vinculin immunofluorescence staining during total in vitro ischaemia in canine heart. *Circ Res*; **60**: 478-486.

Stegemann M., Meyer R., Haas H.G. & Robert-Nicoud M., (1990) The cell surface of isolated cardiac myocytes. A light microscopic study with use of fluorochrome-coupled lectins. *J Mol Cell Cardiol*; **22**: 787-803.

Stein O., Chajek T. & Stein Y., (1976) Ultrastructural localisation of concanavalin A in the perfused rat heart. *Lab Invest*; **35(2)**: 103-110.

Stephenson T.J., Griffiths D.W.R. & Mills P.M., (1986) Comparison of Ulex europaeus I lectin binding and factor VIII-related antegen as markers of vascular endothelium in follicular carcinoma of the thyroid. *Histopathology*; **10**: 251-260.

Stevens A. & Lowe J.S., (1992) Contractile cells. *Histology*. Gower Medical Publishing. London, pp 57-62.

Stewart R.V., Zumwalt R.E., Hirsch C.S. & Kaplan L., (1984) Postmortem diagnosis of myocardial disease by enzyme analysis of pericardial fluid. *Am J Clin Pathol;* **82**: 411-417.

Stossel T.P., (1978) Contractile proteins in cell structure and function. *Annu Rev Med*; **29**: 427-457.

Stoward P.J., Spicer S.S. & Miller R.L., (1980) Histochemical reactivity of peanut lectin-horseradish peroxidase conjugate. *J Histochem Cytochem*; **28(9)**: 979-990.

Strehler B.L., Mark D.M., Mildvan A.S. & Gee M.V., (1959) Rate and magnitude of age pigment accumulation in the human myocardium. *J Gerontol*; **14**: 430-439.

Sulkin N.M. & Sulkin D.F., (1965) An electron microscpic study of the effects of chronic hypoxia on cardiac muscle, hepatic and autonomic ganglion cells. *Lab Invest*; **14(8)**: 1523-1546.

Sumner J.B., (1919) The globulins of the jack bean, Canavalia ensiformis. *J Biol Chem*; **37**: 137-142.

Takeichi M., (1988) The cadherins: cell-cell adhesion molecules controlling animal pathogenesis. *Development*; **102**: 639-655.

Teraoka K., Kaneko N. & Takeishi M., (1991) Clinical and pathologic studies on contraction band lesion: relation to acute myocardial infarction and unexplained sudden death. *Mod Pathol*; **4(1)**: 6-12.

Thompson P.L., Fletcher E.E. & Katavatis V., (1979) Enzymatic indices of myocardial necrosis: Influence on short- and long-term prognosis after myocardial infarction. *Circulation;* **59**: 113-120

Thorgeirsson G. & Robertson A.L., (1978) The vascular endothelium-pathologic significance. *Am J Pathol*; **93**: 803-848.

Tokunaga O., Yamada T. & Fan J., (1991) Age-related decline in prostacyclin synthesis by human aortic endothelial cells. *Am J Pathol*; **138**: 941-949.

Tokuyasu K.T., Dutton A.H., Geigor B. & Singer S.J., (1981) Ultrastructure of chicken cardiac muscle as studied by double immunolabeling in electron microscopy. *Proc Natl Acad Sci USA*; **78(12)**: 7619-7623.

Truong L.D., Rangdaeng S., Cagle P., Ro J.Y., Hawkins S. & Font R.L., (1990) The diagnostic utility of desmin. A study of 584 cases and review of the literature. *Am J Clin Pathol*; **93**: 305-315.

Tsukada T., Tippens D., Gordon D., Ross R. & Gown A.M., (1987) HH35, a muscle-actin-specific monoclonal antibody. *Am J Pathol*; **126**: 51-60.

Väkevä A., Lautila P. & Meri S., (1992) Loss of expression of protectin (CD59) is associated with complement membrane attack complex deposition in myocardial infarction. *Lab Invest*; **67(5)**: 608-616.

Väkevä A., Lautila P. & Meri S., (1993) Regulation of complement membrane attack complex formation in myocardial infarction. *Am J Pathol*; **143(1)**: 65-75.

Van Reempts J., Borgers M. & Renember R.S., (1976) Early myocardial ischaemia: evaluation of the histochemical haematoxylin-basic fuchsin picric acid (HBFP) staining technique. *Cardiovasc Res*; **10**: 262-267.

Varga M. & Zsonda L., (1988) A simple method for postmortem detection of acute myocardial infarction. *Forensic Sci Int;* **37**: 259-263.

Vlaicu R., Rus H. & Niculescu F., (1988) Immunohistochemical localization of the terminal $C5_b$-9 complement complex associated to S-protein and macrophage immunoreactive deposits in human damaged myocardial areas. *Med Interne*; **26(1)**: 21-27.

Walker R.A., (1983) The binding of fluorescent-labelled concanavlin A to human breast tissue-a marker of differentiation. *J Pathol;* **140**: 255-265.

Walker R.A., (1984a) The binding of peroxidase-labelled lectins to human breast epithelium. I. Normal, hyperplastic and lactating breast. *J Pathol*; **142**: 279-291.

Wartman W.B., Jennings R.B., Yokoyama H.O., & Claubagh G.F., (1956) Fatty change of the myocardium in early experimental infarction. *A M A Arch Pathol*; **62**: 318-323.

Watanabe M., Muramatsu T., Shirane H. & Ugai K., (1981) Discrete distribution of binding sites for Dolichos biflorus agglutinin (DBA) and for peanut agglutinin (PNA) in mouse organ tissue. *J Histochem Cytochem*; **29(7)**: 779-790.

Watt S.M., Karhi K., Gatter K., Furley A.J.W., Katz F.E., Healy L.E., Altass L.J., Bradley N.J., Sutherland D.R., Levinsky R. & Greaves M.F., (1987) Distribution and epitope analysis of the cell membrane glycoprotein (HPCA-1) associated with human haemopoietic progenitor cells. *Leukaemia*; **1(5)**: 417-426.

Welch W.H., (1899) Diseases of the coronary arteries and alterations in the muscular walls of the heart. *Proc Trans Pathol Soc Toronto*; **1**: 8-9.

Williams T.J. & Rampart M., (1987) The role of the complement system in acute myocardial infarction. In: Spry C.J.F. (ed.). *Immunology and Molecular Biology of Cardiovascular Diseases.* M.T.P. Press Ltd. Lancaster, pp 119-128.

Wittenberg J.B., (1970) Myoglobin-facilitated oxygen diffusion: role of myoglobin in oxygen entry into muscle. *Physiol Rev*; **50(4)**: 559-636.

Wolfe W.G., Dudley Jr. A.W. & Wallace A.G., (1968) A pathological study of unsuccessful cardiac resuscitation. *Arch Surg*; **96**: 123-126.

Yanagisawa M., Kurihata H., Kimura S., Tomobe Y., Kobayshi M., Mitsui Y., Yasak Y., Goto K. & Masaki T., (1988) A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*; **332**: 411-415.

Yazaki Y., Tsuchimochi H., Kuro-O M., Kurabayashi M., Isobe M., Ueda S., Nagai R. & Takaku F., (1984) Distribution of myosin isozymes in human atrial and ventricular myocardium: comparison in normal and overloaded heart. *Eur Heart J;* **5** (suppl-F): 103-110.

Yee H.F., Kuwata J.H. & Langer G.A., (1991) Effects of neuraminidase on cellular calcium and contraction in cultured cardiac myocytes. *J Mol Cell Cardiol;* **23**: 175-185.

Yokoyama H.O., Jennings R.B., Clabaugh G.F. & Wartman W.B., (1955) Histochemical studies of early experimental myocardial infarction. Periodic acid-Schiff method. *Arch Pathol;* **59**: 347-354.

Yonezawa S., Nakamura T., Tanaka S. & Sato E., (1982) Glycoconjugates with Ulex europaeus agglutinin-I-binding sites in normal mucosa adenoma and carcinoma of the human large bowel. *J Natl Cancer Inst*; **69**: 777-786.

Zabel M., Köster W. & Hohnloser S.H., (1993) Usefulness of CKMB and Troponin T determinations in patients with acute myocardial infarction complicated by ventricular fibrillation. *Clin Cardiol*; **16**: 23-25.

Zanetta J-P., Kuchler S., Lehmann S. & Badache A., (1992) Cerebellar lectins. *Int Rev Cytol;* **135**: 123-148.

Ziegler E., (1883) *A Textbook of Pathological Anatomy and Pathogenesis.* MacAlister D. (trans.). MacMillan & Co. London, pp 403.

Zugibe Jr. F.T. & Zugibe F.T., (1973) Fuchsinophilia and fuchsinorrhagia staining techniques. Use in the early detection of myocardial infarction. *Arch Pathol;* **96**: 243-244.